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# Transcriptional Poising Prior to the Midblastula Transition Underlies Dorsal Cell Fate Specification by the Wnt/Beta-Catenin Pathway

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**Abstract**
Following fertilization in many multicellular organisms, zygotic transcription is suppressed for several hours and cell divisions, until a major embryonic transition termed the midblastula transition (MBT). Nevertheless, steps critical for later patterning of the embryo occur during this early stage. To address this question, we have optimized the chromatin immunoprecipitation technique to allow the investigation of pre-MBT chromatin architecture. We find that, in the context of transcriptional quiescence before the MBT in Xenopus, Wnt signaling through β-catenin primes dorsal gene expression by establishing transcriptionally poised chromatin architecture at target promoters. This is later resolved into active gene expression following the large-scale activation of zygotic transcription at the MBT. During pre-MBT dorsal specification, β-catenin interacts with a histone H3 methyltransferase activity that targets arginine 8 (R8). Recruitment of the arginine methyltransferase Prmt2 to β-catenin target promoters is necessary and sufficient to establish the dorsal developmental program, indicating that Prmt2-mediated histone H3R8 methylation plays a critical role downstream of β-catenin in establishing poised chromatin architecture and marking key organizer genes for later expression. This work demonstrates a mechanism whereby a signal transduction pathway can establish poised chromatin architecture at target genes, which could have implications for the regulation of gene regulatory networks during development. Additionally, our results suggest the possibility that transcriptional poising plays a broader role in maintaining zygotic genome silencing before the MBT.

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# TRANSCRIPTIONAL POISING PRIOR TO THE MIDBLASTULA TRANSITION UNDERLIES DORSAL CELL FATE SPECIFICATION BY THE WNT/β-CATENIN PATHWAY

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# ABSTRACT

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Following fertilization in many multicellular organisms, zygotic transcription is suppressed for several hours and cell divisions, until a major embryonic transition termed the midblastula transition (MBT). Nevertheless, steps critical for later patterning of the embryo occur during this early stage. To address this question, we have optimized the chromatin immunoprecipitation technique to allow the investigation of pre-MBT chromatin architecture. We find that, in the context of transcriptional quiescence before the MBT in Xenopus, Wnt signaling through β-catenin primes dorsal gene expression by establishing transcriptionally poised chromatin architecture at target promoters. This is later resolved into active gene expression following the large-scale activation of zygotic transcription at the MBT. During pre-MBT dorsal specification, β-catenin interacts with a histone H3 methyltransferase activity that targets arginine 8 (R8). Recruitment of the arginine methyltransferase Prmt2 to β-catenin target promoters is necessary and sufficient to establish the dorsal developmental program, indicating that Prmt2-mediated histone H3R8 methylation plays a critical role downstream of β-catenin in establishing poised chromatin architecture and marking key organizer genes for later expression. This work demonstrates a mechanism whereby a signal transduction pathway can establish poised chromatin architecture at target genes, which could have implications for the regulation of gene regulatory networks during development. Additionally, our results suggest the possibility that transcriptional poising plays a broader role in maintaining zygotic genome silencing before the MBT.

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## 1.1: Summary

Embryonic development proceeds as a series of tightly regulated, successive programs of differential gene expression, ultimately establishing the pattern of the adult body plan from a single, totipotent fertilized egg. During oogenesis, eggs are loaded with maternal factors –either proteins or mRNAs– that guide the initial steps of embryonic development until the activation of the zygotic genome. For many organisms, the passage from maternal to zygotic control of development occurs several hours and cell divisions following fertilization, at the midblastula transition (MBT). Before the MBT, transcription from the zygotic genome is largely repressed, yet embryos emerge from this period already having begun the process of regional specification. This observation raises the question of how transcriptionally driven embryonic patterning can proceed under conditions of zygotic genome repression.

During early *Xenopus* embryogenesis, the Wnt/β-catenin signaling pathway functions to activate the gene regulatory network that establishes the dorso-ventral axis. In particular, maternal Wnt/β-catenin signaling functions during the pre-MBT period to activate the expression of the first zygotic genes that function in this network. Several lines of investigation have determined that pre-MBT β-catenin activity is both necessary and sufficient to establish the dorsal gene expression program, leading to the paradox: How can a transcription factor

function to activate transcription under conditions of global transcriptional repression?

To address this question, I hypothesized that β-catenin functions during the pre-MBT period to "mark" target genes for activation at a later time, thus functioning to establish transcriptionally poised chromatin architecture at target gene promoters. According to this model, the early activity of β-catenin at target genes would be sufficient to specify their activation at a later time, thus accounting for how β-catenin can activate gene expression in a repressive environment. To test this hypothesis, I first adapted the technique of chromatin immunoprecipitation to facilitate experimentation on pre-MBT *Xenopus* embryos. With this technique in hand, I determined that β-catenin target genes are indeed "poised" for activation prior to the MBT, and that β-catenin recruits a histone-modifying activity to target genes which is both necessary and sufficient for their eventual activation, perhaps by contributing to the establishment of poised chromatin architecture. This work raises the further questions of what regulatory mechanisms contribute to the establishment of "poised" loci, and whether transcriptional poising is a widespread strategy underlying both the maternal-to-zygotic transition and the specification of embryonic cell fates.

### 1.2: The Midblastula Transition

The earliest of several temporally coordinated, large-scale developmental milestones occurs at the Midblastula Transition (MBT), when embryogenesis

comes to be directed by zygotic rather than maternal factors. Several major biochemical and morphological changes occur during the maternal-to-zygotic transition at the MBT. Generally, newly fertilized embryos undergo several rounds of rapid synchronous mitotic divisions. These initial cell divisions represent a rapid oscillation between DNA Synthesis (S-phase) and mitosis (M-phase), without the intervening gap phases (G1- and G2-phase) observed in the typical somatic cell cycle (Graham and Morgan, 1966). For *Xenopus* and Zebrafish embryos, the rapidly dividing blastomeres lose synchrony and acquire G1- and G2-phases at the MBT, and display a markedly longer period between successive cell divisions (Kane and Kimmel, 1993; Newport and Kirschner, 1982a). In the case of the fruit fly *Drosophila melanogaster*, nuclei rapidly multiply in a syncytium, and the pace of the nuclear division cycles slows at the MBT, as the embryo cellularizes (Edgar et al., 1986). Prior to the MBT, maternal mRNAs and proteins direct embryogenesis while the zygotic genome is maintained in a transcriptionally quiescent state. Zygotic control of development begins with the onset of large-scale transcription from the zygotic genome accompanied by widespread degradation of maternal factors. Also, at the MBT, DNA-damage checkpoints become activated, cells become motile, and cells become competent to undergo programmed cell death (Hensey and Gautier, 1997; Newport and Kirschner, 1982a). In each case, the biochemical and morphological changes that culminate in the MBT are precisely coordinated by one or more timing mechanisms that are still not well characterized.

*1.2.1: Two Developmental Timers*

At least two timers coordinate the transition from maternal to zygotic control of development. The switch from the quick cell cycle of the cleavage divisions to the typical somatic cell cycle after the MBT is timed by the nucleocytoplasmic (N/C) ratio. Since early embryos exponentially multiply their DNA content without increasing their cytoplasmic volume, the increasing DNA is thought to titrate out a cytoplasmic factor that maintains the pre-MBT cell cycle. In *Xenopus* embryos, N/C ratio timer does not require new transcription, as cell cycle lengthening occurs in the presence of the RNA Pol II inhibitor, $\alpha$-amanitin (Newport and Kirschner, 1982a). The N/C ratio timer also regulates the onset of expression for a number of zygotic transcripts, including several abundant type III genes (Lund and Dahlberg, 1987; Newport and Kirschner, 1982a, b). In *Xenopus*, it is unclear whether zygotic expression of *any* type II (mRNA coding) genes is linked to the N/C ratio timer. Notably, expression of several endogenous and exogenous type II genes still occurs at the MBT even under conditions where the N/C ratio timer is stopped by the inhibition of cell cycle progression (Almouzni and Wolffe, 1995; Kimelman et al., 1987; Lund and Dahlberg, 1987). Also, another marker of the MBT, Cyclin E protein degradation, occurs even when embryos are arrested during morula stages by cycloheximide treatment (Howe and Newport, 1996). These observations indicate that at least two independent developmental timers coordinate the multiple transitions that take

place at the MBT, one that measures the N/C ratio, and another that measures the elapsed time following fertilization.

The onset of zygotic transcription in *Drosophila* also depends on measurement of both the N/C ratio and the "absolute time" following fertilization. *Drosophila* embryos typically undergo 14 rapid nuclear divisions prior to the MBT, which is followed by large-scale transcriptional activation and cell cycle lengthening. Haploid embryos will reach the MBT-stage N/C ratio one cell cycle later than their diploid counterparts and undergo an additional, $15^{th}$ nuclear division prior to cell cycle lengthening. Nonetheless, haploid embryos still activate the expression of a subset of genes at the $14^{th}$ nuclear division, albeit at lower intensity due to the abbreviated time between mitoses (Edgar et al., 1986; Yasuda et al., 1991). In support of the two-timer model, genome-wide transcriptional analysis in *Drosophila* has demonstrated that, while some immediate zygotic transcripts are sensitive to the N/C ratio for their activation, the majority of early zygotic genes are activated independently of this ratio, instead becoming active at a certain time after fertilization (Lu et al., 2009). This alternative developmental timer may be driven by regulated translation of the *Drosophila* Smaug protein, which is critical for coordinating both the degradation of maternal mRNAs and the activation of the zygotic genome at the MBT (Benoit et al., 2009). In this model, time is read out by the gradual accumulation of Smaug protein during the pre-MBT period. Smaug is translationally repressed initially, yet accumulates during the pre-MBT period, peaks during syncytial blastoderm stages, and is rapidly cleared at the MBT. Upon reaching a threshold

level of Smaug, the embryo begins clearing maternal transcripts and activating zygotic gene expression. Smaug is required for the destruction of maternal mRNAs that suppress zygotic transcription (*Tramtrack*) and for the zygotic expression of two positive regulators of RNA Polymerase II elongation: Cyclin T and Cdk9. Consequently, Smaug-deficient embryos have reduced RNA Pol II engaged in transcriptional elongation, which could be compounded with the persistence of transcriptional repressors and result in the failure to activate zygotic transcription. While a similar mechanism has not been described in any vertebrate model system, activation of the *Xenopus* zygotic genome can indeed be repressed by translational inhibition if embryos are treated prior to the 32-cell stage of development (A. Rosenberg, S. Blythe, P. Klein, unpublished). This observation suggests that, in addition to the N/C ratio, the embryo interprets the elapsed time after fertilization by the regulated translation of maternal mRNAs such as Smaug.

### 1.3: Transcriptional Control Before the MBT

The mechanisms that maintain the pre-MBT transcriptional quiescence of type II (mRNA coding) loci are poorly understood. Early *Xenopus* embryos are fully *competent* to transcribe DNA. Early embryos contain functional RNA Pol II (Roeder, 1974) and pre-MBT embryonic lysates will transcribe mRNA from plasmid templates *in vitro* (Toyoda and Wolffe, 1992). But despite containing a fully functional transcriptional apparatus, most endogenous loci and exogenous

sources of DNA are silenced in pre-MBT embryos. Certain genes, however, are normally transcribed before the MBT suggesting that this repressive state is not absolute and can be circumvented under the appropriate conditions (Shiokawa et al., 2005; Yang et al., 2002b). Indeed, foci of newly synthesized RNA can be detected in the nuclei of pre-MBT *Xenopus* embryos (Kimelman et al., 1987), indicating that transcription can *at least* be initiated before the MBT. Quantification of radiolabeled UTP uptake further indicates that a low level of mRNA expression occurs as early as the 64-cell stage of development (5 cell divisions, ~2h before MBT at 23°C) (Shiokawa et al., 1981). At least a portion of these newly synthesized pre-MBT transcripts are of high molecular weight, heterogeneous, capped, and polyadenylated (Nakakura et al., 1987; Shiokawa et al., 1981; Yang et al., 2002b). This phenomenon is not limited to *Xenopus* embryos: pre-MBT transcription has also been observed in every developmental model system studied to date that undergoes a distinct MBT (Andéol, 1994; Leung et al., 2003; Mathavan et al., 2005; Pritchard and Schubiger, 1996). Therefore, while the MBT represents the major activation of zygotic transcription, zygotic genome activation is a gradual process that occurs over a period beginning, in *Xenopus*, at the 64-cell stage of development. Additionally, the phenomenon of endogenous pre-MBT transcription underscores the fact that early embryos are transcriptionally competent, and indicates that zygotic genome silencing is not achieved by a wholesale inactivation of the transcriptional apparatus.

*1.3.1: Suppression of Basal Transcription in pre-MBT embryos*

Pre-MBT transcription is suppressed, in part, by chromatin-based mechanisms that are dominant over the basal transcriptional apparatus. Chromatin assembly itself is the major mechanism that represses basal transcription in pre-MBT embryos as it does in somatic cells (Prioleau et al., 1994). Chromatin assembly results in the placement of nucleosomes within genes that will repress transcription by preventing the association of promoter regions with basal transcription factors, such as TATA-binding protein (TBP). Accordingly, interfering with chromatin assembly or supplementing templates with basal transcription factors generally circumvents pre-MBT global transcriptional silencing. Exogenous plasmids containing type II genes are rapidly silenced upon expression in early embryos (Almouzni and Wolffe, 1995; Lund and Dahlberg, 1987; Prioleau et al., 1994), but a temporary burst of pre-MBT transcription is observed if plasmids are pre-loaded with TBP protein (Prioleau et al., 1994). These pre-loaded plasmids are ultimately silenced upon becoming assembled into chromatin, only to be activated at the MBT. Importantly, this does not reflect a deficit in endogenous TBP protein, as overexpression of TBP in the early embryo is not sufficient to cause premature transcription: it must be pre-bound to the plasmid in order to drive early transcription. This suggests that the basal transcriptional apparatus is prevented from interacting with promoters by pre-MBT chromatin structure. If chromatin assembly is competed by co-expressing an excess of “non-specific” DNA, precocious transcription from pre-

loaded plasmids continues throughout the MBT period (Prioleau et al., 1994). Early embryos contain vast stores of core histone proteins, synthesized during oogenesis, sufficient to assemble 20,000 to 40,000 nuclei (Woodland and Adamson, 1977), and to therefore sustain chromatin assembly long past the MBT. Non-specific DNA prevents silencing of pre-loaded plasmids by depleting the maternally supplied pool of core histones, and this effect can be suppressed by adding back exogenous core histone proteins (Almouzni and Wolffe, 1995). These results demonstrate the role of chromatin assembly in the suppression of basal transcription before the MBT, particularly the role of the nucleosome in establishing repressive chromatin architecture.

### *1.3.2: Counteracting Trans-Activator Function*

In general, nucleosome occupancy at promoters is refractory to basal transcription (Lorch et al., 1987; Williamson and Felsenfeld, 1978; Workman and Roeder, 1987). In somatic cells, this repression is counteracted by recruitment of transcriptional activators to gene regulatory domains on chromatin, which function to open chromatin structure and potentiate pre-initiation complex formation at promoters. Therefore, the pre-MBT competition between chromatin assembly and transcriptional complex formation could thus account for the generalized repression of zygotic loci, but does not explain the silencing of genes whose activators are indeed present before the MBT. As a proof of principle, Almouzni and Wolffe (1995) co-expressed the transcriptional activator Gal4-

VP16 in pre-MBT embryos along with a plasmid containing Gal4 consensus binding sequences. Under these experimental conditions, Gal4-VP16 is sufficient for prolonged pre-MBT transcription, leading to the conclusion that pre-MBT transcriptional repression stems from constraints on trans-activator function. It should be noted that the Almouzni and Wolffe (1995) study used a 300-fold molar excess of Gal4-VP16 to Gal4 DNA binding sites. In a subsequent study, Prioleau et al. (1995) were unable to reproduce Gal4-VP16-driven pre-MBT transcription using a more modest 4-fold molar excess of Gal4-VP16, but were able to drive prolonged pre-MBT transcription by pre-loading plasmids with this trans-activator. Therefore, constraints on trans-activator function could account for zygotic genome silencing, either by preventing interaction with regulatory elements on genomic DNA, or by interfering with downstream recruitment of an active transcriptional apparatus.

One example of a constraint on transcriptional activator function is the counteracting repressive effect of DNA methylation. DNA methylation represses transcription at associated genes by serving as a ligand for methylated DNA-binding transcriptional repressors. This is a form of epigenetic control of gene expression, as the pattern of methylated DNA (and hence the associated repressive activity) is inherited by daughter cells following cell division. Epigenetic inheritance of the DNA methylation pattern requires the activity of “maintenance methyltransferases,” such as DNA Methyltransferase I (Dnmt1), which copy the parental pattern of methyl-marks to the non-methylated daughter strand following DNA replication. Interestingly, depletion of Dnmt1 in pre-MBT

*Xenopus* embryos results in the precocious expression of many genes that are normally repressed until the MBT (or shortly thereafter) (Stancheva and Meehan, 2000). This suggests that the activators for these Dnmt1-sensitive genes are otherwise active in early embryos, yet are prevented from activating the expression of their targets due to the counteracting repressive influence of DNA methylation. As Dnmt1 depletion affects only a subset of early zygotic loci, this also suggests that pre-MBT transcriptional silencing may be maintained by several overlapping mechanisms.

Early embryos contain several endogenous transcriptional activators whose activities are required for subsequent embryonic patterning. How, then, do genes whose trans-activators are present in the early embryo maintain their silence? Certain trans-activators are prevented from binding their regulatory regions either by cytoplasmic sequestration or restricted nuclear import (Veenstra, 2002). In addition, trans-activators could be ineffective if their genomic target sites are occluded by nucleosome occupancy. However, at least some zygotic loci are able to interact with trans-activators and even establish active chromatin architecture during the pre-MBT period. For example, the *cMyc* locus establishes typically active patterns of DNase hypersensitivity before the MBT, despite transcriptional repression (Prioleau et al., 1995). Indeed, this has also been observed for several endogenous loci in *Drosophila* pre-blastoderm embryos as well (Lowenhaupt et al., 1983). Since acquisition of DNase hypersensitivity is commensurate in most cases with transcriptional activation, it is intriguing to observe this at transcriptionally silent loci. These observations

raise the possibility that zygotic loci establish and maintain “pre-set” chromatin architecture during the pre-MBT period, reflecting a multi-step process of transcriptional activation where trans-activator binding is temporally uncoupled from mRNA expression. Notably, the formation of active chromatin architecture at promoters leaves open the possibility that these promoters also recruit the basal transcriptional machinery. In this case, transcriptional silencing could be achieved downstream of pre-initiation complex formation, either by regulating RNA Pol II promoter escape or entry into transcriptional elongation. Interestingly, it is not clear whether acquisition of DNase hypersensitivity at pre-MBT promoters reflects either the inheritance of chromatin architecture established during gametogenesis, or if these patterns are established in the zygote by maternal transcription factors.

### *1.3.3: Heritability of active chromatin structure before the MBT*

The pre-MBT embryo is also capable of maintaining patterns of active chromatin architecture despite the rapidity of the cleavage-stage cell cycle. Generally, patterns of DNase hypersensitivity are rapidly re-established following DNA replication, within 20 nucleosomes of the passing replication fork (Weintraub, 1979). This indicates that rapid DNA replication *per se* does not limit the propagation of established chromatin architecture. Furthermore, the rapidity of the early cell cycle does not interfere with *active* chromatin structure, as transplanted, transcriptionally active, post-MBT nuclei retain an epigenetic

“memory” of their parental gene expression program (Ng and Gurdon, 2005). These donor nuclei will have established active chromatin architecture and, upon transplantation, at least some of the “marks” of transcriptional activity are propagated through the pre-MBT period. For example, a cloned embryo generated by transplantation of a nucleus from the neural ectoderm of a tailbud-stage embryo will “remember” its origin and express the neural gene NCAM well in advance of neural induction, when NCAM expression is normally activated (Ng and Gurdon, 2005). Remarkably, this precocious “remembered gene” expression is observed before the MBT in cloned embryos, indicating that establishment of transcriptionally active chromatin architecture is sufficient to drive pre-MBT transcription. This transcriptional memory is linked to chromatin modifications that correlate with active gene expression, the incorporation of histone variant H3.3 (Ng and Gurdon, 2008). Because these transplanted nuclei enter the pre-MBT cell cycle, these observations reveal that chromatin assembly in the pre-MBT embryo *per se* is not sufficient to cause global transcriptional silencing. In fact, establishment of active chromatin architecture can be propagated in early embryos, indicating that these mechanisms are intact and functional before the MBT.

In conclusion, the pre-MBT embryo is not transcriptionally inert. In fact, mechanisms for establishing and propagating active chromatin architecture function during this time much as they would in somatic cells. At least some zygotic loci do, in fact, establish active chromatin architecture during this period, and are maintained in a pre-set or poised state until the MBT. Some genes are

even normally expressed prior to the MBT. It is unknown whether establishment of a poised state is typical of most “immediate-early” zygotic loci, or whether this is only true for a subset of these genes. Several potentially overlapping repressive mechanisms suppress these pro-transcriptional activities in order to prevent the expression of most zygotic loci. This repression has been shown to function at several levels: interfering with the assembly of the basal transcriptional machinery, and constraining transcriptional activator function. However, the formation of active chromatin architecture in the pre-MBT embryo leaves open the possibility that transcriptional repression takes place farther into the transcriptional cycle, perhaps by interfering with entry of RNA Pol II into the elongation phase of transcription. Interfering with these repressive mechanisms results in precocious transcription of zygotic loci, supporting the idea that pre-MBT genomic silencing is achieved by balancing pro- and anti-transcriptional mechanisms.

### 1.4: Transcriptional Poising

In somatic cells, genes which can be activated fall into two general classes based on their chromatin structure: “pre-set” or “remodeling” [after (Wallrath et al., 1994)]. A pre-set (or poised) locus can produce transcripts quickly due to maintenance of open chromatin structure, which reveals trans-activator interaction sites allowing for immediate binding and activation. A remodeling locus requires some kind of chromatin remodeling event prior to

activation, which facilitates the ultimate binding of trans-activators to otherwise concealed regulatory sites. In addition, genes requiring remodeling will typically have nucleosomes covering the transcriptional start site [e.g. (Richard-Foy and Hager, 1987)]. On the other hand, poised loci usually contain paused or stalled RNA Pol II within the transcriptional start site, in addition to other chromatin modifications that correlate with transcriptional activity (Margaritis and Holstege, 2008). Consequently, these loci are kept silent by different mechanisms. While chromatin remodeling and trans-activator binding is limiting for the activation of a remodeling locus, poised loci are likely kept silent by regulating the entry of RNA Pol II into the elongation phase of transcription (Saunders et al., 2006). Furthermore, genome-wide analyses of RNA Pol II promoter occupancy in diverse organisms have established that a significant proportion of protein-coding genes are maintained in a poised state, which likely facilitates a rapid response to biological or environmental stimuli (Baugh et al., 2009; Guenther et al., 2007; Hargreaves et al., 2009; Muse et al., 2007; Radonjic et al., 2005; Zeitlinger et al., 2007). The roster of poised genes in early *Drosophila* embryos and in mammalian embryonic stem cell systems also reflects the developmental potential of each system, suggesting that transcriptional poising could be an evolutionarily conserved mechanism underlying cell fate specification during embryogenesis.

During *Drosophila* embryogenesis, for example, genes can be categorized according to the extent to which they associate with RNA Pol II. “Active” genes are found to interact with Pol II across the entire coding region; “stalled” genes

have Pol II bound only to the 5' promoter-proximal region; and "absent" genes display no detectable Pol II binding. Both stalled and absent genes are transcriptionally repressed, but stalled genes are typically associated with developmental genes whereas absent genes are typically expressed much later in development (Zeitlinger et al., 2007). It is conceivable that stalled Pol II functions in this situation to poise genes for rapid activation later in development. Indeed, genomic loci that respond rapidly to biological and environmental stimuli tend to fall into the poised class (Baugh et al., 2009; Hargreaves et al., 2009; Muse et al., 2007; Radonjic et al., 2005; Rougvie and Lis, 1988), and transcriptional poising facilitates the synchronous, rather than stochastic, activation of genes in response to developmental cues (Boettiger and Levine, 2009). Similarly, in human embryonic stem cells, approximately 75% of protein-coding genes associate with Pol II, but only 50% of that set are transcriptionally active (Guenther et al., 2007). The remaining 50% of genes have RNA Pol II accumulated in the promoter proximal region, and many undergo abortive transcription, without transcript completion. In addition to interacting with stalled Pol II, poised loci also associate with histone modifications that typically correlate with active transcription: histone H3 lysine 4 trimethylation (H3K4me3), and histone H3 lysine 9/14 acetylation (H3K9/14Ac) (Guenther et al., 2007). These modifications are likewise restricted to the 5' end of protein-coding genes, and have been implicated in the regulation of early phases of the transcriptional cycle (See Chapter 4). However, the role of specific histone modifications –such as H3K4me3– in the establishment or maintenance of a poised state is unclear.

In pre-MBT *Xenopus* embryos, an exogenous plasmid containing the *cMyc* locus will adopt an active pattern of DNase hypersensitivity, and this locus has been demonstrated in other model systems to undergo transcriptional poising (Krumm et al., 1992; Strobl and Eick, 1992). If the *cMyc* locus is indeed poised in pre-MBT embryos, this suggests that the embryo contains the maternal factors to both establish and maintain this type of chromatin architecture before the MBT. Does the establishment of transcriptional poising reflect inherited parental chromatin structure, or is it the product of developmental patterning events carried out after fertilization? There is evidence that suggests that chromatin structure can, in fact, be established in the gametes, and propagated epigenetically during early embryogenesis (Hammoud et al., 2009). In human spermatocytes, the promoters of many genes required for future embryogenesis maintain their association with somatic nucleosomes, whereas the rest of the genome is packaged in protamines. These retained somatic nucleosomes also bear chromatin modifications that typically correlate with transcriptional activity, although sperm are transcriptionally repressed. This results in the preferential marking of developmental promoters, which could serve as a basis for establishing “poised” chromatin conformation in the resulting zygote. Indeed, many of the genes that are found to be poised in human embryonic stem cells are also found to retain somatic nucleosomes in spermatocytes, allowing for the possibility that the poised state is initially specified in the gametes, and epigenetically inherited through the early cell divisions of the embryo, presumably aided by pro-transcriptional histone modifications. In species that silence their

genomes before the MBT, the establishment of a poised state could also serve as a mechanism to counteract any default state of transcriptional repression, as poised loci have also been shown to function as insulator elements (Chopra et al., 2009). Thus the establishment of a poised state could reflect the intrinsic maintenance of a genetic locus in a ready state that can be epigenetically inherited and established well in advance of mRNA expression in order to provide for a rapid and synchronous transcriptional response to developmental cues.

### 1.5: Dorsal Specification by the Maternal Wnt/β-catenin Pathway

The Wnt signaling pathway plays essential roles during embryonic development, tissue homeostasis, and oncogenesis. The “canonical” Wnt (Wnt/β-catenin) signaling pathway, in particular, represents a critical mechanism for converting extracellular stimuli into intracellular transcriptional responses. In the absence of a Wnt signal, β-catenin protein is translated, but rapidly degraded. Upon activation of the Wnt pathway, β-catenin protein is stabilized in the cytoplasm and ultimately translocates to the nucleus where it interacts with its genomic targets by binding members of the Tcf/Lef family of DNA-bound transcription factors. Within the nucleus, β-catenin functions as a versatile scaffold for recruiting a wide array of chromatin remodeling factors to target loci, and thereby activating their expression.

The Wnt/β-catenin signaling pathway is activated in *Xenopus* embryos prior to the first cell division and functions to initiate the gene expression program that will establish the embryonic dorso-ventral axis. This represents the earliest cell fate decision made in the embryo, and it occurs entirely before the MBT. Following fertilization, rotation of the cortical cytoplasm relative to the inner cytoplasm of the egg asymmetrically displaces a vegetally localized pool of Wnt pathway effectors (Miller et al., 1999; Tao et al., 2005). Blastomeres inheriting this cytoplasm activate Wnt signaling, which results in the establishment of the gene expression program that will give rise to the critical inductive center for dorsally derived tissues, Spemann's organizer. The genes for the homeodomain proteins *Siamois* and *Twin* are two targets of maternal Wnt signaling, and they are both necessary and sufficient to drive dorsal development downstream of β-catenin [(Ishibashi et al., 2008; Laurent et al., 1997; Lemaire et al., 1995), also D. S. Kessler unpublished, and Chapter 3]. Expression of *Siamois* and *Twin* does not begin until the MBT, but several observations indicate that their expression is activated shortly after fertilization, prior to the 32-cell stage (2.5h or six cell divisions before the MBT).

The embryo is only competent to activate the dorsal developmental program in response to the Wnt/β-catenin pathway until the MBT, after this point activation of Wnt signaling activates a distinct, non-dorsal gene expression program (Hamilton et al., 2001; Kao et al., 1986; Yamaguchi and Shinagawa, 1989). The maternal (pre-MBT) Wnt pathway is restricted to dorsal progenitors (Tao et al., 2005), and early zygotic (post-MBT) Wnt pathway activity is activated

in the ventro-lateral marginal zone of the embryo (Christian et al., 1991; Smith and Harland, 1991). Correspondingly, stimulating the Wnt/β-catenin pathway before the MBT activates the dorsal gene expression program, and this response is abruptly lost after the MBT (Darken and Wilson, 2001; Fredieu et al., 1997; Hamilton et al., 2001). Therefore, the embryo is competent to activate dorsal gene expression in response to the Wnt/β-catenin pathway before the MBT, but not after. Additionally, loss-of-function studies for β-catenin indicate that dorsal specification is complete by the end of the 32-cell stage ($5^{th}$ cleavage, 2.5h before the MBT). β-catenin function must be blocked, either by translational inhibition or by expression of a dominant-negative Tcf protein, prior to the 32-cell stage in order to prevent the induction of β-catenin-dependent transcription after the MBT (Heasman et al., 2000; Yang et al., 2002b). These results indicate that β-catenin ostensibly specifies dorsal cell fates in the absence of transcriptional activity. Interestingly, transiently interfering with RNA Pol II function during the pre-MBT period prevents dorsal specification by β-catenin (Yang et al., 2002b). This indicates that, despite occurring during a period of transcriptional quiescence, dorsal specification by β-catenin nonetheless requires RNA Pol II function. It is unclear whether the requirement for RNA Pol II suggests that β-catenin-directed pre-MBT transcription is absolutely required for dorsal specification, or if RNA Pol II function is required for the establishment of a poised chromatin structure at β-catenin target genes before the MBT. Indeed, two β-catenin target genes, Nodal-related 5 and 6 (Xnr5/6) are transcribed before the MBT (Yang et al., 2002b), however no studies have addressed whether their

pre-MBT expression, *per se*, is required for dorso-ventral patterning. Additionally, it is not clear how precocious expression of Xnr5/6 could activate the expression of Siamois and Twin, without which dorsal development fails. This raises the possibility that β-catenin functions via at least two distinct mechanisms during the pre-MBT period, one that results in the early activation of Xnr5/6, and another that specifies the eventual activation of Siamois/Twin at the MBT.

*In vitro*, chromatin structure is essential for β-catenin/Tcf transcriptional activation (Tutter et al., 2001), thus raising the possibility that β-catenin activates transcription solely by mediating the recruitment of chromatin-directed factors to target loci. β-catenin has been shown to interact with members of three classes of chromatin-modifying enzymes: ATP-dependent chromatin remodelers, histone acetyl-, and methyl-transferases. The majority of these interactions take place through β-catenin's C-terminal trans-activation domain, which is necessary and sufficient for transcriptional activation by β-catenin [reviewed in (Mosimann et al., 2009)]. In addition, β-catenin also interacts with the Mediator and the Paf1 complexes, which ostensibly link β-catenin to both initiating and elongating RNA Pol II complexes at target genes. The Wnt pathway even has a dedicated histone-modification "reader," the PHD-domain containing protein Pygopus, which interacts indirectly (via Legless/Bcl9) with β-catenin, and is necessary for Wnt pathway transcriptional output. Pygopus also binds histone H3, trimethylated at lysine 4 (H3K4me3), which is a modification associated with both transcriptionally poised and active loci, and could thus serve as a Tcf-independent tether for β-catenin at H3K4me3-marked loci. In light of this broad

roster of chromatin-directed β-catenin interacting proteins, it is reasonable to conclude that activation of the Wnt/β-catenin pathway results in chromatin remodeling and modification at target gene promoters as an initial step to activate their expression.

Since induction of *Siamois/Twin* is the critical downstream target of the maternal Wnt/β-catenin pathway, and expression of these genes does not commence until after the MBT, β-catenin's activity is temporally uncoupled from the transcriptional response of these essential targets. As such, β-catenin could function before the MBT to poise target gene activation after the MBT. In this model, β-catenin interacts with target gene promoters prior to the 32-cell stage and functions to establish a heritable, transcriptionally poised state. As such, it is predicted that several molecular markers common to poised loci would be present at β-catenin target genes before the MBT, including pre-bound RNA Pol II, and histone H3 K4me3, and K9/14Ac. Additionally, it is predicted that β-catenin plays an integral role in establishing this poised chromatin architecture, functioning either in a "pioneering" role by initially relaxing chromatin structure to facilitate binding of the stalled Pol II complex, or by recruiting additional factors to already poised promoters in order to facilitate their eventual release into a *bona fide* active state at the MBT.

### 1.6: Research Summary

To address the question of how β-catenin functions before the MBT to activate the dorsal gene expression program, I first adapted a standard chromatin immunoprecipitation (ChIP) technique for use with a manageable number of pre-MBT embryos. This work is presented in Chapter 2 as published in Blythe et al., (2009). Upon optimizing the *Xenopus* ChIP protocol, I first evaluated the status of pre-MBT promoters with respect to binding of both β-catenin and RNA Pol II, and association of pro-transcriptional histone modifications (H3K9/14 acetylation and H3K4 methylation). This work confirmed that β-catenin target genes are indeed poised before the MBT, and that β-catenin is particularly required for establishment of H3K4me3. Subsequently, I tested the hypothesis that β-catenin directly recruits a histone methyltransferase (HMT) activity to promoters before the MBT. While β-catenin does indeed recruit an HMT, surprisingly this activity was found to modify H3R8, not H3K4. Thus, I identified the β-catenin associated HMT to be Prmt2, an arginine methyltransferase with no previously known catalytic activity (or catalytic targets). Prmt2 is recruited to β-catenin target genes before the MBT, and this recruitment is sufficient to drive dorsal development downstream of β-catenin. Additionally, Prmt2 is necessary for dorsal specification, and we find evidence that Prmt2 functions to prevent transcriptional repression of β-catenin target genes, suggesting a role for H3R8 methylation as an anti-repressive histone modification. These latter observations are presented in Chapter 3. A broad discussion of conclusions and future directions follows in Chapter 4.

# Chapter 2: Chromatin Immunoprecipitation in Early *Xenopus Laevis* Embryos*

## 2.1: Introduction

Chromatin immunoprecipitation (ChIP) has emerged as an invaluable tool for the study of the mechanisms of transcriptional control and chromatin dynamics. ChIP allows an investigator to determine whether a genomic locus is occupied by chromatin-bound factors such as transcription factors, chromatin remodeling complexes, and modified histones. The most widespread ChIP procedure uses formaldehyde-crosslinked, sheared chromatin from $10^6$ to $10^7$ cells as the input material for an immunoprecipitation (IP), which is followed by several rounds of washing, crosslink reversal, and DNA purification. Because the genomic DNA is sheared to an average size of 1000 base pairs or less, the IP results in the purification of discrete genomic DNA fragments that associate with the antigen of interest. Thereafter, the purified DNA from the experimental IP is queried for enrichment relative to a control IP, either by PCR—for small numbers of target genes—or by one of several genome-wide analysis methods (microarray, high-throughput sequencing, library screening). Thus, ChIP represents a powerful method for investigating *in vivo* protein-DNA interactions.

* The text of this chapter has been published (Blythe et al. Chromatin immunoprecipitation in early Xenopus laevis embryos. Dev Dyn (2009) vol. 238 (6) pp. 1422-32). The data presented in Figure 2.4 (and the accompanying text in section 2.3.6) were provided by co-authors Christine Reid and Dr. Daniel Kessler.

For molecular embryologists, however, the typical ChIP protocol poses a number of challenges. Embryos represent heterogeneous populations of cells containing limiting amounts of genomic material. In addition, fractionation of embryos under the denaturing conditions commonly used in ChIP releases a large amount of non-chromatin-associated proteins, such as yolk, that complicate sample preparation and can increase nonspecific background. Consequently, molecular embryologists have been slow to adopt ChIP as a routine assay, especially for the analysis of early embryos. However, ChIP analysis of early embryos could help forge new frontiers in developmental biology. Whether by providing for the enhanced analysis of transcription factor function in gene regulatory networks, or by investigating the function of histone modifications and how their patterns unfold during embryogenesis, numerous new avenues of investigation will require the establishment of a ChIP protocol amenable to embryonic tissues.

Our goal was to develop a ChIP procedure that would be sensitive enough to detect transcription factor occupancy at promoters in cleavage-stage *Xenopus laevis* embryos (stage 7.5 to 8: approximately $1x10^3$ cells per embryo). In addition, we wanted the protocol to be amenable to typical embryological manipulations, such as microinjection, and therefore optimized the protocol to use as few as 50 embryos per sample from these early stages. Finally, we wanted to circumvent several foreseeable problems with sample preparation by minimizing non-chromatin proteins in the ChIP samples, optimizing the crosslinking and sonication steps, and optimizing DNA extraction and PCR

conditions to maximize sensitivity. While this protocol was in development, a number of ChIP experiments on *Xenopus laevis* embryos were reported (Jallow et al., 2004; Kim et al., 2004; Messenger et al., 2005; Morgan et al., 2004; Ng and Gurdon, 2008; Park et al., 2005), representing four different protocols. While all of these protocols are similar in principle to ours, we have concentrated on maximizing the sensitivity of this procedure for the analysis of earlier stages of development with a small number of embryos. Here, we present our optimized protocol in detail, with some examples of its implementation. By demonstrating the basic method, and describing how the protocol was optimized, we aim to facilitate the adoption of ChIP as a routine assay in the *Xenopus* embryological laboratory.

While our method is similar to now-standard ChIP protocols in use with other model systems (Kuo and Allis, 1999), some critical differences may be particular to the *Xenopus* system. First, we report optimized fixation and sonication techniques that yield chromatin crosslinked and sheared enough to detect transcription factor occupancy at promoters with at least 1000bp resolution. Second, because the standard 1% SDS lysis buffer used during sonication tended in our hands to produce low-quality chromatin from early embryos, we used a low-SDS (0.1%) radio-immunoprecipitation assay (RIPA) buffer. RIPA buffer produces high quality sheared chromatin samples while reducing yolk solubilization, thus limiting background by preventing protein precipitation. Doubling the number of washes further reduces background. Finally, we have optimized DNA purification and PCR conditions to allow for the

reliable detection of as little as 30 copies of a target sequence per reaction, facilitating the use of as few as $5x10^4$ cells in the starting sample, an improvement of two orders of magnitude from the typical ChIP protocol. We also demonstrate approaches for quantitative PCR and statistical analysis of ChIP results, which can offer several advantages over endpoint PCR detection strategies for detecting differences between samples. Interestingly, several protocols for ChIP using either cultured cells, early mouse embryos, or tissue biopsies have been described that use as few as $1x10^2$ cells (Acevedo et al., 2007; Dahl and Collas, 2008; O'Neill et al., 2006), suggesting that, with modification, the sensitivity of this procedure could be enhanced even further. The protocol we present here is well suited to the *Xenopus* embryologist: it facilitates the use of microinjected embryos and explanted tissues by decreasing the amount of genomic material required to obtain meaningful data. Thus, we present this work with the hope that it will help advance the use of ChIP in embryological experiments and lead to new avenues of research in developmental biology.

### 2.2: Results and Discussion

In this section, we discuss the critical parameters for optimization and validation as well as general guidelines for ChIP in *Xenopus*. A step-by-step protocol follows in “Experimental Procedures”. The most critical parameter in our experience is the method used to generate the sheared chromatin sample. Several factors need to be considered: extent of crosslinking, duration/strength of

sonication, and yield. In general, the greater the extent of crosslinking, the greater the amount of sonication that will be required to generate ideal (<1000bp) fragments. However, prolonged crosslinking will render chromatin impervious to fragmentation (Orlando et al., 1997), and over-sonication will result in reduced overall yield of genomic DNA. In addition, the target antigens should be considered. For example, nucleosomes can be immunoprecipitated with sheared genomic DNA without crosslinking (O'Neill and Turner, 2003), while transcription factors and secondarily-associated protein complexes may require extended crosslinking times (or different crosslinking reagents) to achieve sufficient co-immunoprecipitation of genomic DNA (Zeng et al., 2006).

These factors should also be considered when customizing this protocol to particular applications. The following procedure was used to optimize crosslinking and shearing for blastula and gastrula stage *Xenopus* embryos. All sonication steps were performed using a Branson Sonifier 250 equipped with a 1/4" microtip horn, set at 20% output. Other makes of sonicators will have different efficiencies, making these optimization steps even more critical.

### *2.2.1: Crosslinking Optimization*

To optimize crosslinking time, we performed a fixation time course on gastrula stage (stage 10) embryos. Using this stage ensures that enough DNA will be recovered for analysis by agarose gel electrophoresis. We collected embryos fixed in 1% formaldehyde / PBS for 15, 30, 45, and 60 minutes, as well

as control, non-fixed embryos. Samples were prepared according to the ChIP Day 1 protocol up to step 8 (see Experimental Procedures). We sonicated the samples minimally using conditions (3x 20 seconds, 20% output, 20% duty cycle) that would solubilize the genomic DNA from the insoluble pellet—effectively shearing native DNA— but would minimally shear crosslinked DNA. Post-sonication supernatants were adjusted to 1% SDS, 10mM EDTA prior to crosslink reversal and DNA purification, as described in the Experimental Procedures, except that following RNase treatment, DNA was ethanol precipitated, resuspended in 50μl $H_2O$ and analyzed by agarose gel electrophoresis.

While genomic DNA from embryos fixed for 15 minutes showed no resistance to shearing as compared to control, DNA from embryos fixed for as little as 30 minutes showed evidence of crosslinking, indicated by the detection of slower-migrating, sonication-resistant DNA (Figure 2.1A). Subsequently, the extent of crosslinking was increased incrementally until the 60-minute timepoint. Although 60-minute fixation times have been reported (Orlando et al., 1997), typical ChIP protocols performed on yeast and cultured cell systems fix samples for 10 to 15 minutes (Kuo and Allis, 1999; Luo et al., 1998), with similar amounts of formaldehyde in PBS or culture medium. This result suggests that the kinetics of nucleoprotein crosslinking by formaldehyde is different in the case of the *Xenopus* embryo, perhaps due to a greater non-chromatin protein to DNA ratio compared to other systems. Therefore, it will also be important to optimize

FIGURE 2.1

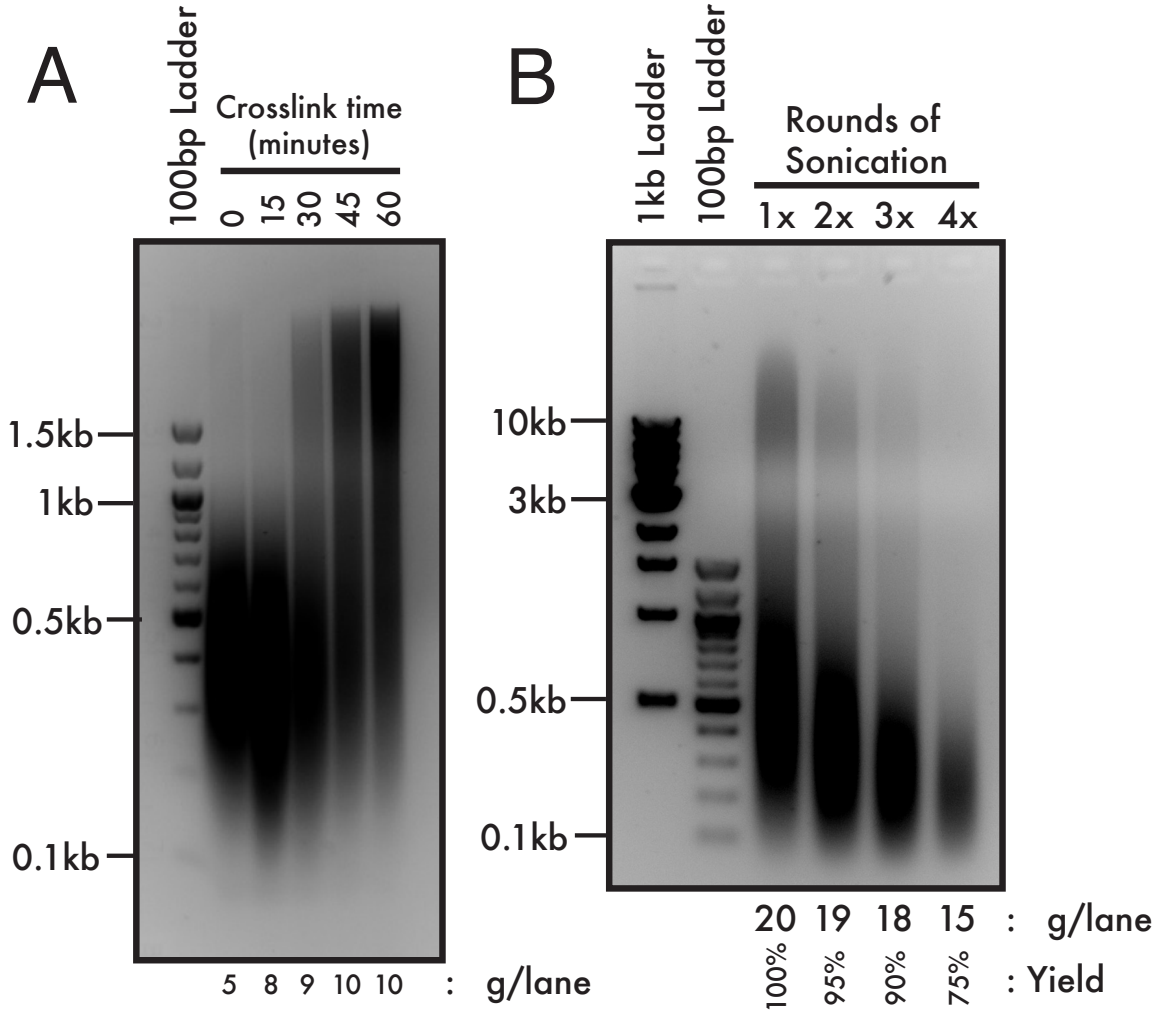

A
100bp Ladder
Crosslink time (minutes)
0
15
30
45
60
1.5kb
1kb
0.5kb
0.1kb
5 8 9 10 10 : g/lane
B
1kb Ladder
100bp Ladder
Rounds of Sonication
1x 2x 3x 4x
10kb
3kb
0.5kb
0.1kb
20 19 18 15 : g/lane
100% 95% 90% 75% : Yield

**Figure 2.1: Crosslinking and Sonication Optimization**.

(A) Minimally-sonicated, gastrula stage (stage 10) DNA from a 1% formaldehyde/ 1XPBS time course was resolved by 2% agarose gel electrophoresis. Onset of crosslinking is observed by the recovery of sonication-resistant, high molecular weight DNA (>1kb). (B) 60-minute crosslinked chromatin from gastrula stage embryos was fully sonicated for one to four rounds of 20 seconds each and resolved by 1.2% agarose gel electrophoresis. Three rounds of sonication balances optimal yield (90%) with maximal shearing (<1kb) of the crosslinked chromatin.

crosslinking times for later embryonic stages when this ratio begins to approach typical somatic levels.

### *2.2.2: General Sonication Guidelines*

1) Perform sonication on ice to prevent overheating. Place the sample to be sonicated in a beaker filled with ice, and hold this under the sonicator horn during shearing.
2) Sonicate in short bursts. 20-second rounds of sonication prevent sample overheating. Let the samples rest for at least 1 minute before the next round.
3) Center the horn in the sample and avoid contact with the walls of the microcentrifuge tube. This will improve reproducibility.
4) Avoid sample foaming, which happens when the tip of the horn draws air into the sample because it was brought too close to the surface.

### *2.2.3: Shearing Optimization*

To determine whether we could generate sufficiently small average DNA fragment size with a longer fixation time, we crosslinked gastrula stage (stage 10) embryos for 60 minutes and performed one to four rounds of full-strength sonication (20 seconds each, 20% output, 100% duty cycle) and repeated the crosslink reversal and DNA purification as described below (see Experimental

Procedures). One round of sonication generated a majority of <1000bp fragments, but we also noted a population of high molecular weight DNA that was reduced with each successive round of sonication (Figure 2.1B). By four rounds of sonication, the high molecular weight DNA was virtually undetectable, but overall DNA yield was also reduced by 25%. Therefore, we concluded that three 20-second rounds of sonication balances optimal yield (90%) with average fragment length (<1000bp). The efficiency and specificity of this method of crosslinking and shearing was further validated as described below.

### *2.2.4: Chromatin Immunoprecipitation in Blastula Stage Embryos*

We tested the ChIP protocol by scanning for occupancy of the transcription factor β-catenin within a 2.5kb upstream portion of the *Xenopus nodal-related 6a* (*Xnr6*) locus that contains several predicted Tcf/Lef binding sites (Figure 2.2A). Wnt/β-catenin pathway activity is required for *Xnr6* expression (Rex et al., 2002; Takahashi et al., 2000; Xanthos et al., 2002; Yang et al., 2002b), so we predicted that some of these sites would be occupied by β-catenin in blastula stage embryos. In addition, we performed ChIP for a euchromatin marker, di-acetylated [K9/K14] histone H3 (AcH3), reasoning that an active locus should be positive for AcH3 (Roh et al., 2005). A portion of the related *Xnr1* locus that is not predicted to bind β-catenin was analyzed as an additional control (Figure 2.2A).

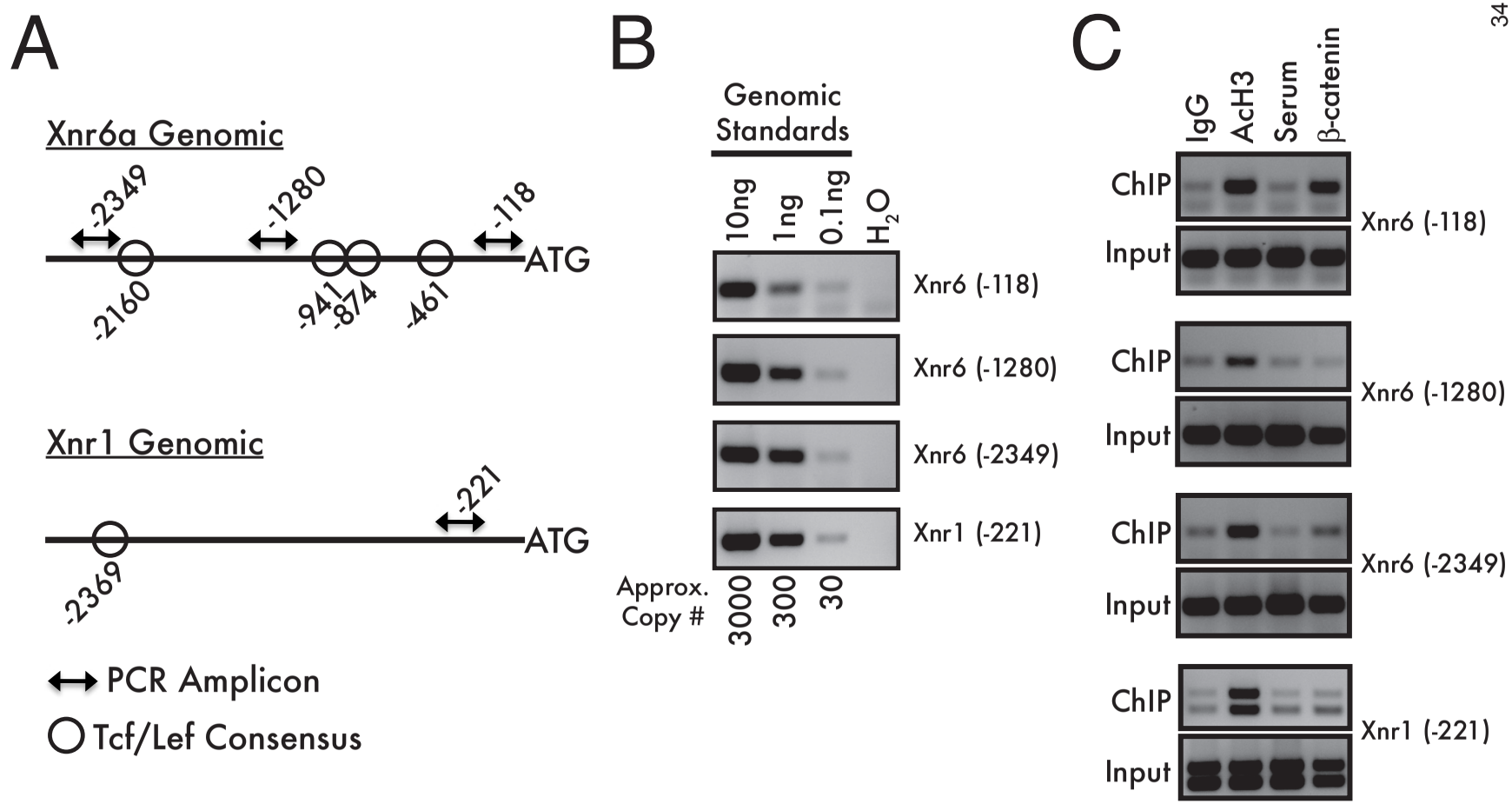

A
Xnr6a Genomic
-2349
-1280
-118
ATG
-2160
-941
-874
-461
Xnr1 Genomic
-221
ATG
-2369
PCR Amplicon
Tcf/Lef Consensus
B
Genomic Standards
10ng
1ng
0.1ng
H2O
Xnr6 (-118)
Xnr6 (-1280)
Xnr6 (-2349)
Xnr1 (-221)
Approx. Copy #
3000
300
30
C
IgG
AcH3
Serum
β-catenin
ChIP
Input
Xnr6 (-118)
Xnr6 (-1280)
Xnr6 (-2349)
Xnr1 (-221)

FIGURE 2.2

## Figure 2.2: Chromatin immunoprecipitation in blastula-stage *Xenopus* embryos

Schematic representations of the *Xnr6a* and *Xnr1* genomic loci (A) demonstrate the locations of predicted Tcf/Lef consensus sequences relative to the ChIP PCR amplicons. (B) These primer sets amplify standard genomic DNA by PCR with similar efficiencies, with a limit of detection at approximately 30 haploid genomes. **(**C) Blastula-stage embryos (Stage 9) were processed for ChIP using either anti-acetylated histone H3, or anti-β-catenin antisera (with the corresponding negative controls). The PCR products were visualized by 3% agarose gel electrophoresis and ethidium bromide staining. Co-immunoprecipitation of associated genomic DNA is observed when the PCR signal is greater for the experimental IP (AcH3, β-catenin) than in the control IP (IgG, Serum). Note that the *Xnr1 (-221)* product occasionally amplifies as a doublet (seen in panel C): this represents genetic variation at this locus within our colony.

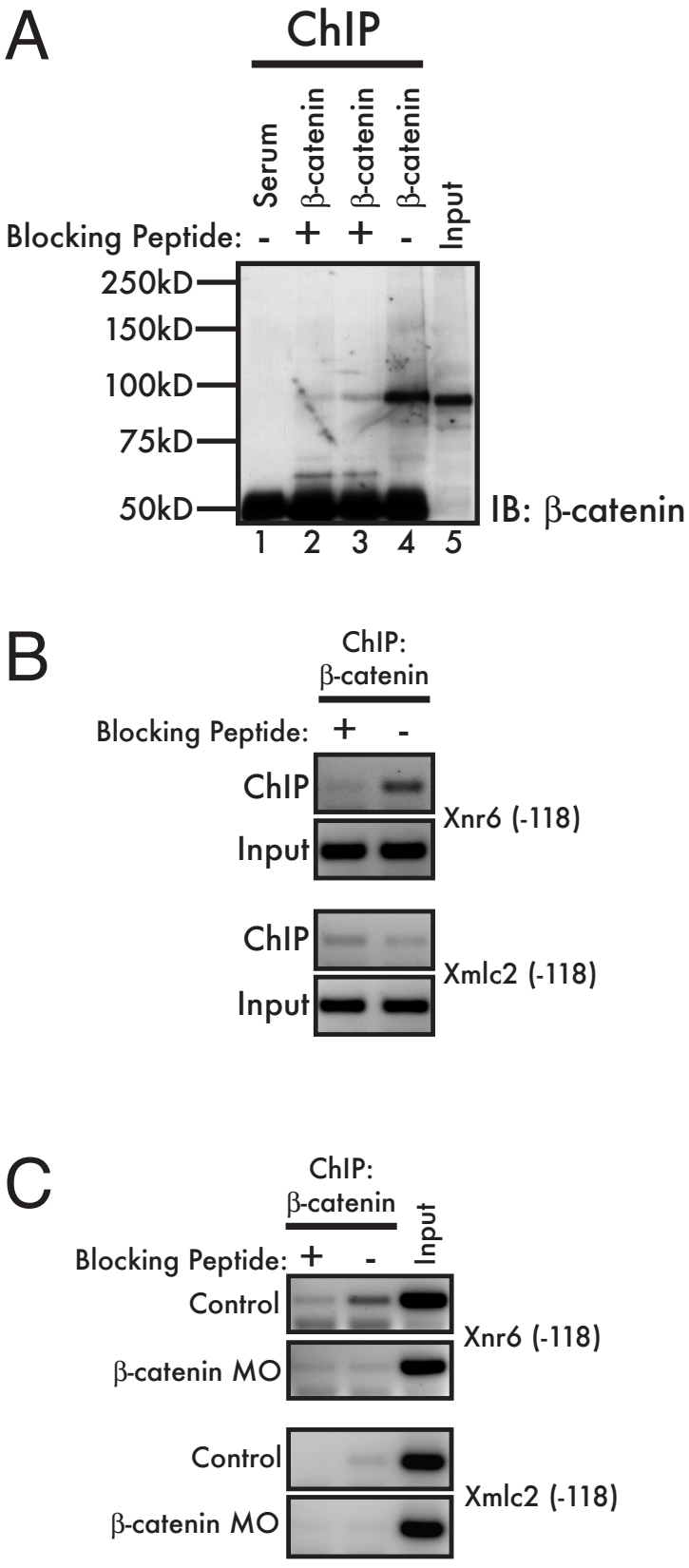
PCR conditions were optimized to ensure detection of immunoprecipitated DNA present in limiting quantities. To maximize detection, we performed nested PCR, using two rounds of 20 cycles each to amplify target sequences from genomic DNA standards. Nested PCR has the twofold advantage of replenishing the polymerase, primers, and dNTPs available for amplification while increasing priming specificity by using a second, internal primer set for the second round of amplification. By this method, we are able to detect PCR products from as little as 100pg of genomic DNA, corresponding to approximately 30 haploid genomes (Figure 2.2B). Radiolabeling the second, inner PCR reaction further increases sensitivity (not shown).

We performed ChIP on blastula stage embryos with anti-AcH3 and anti-β-catenin (Figure 2.2C). All loci tested were associated with AcH3, while only the *Xnr6* (-118) and (-2349) amplicons were bound by β-catenin. Notably, an intermediate amplicon (-1280) was negative for β-catenin binding. Therefore, the fixation and sonication method in this ChIP protocol sufficiently fragments chromatin, and has at least a ~1000bp resolution. We expect that this approach will be useful for scanning intergenic regions for transcription factor binding sites and occupancy of modified histones. Additionally, these results confirm an expected result, namely, that *Xnr6* is a direct target of the Wnt/β-catenin signaling pathway.

*2.2.5: Controls for Antibody Specificity*

Several control experiments confirmed the specificity of our β-catenin antibody under the conditions used for ChIP. To demonstrate antibody specificity, we both confirmed that the antibody could be competed by the immunizing peptide and tested that depletion of β-catenin would reduce the amount of co-immunoprecipitated chromatin. When possible, this latter approach is a powerful method for antibody validation, as it will reveal off-target antibody recognition that could be overlooked by peptide competition alone. First, we optimized conditions for peptide competition of antibody-antigen binding using western blotting of protein recovered from immunoprecipitated chromatin. The β-catenin antibody was raised against the 145 N-terminal amino acids of the *Xenopus laevis* β-catenin protein. We therefore used a 6xHis-tagged peptide corresponding to the immunizing peptide to compete for β-catenin binding in the ChIP assays, either by pre-incubation of the peptide with the antibody prior to the addition of sheared chromatin (Figure 2.3A, lane 2) or by addition of the peptide to the sheared chromatin before the antibody (Figure 2.3A, lane 3). For this experiment, the ChIP protocol was followed through Day 2, step 9, whereupon samples were analyzed by 8% SDS-PAGE followed by western blotting (see Experimental Procedures). Both methods of peptide competition reduced immunoprecipitated β-catenin in the ChIP samples, confirming the specificity of the antiserum. Notably, a 1/5$^{th}$ embryo equivalent was loaded in the input lane, while 2 embryo

FIGURE 2.3


A
ChIP
Serum
β-catenin
β-catenin
β-catenin
Input
Blocking Peptide: - + + - -
250kD
150kD
100kD
75kD
50kD
IB: β-catenin
1 2 3 4 5
B
ChIP:
β-catenin
Blocking Peptide: + -
ChIP
Input
Xnr6 (-118)
ChIP
Input
Xmlc2 (-118)
C
ChIP:
β-catenin
Input
Blocking Peptide: + -
Control
β-catenin MO
Xnr6 (-118)
Control
β-catenin MO
Xmlc2 (-118)

## Figure 2.3: Controls for antibody specificity

(A) The specificity of the β-catenin antiserum was confirmed by performing ChIP on blastula-stage (stage 9) embryos and competing with an excess of immunizing peptide (lane 2: pre-incubation of antibody with peptide; lane 3, addition of peptide directly to sheared chromatin sample). Following IP, samples were processed as described in the text and separated by 8% SDS-PAGE. Immunoprecipitated β-catenin was analyzed by a standard western blot using the β-catenin antiserum. (B) Peptide competition of the β-catenin ChIP and (C) knockdown of β-catenin by morpholino injection (20ng/cell at the 2-cell stage) confirms the specificity of the interaction between β-catenin and the *Xnr6* promoter. The competitions in (B) and (C) were performed by pre-incubation of the peptide with the antiserum for 1 hour before addition to the sheared chromatin sample.

equivalents were loaded in the IP lanes, but similar band intensities for β-catenin are observed by western. This indicates that, following ChIP for β-catenin, as little as 10% of the available antigen is recovered, although these conditions effectively deplete lysates of antigen under native conditions (not shown).

Additionally, we tested whether the immunizing peptide would compete for co-immunoprecipitation of the *Xnr6* genomic locus. Indeed, when the β-catenin antibody is competed with the immunizing peptide, only background levels of *Xnr6* (-118) are co-immunoprecipitated, thus confirming the specificity of this interaction (Figure 2.3B). Likewise, only background levels of signal were detected with a negative control locus, *Myosin Light Chain 2* (*Xmlc2*) (Park et al., 2005). Finally, in Figure 2.3C, we demonstrate that knockdown of β-catenin by microinjection of a morpholino oligonucleotide (Heasman et al., 2000) also results in a loss of β-catenin binding to the *Xnr6* locus, comparable to the reduction seen by peptide competition. This latter result is notable, insofar as the experiment was performed with a single set of microinjected embryos (100 total, plus 100 non-injected controls), demonstrating that this approach is amenable to typical embryological manipulations in common use within the *Xenopus* community. These observations validate the specificity of the ChIP protocol and the observation that β-catenin binds to the *Xnr6* genomic locus in blastula stage embryos.

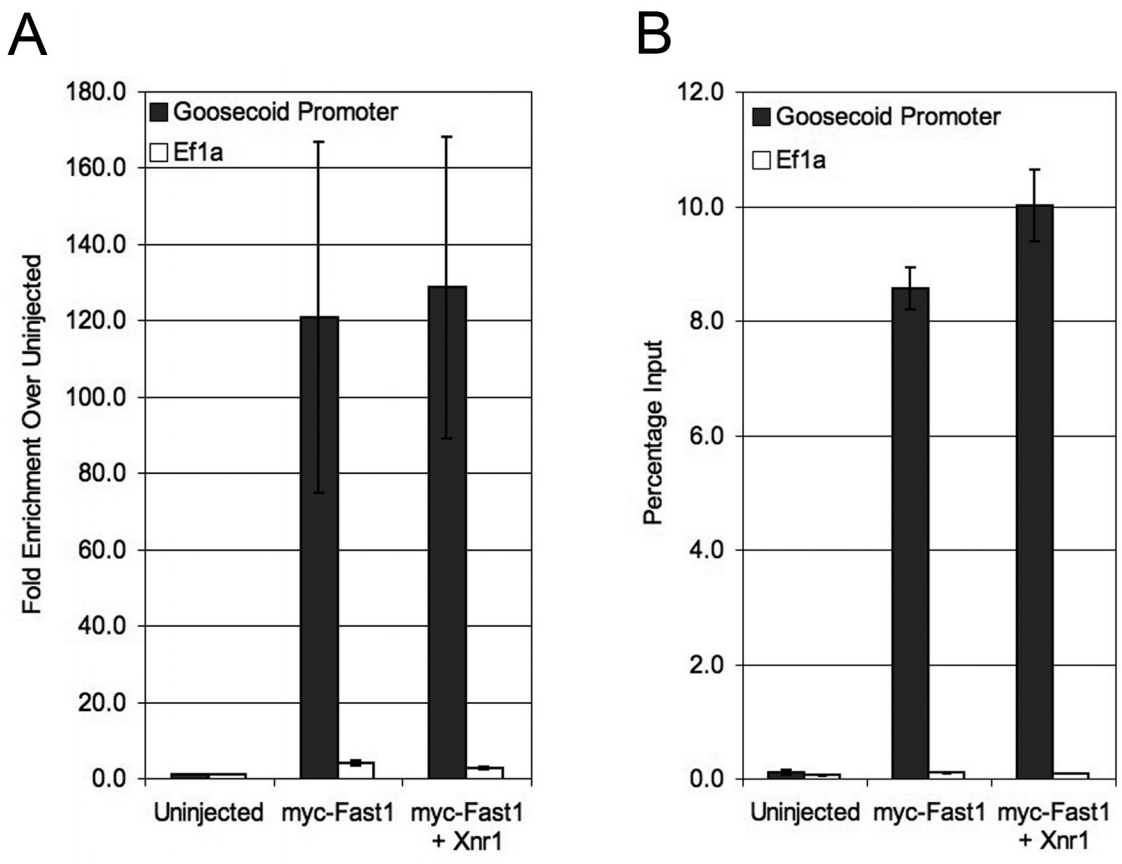
*2.2.6: Quantitative PCR Analysis*

To determine whether ChIP samples produced by this protocol would be amenable to quantitative PCR analysis, we designed an experiment to evaluate the binding of Fast-1 (FoxH1) to the endogenous *Goosecoid* promoter. *Goosecoid* is an organizer gene with a well-defined promoter region responsive to both Wnt and Nodal signals (Watabe et al., 1995). During early embryogenesis, the Nodal pathway signals through the DNA-bound effector Fast-1 (Shen, 2007).

Embryos were injected at the one-cell stage with mRNA encoding myc-tagged Fast-1 (250pg) alone or in combination with Xnr1 mRNA (50pg), a *Xenopus* Nodal-related gene. Embryos were collected at the mid-gastrula stage (stage 10.5) and processed according to the ChIP protocol, using a polyclonal anti-myc antibody to immunoprecipitate myc-Fast-1 containing complexes, followed by QPCR. As a control for non-specific binding of the antibody, uninjected embryo samples were analyzed in parallel. As a negative control, binding of myc-Fast1 to the *Ef1*$\alpha$ coding region, which is not expected to bind Fast-1, was also examined.

As shown in Figure 2.4, myc-Fast1 binds to the endogenous *Goosecoid* promoter, and not to *Ef1*$\alpha$. While the signal from the *Goosecoid* promoter is high, the background from *Ef1*$\alpha$ is low, indicating that the binding of myc-Fast1 to the *Goosecoid* promoter is quite robust, as predicted. Similar results were obtained

Figure 2.4*




*Contributed by Christine Reid and Daniel Kessler

## Figure 2.4: Quantitative PCR analysis of ChIP

Quantitative PCR for the *Goosecoid* promoter (gray bars) and *Ef1*$\alpha$ (white bars) normalized to uninjected embryo control (A) or quantified as a percentage of input DNA (B). Graphs represent average relative quantification for four independent experiments. An average of 45 one-cell embryos were injected with myc-Fast1 (250pg) alone or in combination with Xnr1 (50pg) and harvested at gastrula stage (stage 10.5) for ChIP analysis according to this protocol. QPCR was performed using SYBR green and relative quantification was performed using the $\Delta\Delta$C(t) method. Error bars shown represent standard error.

with injection of as little as 25pg of myc-Fast-1 mRNA (data not shown). From these results we conclude that myc-Fast-1 indeed binds to the endogenous *Goosecoid* promoter and that QPCR provides a sensitive and quantitative method for analyzing ChIP samples obtained using this protocol.

Figure 2.4 presents two approaches to data normalization. Figure 2.4A represents the fold enrichment when experimental samples are normalized to uninjected control samples and Figure 2.4B shows the quantity of each immunoprecipitated target sequence as a percentage of total input DNA. In this experiment, PCR amplification was linear over a range of 5% (the highest amount tested) to 0.01% input material. By comparison to genomic DNA standards, this corresponds to a range between 65 and 0.1ng genomic DNA (data not shown). Thus, in these experiments, single round QPCR is as sensitive as the nested PCR shown in Figure 2.2B. As such, we have not investigated whether QPCR is amenable to a nested PCR approach, but it is conceivable that a limited, initial (conventional) PCR amplification could enhance the sensitivity of a subsequent QPCR analysis.

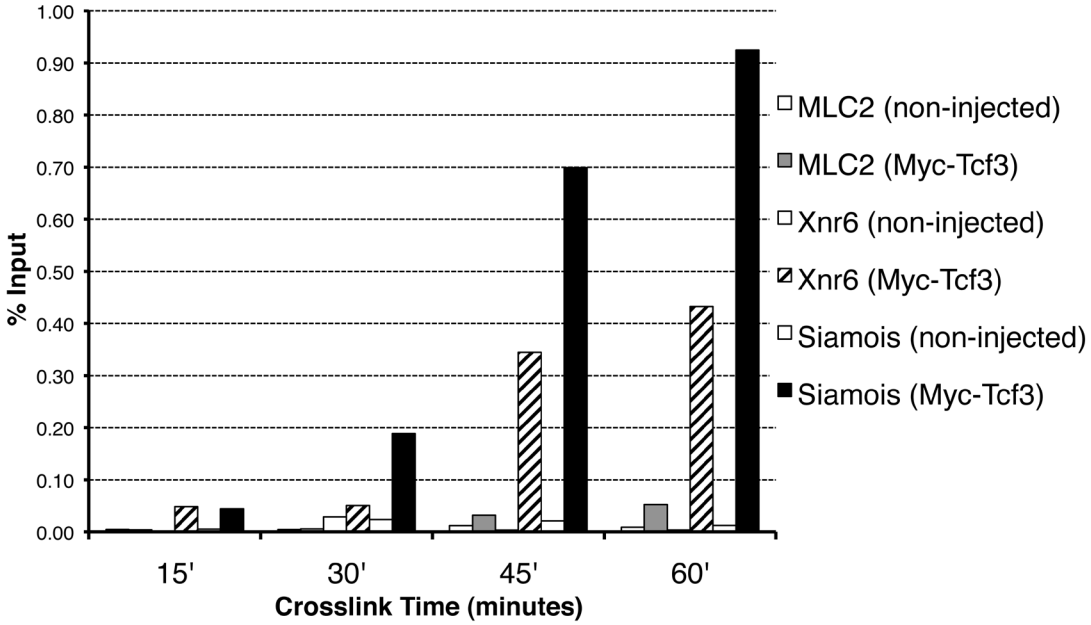
Finally, β-catenin binds to chromatin indirectly through interaction with Tcf/Lef family members, raising the possibility that prolonged crosslinking (60 min) is only required for indirect binding proteins, whereas proteins that bind directly to DNA may be crosslinked more efficiently. We therefore evaluated the effect of crosslinking time on the recovery of Tcf3 at target gene promoters by ChIP. Tcf3 is a member of the Tcf/Lef family of transcription factors that is predicted to bind directly the promoters of β-catenin target genes during early

embryogenesis (Molenaar et al., 1996). Embryos were injected with 25pg Myc-tagged Tcf3 per blastomere at the two-cell stage and fixed at stage 10 for 15, 30, 45, or 60 minutes. Following ChIP, samples were analyzed by QPCR for recovery of promoter sequences for the β-catenin target genes *Siamois* and *Xnr6*. Tcf3 has been shown previously to bind the *Siamois* promoter in gastrula stage embryos, and thus serves as a positive control in this experiment (Park et al., 2005). Recovery of *Xmlc2* was also measured as a negative control for Tcf3 binding. Figure 2.5 demonstrates that recovery of Tcf3-bound promoter sequences is maximal with 60 minutes of crosslinking time, and that crosslinking for 30 minutes or less leads to little or no recovery of target gene promoter sequences. Notably, crosslinking for up to 60 minutes does not result in a large increase of non-specific encrichment for the negative control locus. In addition, a 45-minute crosslinking time is also sufficient to recover bound promoters (albeit less efficiently), with the advantage of slightly less signal from the negative control ChIP. These results underscore the observation in Figure 2.1A that extended crosslinking times are required to perform ChIP for DNA-bound transcription factors in early *Xenopus* embryos.

### *2.2.7: Further considerations*

In addition to the factors described above, several additional considerations should be made when designing a *Xenopus* ChIP experiment. Particularly, since embryos represent a heterogeneous population of cells,

FIGURE 2.5


1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
% Input
15'
30'
45'
60'
Crosslink Time (minutes)
MLC2 (non-injected)
MLC2 (Myc-Tcf3)
Xnr6 (non-injected)
Xnr6 (Myc-Tcf3)
Siamois (non-injected)
Siamois (Myc-Tcf3)

## Figure 2.5: Effect of crosslinking times on ChIP

Anti-Myc-Tag ChIP was performed on sets of 37 gastrula stage (stage 10), non-injected or Myc-Tcf3 injected (50pg) embryos, which were crosslinked for 15, 30, 45, or 60 minutes, as indicated. Samples were analyzed by QPCR for enrichment of *Xmlc2 (-118)*, *Xnr6 (-118)*, and *Siamois (-303)*, represented as % Input recovery. Crosslinking times between 45 and 60 minutes are required for the specific enrichment of the promoters for both *Siamois* (black bars) and *Xnr6* (hatched bars) by Myc-Tcf3 ChIP compared to the *Xmlc2* promoter (gray bars). All non-injected (negative control) ChIPs yielded a comparatively low signal and are represented as white bars. Recovery of input material was similar for each timepoint.

certain protein:DNA interactions may occur in only a small fraction of the experimental sample. This will result in an overall reduction in the number of available binding events for analysis. In cases where a minimal amount of starting material is used, this heterogeneity could lead to “false negative” results. Several approaches are available to circumvent this problem: increasing the amount of starting material, increasing the amount of material in the PCR analysis, and enriching for the subpopulation of cell types with the predicted protein:DNA interaction. *Xenopus* embryos are particularly well suited for this latter approach. For example, using explanted tissues containing the lineage of interest can enrich for particular cell types. Alternatively, molecular techniques for expanding cell lineages can be used to troubleshoot negative results. Finally, in certain cases (and also when a suitable antibody is unavailable), it may be possible to overexpress the DNA binding factor of interest throughout the embryo to increase the number of protein:DNA binding events available for analysis. However, care must be taken in the interpretation of overexpression experiments, particularly due to the difficulty in controlling the spatio-temporal expression of injected mRNAs. In this case, it may be possible to refine further overexpression approaches by driving tissue-specific expression of the DNA-binding factor of interest, either by plasmid DNA injection or transgenesis.

*2.2.8: Concluding Remarks*

We have reported a ChIP assay protocol amenable to early *Xenopus* embryo research, have demonstrated examples of its implementation, and have outlined methods to optimize several aspects of the protocol. We strongly encourage repeating the optimization experiments shown in Figure 2.1 when different conditions or embryo stages are used, as we find this is the most critical aspect of the protocol in our hands. We expect that ChIP will become a useful tool within the *Xenopus* community, allowing for the identification of direct protein-DNA interactions, which, in the past, have been indirectly surmised, usually by plasmid-based reporter gene assays or by the combination of overexpressed, hormone inducible chimeras and cycloheximide treatment. In addition, this technique will allow for many unanswered questions in developmental biology to be addressed, including the systematic analysis of gene regulatory networks and the investigation of the function of chromatin modifications during early vertebrate embryogenesis.

## 2.3: Experimental Procedures

ChIP experiments are designed similarly to common immunoprecipitations (IPs), which include both experimental and control IPs and a sample of the pre-IP (input) material, generally 1%. This protocol has been used successfully with as few as 50 stage 8, 1000-cell embryos ($5x10^4$ cells total) per IP (or an equivalent

amount of explanted tissues from later-stage embryos). Therefore, 100 embryos are required for a basic ChIP if early blastula stage is used. If preliminary experiments demonstrate weak signal strength, the number of embryos can be increased at the investigator's discretion.

ChIP requires high-quality antibodies that will specifically immunoprecipitate the antigen in 0.1% SDS and maintain the interaction throughout several washes of increasing stringency. Because the IP material is also highly crosslinked, certain antigens may be masked to a greater extent than under native conditions. Since this protocol is amenable to the use of microinjected samples, N- or C- terminal tagged proteins of interest can be used if an antibody doesn't perform as expected, or if one is unavailable. We have successfully used both Myc- and HA-tagged proteins in this way, and recommend the use of polyclonal rather than monoclonal antisera, when possible.

### *2.3.1: ChIP Protocol*

This protocol was developed to detect occupancy of transcription factors at target gene promoters during blastula and early gastrula stages. It is divided into five sections, each of which can be completed easily in one day: "Sample Preparation," "Days 1-3," and "PCR." Points that can be shortened to accelerate sample preparation will be noted.

**Sample Preparation:** Embryos are cultured to the desired stage and fixed in formaldehyde. This will crosslink proteins to one another and to DNA (Orlando et al., 1997). Fixation is quenched by washing with glycine, yielding a sample ready for ChIP.

**Materials:**

-Standard *Xenopus* embryo culture and microinjection materials (Sive et al., 2000)

-Phosphate Buffered Saline (PBS): (Per liter) 8g NaCl, 0.2g KCl, 1.44g $Na_2HPO_4$, 0.24g $KH_2PO_4$, pH 7.4 (Maniatis et al., 1982).

-Formaldehyde (37% stock, Molecular Biology Grade, Thermo Fisher BP531); 1% Formaldehyde in PBS working solution: 676μl formaldehyde per 25ml PBS

-0.125M Glycine in PBS: Mix 235mg Glycine (Sigma G7125) per 25ml PBS

-1.5ml microcentrifuge tubes (Eppendorf 02236411)

**Time:** Following culture to the desired stage, up to 1 hour to crosslink, and 20 minutes to quench, wash, and distribute samples to tubes.

**Procedure:** At the desired stage, embryos are fixed/crosslinked in 1% formaldehyde/ PBS for up to 1 hour (see Results and Figure 2.1a). Agitation is not necessary. Crosslinking is stopped by a 10-minute wash in 0.125M Glycine/

PBS, followed by three washes in PBS. Fixed embryos are transferred to microcentrifuge tubes (50 per tube), excess PBS is removed, and they may be either frozen at -80°C for at least 3 months or processed for ChIP immediately (Day 1 protocol).

**Day 1:** Samples are homogenized and crosslinked chromatin is sheared to <1000bp fragments by sonication. Samples are then pre-cleared and the primary antibody incubation is performed overnight.

**Materials:**

-RIPA buffer (4°C, 1.25ml per set of 50 embryos): 50mM Tris-HCl, pH 7.4, 1% Igepal CA-630 (NP-40) (Sigma I3021), 0.25% Na-Deoxycholate, 150mM NaCl, 1mM EDTA, 0.1% SDS, 0.5mM DTT, 5mM Na-Butyrate, Protease Inhibitor Cocktail (Sigma P8340), Phosphatase Inhibitor Cocktail I (Sigma P2850), Phosphatase Inhibitor Cocktail II (Sigma P5726).

-20ml 5% Bovine Serum Albumin (BSA fraction V, Sigma A9647) in PBS (see above)

-15ml conical tubes with caps (Thermo Fisher 14-959-70C)

-Recombinant Protein G-Agarose (100μl per set of 50 embryos) (Invitrogen 15920-010)

-Kontes Pellet Pestles (Thermo Fisher K749521-1590)

-Cold centrifuge capable of 14,000g

-Kimwipes (Thermo Fisher 06-666)

-Nutator or end-over-end rotator (at 4°C) to mix samples

-Sonicator (Branson Sonifier 250 or equivalent, with a microtip horn)

-Safe-Lock 1.5ml microcentrifuge tubes (Eppendorf 2260002-8)

-TES Buffer: 50mM Tris-HCl pH 8.0, 10mM EDTA, 1% SDS (store at room temperature)

-Control and Experimental Antibodies

**Time:** 1.5 hours for homogenization and shearing, 1 hour preclear, overnight primary incubation

**Procedure:**

1) Thaw crosslinked embryos for 15 minutes on ice.
2) Prepare blocked protein-G agarose while the embryos are thawing. Dispense enough protein-G agarose for each IP (plus an extra 50μl) into each of two 15ml conical tubes. Mark the volume's height on the side of the tube with a marker. Add 10ml 5%BSA/ PBS to each tube and incubate at 4°C with mixing for at least 1 hour prior to use.
3) Add 600ul 4°C RIPA buffer to the fixed embryos.
4) Homogenize the fixed embryos with a pellet pestle: gently disrupt the embryo pellet, and then vigorously homogenize each sample for 30 seconds. Proceed through all the samples, using different pestles for each set of embryos, and then repeat the homogenization once again. The

sample will become a uniform gray color with a fine particulate consistency. No large embryo fragments should remain.

5) Incubate embryos on ice for at least 10 minutes beginning from the time of initial homogenization.
6) Centrifuge at 14,000g for 10 minutes at 4°C.
7) Discard the supernatant and wipe the wall of the tube above the pellet with a kimwipe to remove lipid residue.
8) Add 650μl 4°C RIPA to the pellet and re-homogenize vigorously, ensuring that no coarse fragments of pellet remain.
9) Sonicate sample (see Results and Discussion).
10) Centrifuge for 10 minutes at 4°C at 14,000g.
11) Transfer the supernatant (600μl recovery is typical) into pre-chilled, clean 1.5ml microcentrifuge tubes. The supernatant contains the sheared chromatin and should appear medium-yellow without any debris. The pellet should be compact, and will have a top layer of dark pigment, with a bottom yellowish, yolky layer.
12) To prepare Input Samples: In a safe-lock tube, combine 195μl TES buffer and 5μl sheared chromatin. These samples will be processed once the IPs are complete. Freeze at -80°C.
13) Centrifuge one of the 15ml conicals containing the blocked protein-G agarose at 1000g for 5 minutes. Remove excess 5%BSA/ PBS to the level of the original bead volume (marked on the side of the tube).
14) Gently resuspend the blocked beads by low speed vortexing.

15) Pre-clearing: Dispense 50μl blocked beads to each sample of sheared chromatin, mixing the beads by pipetting before removing them from the 15ml conical to ensure equal distribution between samples. Mixing by vortexing each time is not recommended.
16) Incubate the samples at 4°C with mixing for 1 hour.
17) Centrifuge at 1000g for 1 minute at 4°C.
18) Transfer 580μl pre-cleared, sheared chromatin to a pre-chilled 1.5ml tube. Avoid transferring any beads.
19) Begin the immunoprecipitation: Add the appropriate amount of antibody or control serum to each sample. Amounts will need to be determined empirically. In the examples here, 1μg affinity purified IgG, or 5μl whole serum, per sample was optimal.
20) Incubate samples overnight at 4°C with mixing. (To shorten sample preparation time, the antibody incubation could be reduced to a minimum of 1 hour, followed by continuation with the Day 2 Protocol. Appropriate minimum conditions will have to be determined empirically.)

**Day 2:** Immunocomplexes are precipitated, washed extensively, and eluted. Crosslinks are reversed and proteins are digested.

**Materials:**

-Blocked protein-G agarose (see above)

-Wash Buffer I: 20mM Tris-HCl pH 8.0, 0.1% SDS, 1% Triton X-100, 2mM EDTA, 150mM NaCl

-Wash Buffer II: (Wash Buffer I, with 500mM NaCl)

-Wash Buffer III: 10mM Tris pH 8.0, 0.25M LiCl, 1% Igepal CA-630 (NP-40), 1% Na-Deoxycholate, 1mM EDTA

-Wash Buffer IV: (TE buffer) 10mM Tris-HCl pH 8.0, 1mM EDTA

-1ml syringe fitted to a vacuum aspirator

-20-gauge syringe needles

-26-gauge syringe needles

-TES Buffer (see above)

-Safe-lock tubes (see above)

-Hybridization oven or water bath set at 65°C

-Proteinase K/ Glycogen Solution: 15mg/ml Proteinase K, 6mg/ml GlycoBlue (Ambion AM9515), PBS, 30% Glycerol

**Time:** 1 hour for immune complex precipitation, 2 hours for the washes and elution, followed by an overnight crosslink reversal/ proteinase K digestion.

**Procedure:**

1) Centrifuge samples at 14,000g for 5 minutes at 4°C and transfer supernatants (560μl) to pre-chilled 1.5ml microcentrifuge tubes. This will remove insoluble precipitates that may have formed during the overnight incubation.

2) Repeat steps 13-17 of the Day 1 procedure with the second 15ml conical tube of pre-blocked protein-G agarose.
3) To Wash: Each wash consists of a 1-minute spin at 1000g in a 4°C centrifuge to pellet the immunocomplexes, removal of supernatant via aspiration with a 20-gauge needle, addition of 1ml wash buffer (see below), and incubation at 4°C with mixing for 5 minutes.
    a. Wash for a total of 8 times, using 2 washes each with buffers I through IV.
    b. If high background is observed, it may help to do 3 washes with each of the buffers, or to increase the wash time to 10 minutes.
4) Following the washes, aspirate the supernatant with a 26-gauge needle, inserting it into the beads at the end to completely remove any residual wash buffer. If necessary, perform a quick additional spin and repeat aspiration to ensure all of the supernatant has been removed.
5) Add 220μl TES buffer to the beads and vortex vigorously for 5 seconds.
6) Elution: Incubate the samples at 65°C for 10 minutes, vortexing each sample vigorously for 5 seconds every 2 minutes.
7) During this time, the frozen input samples should be thawed and vortexed to resuspend any precipitated SDS. IP and input samples are processed in the same manner for the rest of the procedure.
8) Centrifuge the samples at 14,000g at room temperature for 1 minute.
9) Transfer 200ul of the eluted IP supernatant to a safe-lock tube. Do not transfer any beads.

10) Add 7μl Proteinase K/ Glycogen solution to each sample.

11) Incubate the samples at 65°C overnight to reverse crosslinks and digest proteins. (Other protocols suggest that a 4-hour incubation is sufficient to reverse crosslinks and digest. One could try this to shorten this step.)

**Day 3:** DNA is purified from the samples, RNase-treated, and finally re-purified through a spin-column.

**Materials:**

-Phenol/Chloroform/Isoamyl Alcohol (Thermo Fisher BP1752)

-Sterile MilliQ (or equivalent) $H_2O$

-TE-Saturated Chloroform (Mix equal parts TE buffer (10mM Tris-HCl pH8.0, 1mM EDTA) and Chloroform (Thermo Fisher BP1145) and allow the phases to separate. Use the lower, organic phase)

-5M NaCl

-100%, 70% Ethanol

-RNase A/ TE buffer (Dilute RNase A (100mg/ml stock solution, Roche 10109169001) to 2mg/ml in TE buffer)

-Qiagen PCR Purification Kit (Qiagen 28104)

**Time:** 1.5 hours for phenol/chloroform extraction and ethanol precipitation, 1 hour for RNase treatment, and 15 minutes for spin-column purification.

**Procedure:**

1) Add 200μl phenol/chloroform/isoamyl alcohol to each sample, vortex vigorously, and centrifuge at 14,000g for 1 minute at room temperature.
2) Transfer 200μl of the upper, aqueous phase to a clean 1.5ml microcentrifuge tube.
3) Add 200μl sterile milliQ water to the lower, organic phase. Vortex the sample vigorously and centrifuge at 14,000g for 1 minute at room temperature.
4) Add 200μl of the second aqueous phase to the first aqueous phase.
5) Add 350μl TE-saturated chloroform to each sample, vortex vigorously, shake, and centrifuge at 14,000g for 1 minute at room temperature.
6) Transfer 350μl of the aqueous phase to a clean 1.5ml tube.
7) Add 14μl 5M NaCl to each sample. Vortex to mix and do a quick spin.
8) Add 920μl room temperature, 100% ethanol to each sample.
9) Mix well by inversion and incubate the samples at –80°C for 30 minutes.
10) Centrifuge at 14,000g for 15 minutes at 4°C. A blue pellet should form at the bottom of the tube. If not, vortex the tube and repeat centrifugation.
11) Discard the supernatant.
12) Wash samples by adding 1ml 70% ethanol, mixing by inversion, and centrifuge at 14,000g for 5 minutes at 4°C.
13) Discard the supernatant.
14) Resuspend pellets in 100μl RNase A / TE.
15) Incubate samples at 37°C for 1 hour.

16) Add 500μl Qiagen Buffer PB to each sample.

17) Mix well and briefly spin down.

18) Transfer each 600μl sample to a Qiagen PCR Purification Kit column.

19) Follow the kit protocol for PCR Purification, eluting in 50μl buffer EB.

20) The eluate from the Qiagen PCR Purification kit is now ready for analysis by PCR.

*2.3.2: PCR*

**PCR Primers:** For nested PCR, primer sequences were designed with MacVector 9.0, with default settings except that the primer melting temperature was reduced from 55°C to 50°C. Amplicon length is ideally between 100-180bp. Primers are defined as "nested" if the inner primer pairs have 5 or more unique 3' nucleotides. The numbering of the primer sets represents the midpoint of the amplicon, relative to the predicted translational start site (ATG). The inner *Xmlc2* primer was previously reported (Park et al., 2005). *Xnr6 (-118):* Outer/Forward 5'- TCT GAG GTG TGA GGT ATA TGA AAG G -3'; Outer/Reverse 5'- TGG GGC TCT TGA AAA CTG AAA TG -3'; Inner/Forward 5'- GGT AGA TGA AAG GCT GAC AGG TGT G -3'; Inner/Reverse 5'- GGC TGT TGA AAA CTG AAA TGA AGC -3'. *Xnr6 (-1280):* Outer/Forward 5'- AAA AGG AGT CTA TGA GAA GTG GC -3'; Outer/Reverse 5'- TGA GAA TAC AGT AAG GAG GGG C -3'; Inner/Forward 5'- GGA TAA TGG GTT TCT GGA TAA CTG -3'; Inner/Reverse 5'- GGT GAT GCT AAA GGT GAG ATG G -3'. *Xnr6 (-2349):* Outer/Forward 5'-

ACA CCC CCT GCT CCC CCG -3'; Outer/Reverse 5'- GCA AAA CAA TCC CAC CCA G -3'; Inner/Forward 5'- GGT ACT TCC GCC ACT GAA AG -3'; Inner/Reverse 5'- AGA CCC CTA TCC AGA AAA TCT C -3'. *Xnr1 (-221):* Outer/Forward 5'- TCT GAG GTG TGA GGT ATA TGA AAG G -3'; Outer/Reverse 5'- TGG GGC TCT TGA AAA CTG AAA TG -3'; Inner/Forward 5'- GGT AGA TGA AAG GCT GAC AGG TGT G -3'; Inner/Reverse 5'- GGC TGT TGA AAA CTG AAA TGA AGC -3'. *Xmlc2 (-118):* Outer/Forward 5'- TGG GAT ATT TTA CTG AAC ACA ATG -3'; Outer/Reverse 5'- CGT CCT GTG CCA CCT AAT G -3'; Inner/Forward 5'- GAA TGT TAG CCC TTG TGC TCT T -3'; Inner/Reverse 5'- GGA AAG TTC TCT TGA TCA TTT TA -3'.

For QPCR, primers were 18 to 30 nucleotides long with an approximate melting temperature of 60°C. Amplicon length was between 80-120bp. The following QPCR primer sets were used. *Goosecoid (-224):* Forward 5'- AAT GAC AGC CAA CAG CTC AGA GGA CA -3'; Reverse 5'- TCG CAG ACT CTC CCT GTA GTT ATT CAC A -3'. *EF1$\alpha$ (-335):* Forward 5'- GTC TCG GCC CCT AAA TAT GA -3'; Reverse 5'- CAG CTC CCA GCT CTT TTG TC -3'. *Siamois (-303):* Forward 5'- GGG ACT TTG AAG TCT TGC CA -3'; Reverse 5'- TCT GAT GAC ACG TGT TTC CC -3'. For QPCR of *Xnr6 (-118)* and *Xmlc2 (-118)*, the "outer" primer sets were used (see above).

**Nested PCR:** ChIP DNA is subjected to two sequential PCR reactions of 20 cycles each using Promega GoTaq Flexi DNA Polymerase (M8295). For the first, "outer," PCR reaction, the final conditions are: 25$\mu$l total volume, containing 2$\mu$l

ChIP DNA sample, 1X Clear Reaction Buffer, 1.5mM $MgCl_2$, 0.1mM dNTP (each), 0.63U GoTaq Polymerase, and 4ng/μl each "outer" primer.

PCR was performed in a MJ Research Tetrad thermal cycler. Cycling parameters are as follows: 95°C for 3 minutes, followed by 20 cycles of [95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 60 seconds], ending with 72°C for 10 minutes. The low annealing temperature is to accommodate the low C/G content of *Xenopus laevis* intergenic DNA.

The second, "inner" PCR reactions are identical to the "outer" PCR reactions except the template is 2μl of the complete "outer" PCR reaction, and the "inner" primer sets are used instead of the "outer" ones. In addition, the green PCR reaction buffer that comes with the polymerase can be used instead of the clear one, since it doubles as a loading buffer for electrophoresis. PCR products (15-20μl) are visualized by 3% agarose electrophoresis in 1x TAE with ethidium bromide. To facilitate handling of high-percentage agarose, a 1 to 3, low-melt to normal agarose mix is used.

Radiolabeling the "inner" PCR reaction can increase the limit of detection. To radiolabel, add 0.1μl $\alpha^{32}$P-dCTP (3pmol at 3000Ci/mmol) to each "inner" PCR reaction. PCR products (10μl) are then resolved by 6% polyacrylamide electrophoresis in 0.5x TBE and visualized with a Phosphorimager.

It may be useful to include genomic DNA standards to assess the efficiency of PCR amplification. For the experiment in Figure 2.2B, genomic DNA was isolated from adult male liver (~0.5mg fragment) similarly to the ChIP DNA purification, except samples were ethanol precipitated following RNase treatment

and resuspended in 200μl TE buffer. The DNA concentration was determined by absorbance at 260nm. The estimated mass of a karyotypically "haploid" *Xenopus laevis* genome (3.175pg) (Green and Sessions, 1991; Wickbom, 1945) was used to calculate approximate copy number. The possible amplification by PCR of duplicated genomic loci was not considered in this estimate.

**Quantitative PCR and Analysis:** The QPCR reaction conditions are: 20μl total volume with 1X SYBR green master mix (Applied Biosystems, 4367659), 1μl ChIP DNA sample, and 5ng/μl each primer. If the QPCR yield is low, the amount of ChIP DNA per reaction can be increased. Amplification was performed with an Applied Biosystems Step One Plus machine, using the standard SYBR green program with an initial melt stage at 95°C for 10 minutes, followed by at least 40 cycles of 95°C for 15 seconds and 60°C for one minute. The run is finished by a melt curve from 95°C to 60°C to ensure PCR product purity.

Data are analyzed by the ΔΔC(t) (or Livak) method (reviewed in (Taneyhill and Adams, 2008)). Once a threshold cycle (C(t)) number is calculated for each sample, ΔC(t) is calculated by normalizing the IP values to the input values for each condition by subtracting input values from each corresponding ChIP value [ΔC(t) = ChIP C(t) – Input C(t)]. ΔΔC(t) is next calculated by subtracting the ΔC(t) for uninjected samples from the ΔC(t) for experimental samples [ΔΔC(t) = $\Delta C(t)_{experimental} - \Delta C(t)_{uninjected}$]. Once ΔΔC(t) is determined, the fold change between samples can be determined by using the formula [Fold Change (FC) =

$2^{[-\Delta\Delta C(t)]}$] (Taneyhill and Adams, 2008). To calculate the quantity of IP-ed DNA as a percentage of the original input material, the following formula is used: [% Input = $2^{[-\Delta C(t)]}$ x initial percentage input] (Frank et al., 2001; Taneyhill and Adams, 2008). Once several experiments have been analyzed, data can be combined to calculate standard error and be subjected to further statistical analysis

*2.3.3: Cloning of additional Xnr6a genomic sequence*

Additional genomic sequence for the *Xnr6a* locus was obtained by screening a *Xenopus laevis* genomic library (kind gift of Dr. Steven Klein) with a probe corresponding to the 5' end of the available *Xnr6* sequence (Genbank AY050648, bases 292-712). A 15kb clone containing the *Xnr6a* genomic locus was isolated, sequenced, and deposited in Genbank (accession number: FJ468558).

*2.3.4: Western Blotting*

Following elution of the immunoprecipitates (Day 2, step 9) a portion of the eluted material can be analyzed by western blot to determine IP efficiency. Western blotting is performed according to standard protocols, except that samples are mixed 1:1 with standard 2x Laemmli Sample Buffer (4% SDS, 20% Glycerol, 0.125M Tris-HCl pH 6.8, 0.004% Bromophenol Blue, 0.2M Dithiothreitol) and

heated to 95°C for 30 minutes to reverse the crosslinks. A portion of the input material can also be included as a control.

*2.3.5: Antibodies*

The rabbit anti-β-catenin antiserum was raised against the 145 N-terminal amino acids of *Xenopus* β-catenin (Cocalico Laboratories; Reamstown, PA). Anti acetylated H3 (di-acetylated H3K9/K14, (06-599)) and polyclonal anti-myc (06-549) were purchased from Millipore. Purified normal rabbit IgG was purchased from Pierce (31235).

# Chapter 3: β-catenin Primes Organizer Gene Expression By Recruiting a Histone H3 Arginine 8 Methyltransferase, Prmt2

## 3.1: Introduction

The earliest events in embryonic development are controlled by maternal factors until the activation of the zygotic genome. In *Xenopus, Drosophila,* and Zebrafish, zygotic genome activation occurs several hours and cell divisions following fertilization, at the midblastula transition (MBT) (Edgar et al., 1986; Kane and Kimmel, 1993; Newport and Kirschner, 1982a). Before the MBT, the zygotic genome is largely repressed, stemming in part from competition between chromatin deposition and transcription complex assembly at promoters (Almouzni and Wolffe, 1995; Prioleau et al., 1995; Prioleau et al., 1994). Nevertheless, essential steps in embryonic patterning are accomplished during this time and embryos emerge from this period having begun the process of regional specification.

The Wnt/β-catenin pathway mediates the earliest cell fate decision in amphibian embryogenesis, the establishment of the dorso-ventral axis. During the first cell cycle, a displacement of cortical cytoplasm asymmetrically activates Wnt signaling in the future dorsal half of the embryo (Miller et al., 1999; Tao et al., 2005). This stabilizes β-catenin, resulting in its accumulation and nuclear translocation. In the nucleus, β-catenin localizes to target genes by interacting with Tcf/Lef DNA-binding proteins (Molenaar et al., 1996) and activates transcription by recruiting transcriptional coactivators to these sites (Barker et al.,

2001; Hecht et al., 2000; Koh et al., 2002; Major et al., 2008; Mosimann et al., 2006; Sierra et al., 2006). β-catenin is required during pre-MBT stages for the later expression of dorsal determinants that give rise to the Spemann organizer (Heasman et al., 2000). Thus, dorsal specification by β-catenin occurs under conditions of global transcriptional repression.

Several observations suggest that β-catenin primes organizer gene promoters for activation well in advance of the large-scale initiation of zygotic gene expression such that they reach the MBT poised for immediate expression. Activation of β-catenin before the MBT initiates a dorsal gene expression program, whereas activation at the MBT or beyond results in ventrolateral gene expression, reflecting the different roles of the maternal (pre-MBT) and zygotic (post-MBT) Wnt pathways (Christian and Moon, 1993; Kao et al., 1986; Yamaguchi and Shinagawa, 1989). During the pre-MBT period, β-catenin is asymmetrically localized in dorsal progenitors prior to the 64-cell stage (Kofron et al., 2007; Larabell et al., 1997). Furthermore, dorsal specification by β-catenin is complete by the end of the 32-cell stage of development, six cell divisions before the MBT, as β-catenin activity must be inhibited before the 32-cell stage in order to block dorsal specification (Heasman et al., 2000; Yang et al., 2002b). Interestingly, transient inhibition of RNA Polymerase II (Pol II) activity between the 4- and 32-cell stages delays dorsal specification by β-catenin, further suggesting a requirement for Pol II function in dorsal specification before the MBT. In support of this observation, the β-catenin target genes *Xnr5* and *Xnr6* are transcribed before the MBT, shortly after the completion of dorsal

specification (Yang et al., 2002b). However, not all maternal β-catenin target genes are transcribed early, including the homeobox transcription factors *siamois* and *twin*, and *nodal related 3* (*xnr3*) (Yang et al., 2002b). These observations raise the possibility that β-catenin functions early to establish a heritable, transcriptionally poised state that results in the later expression of dorsal determinants such as *siamois* and *xnr3*.

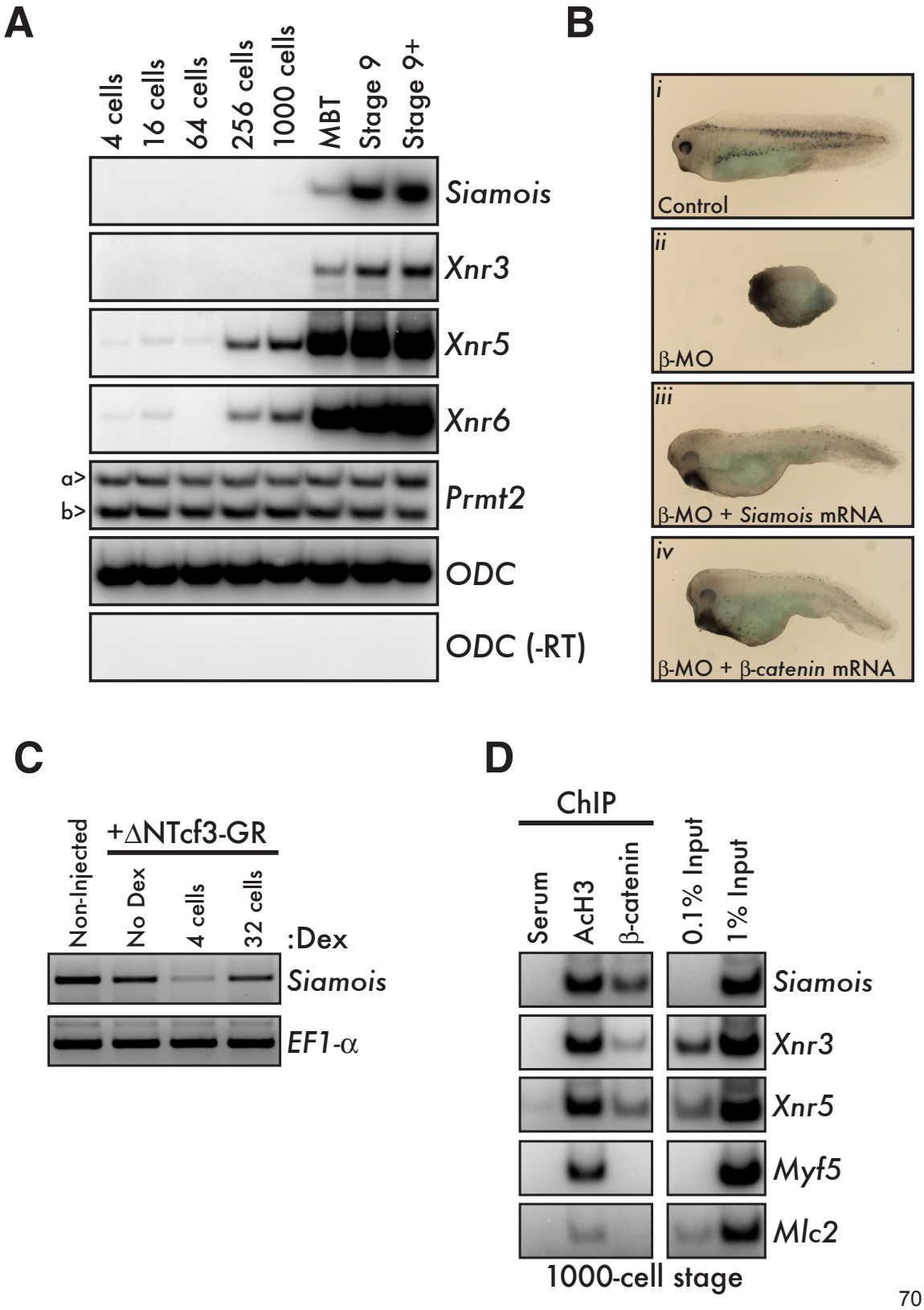
We have investigated the chromatin architecture of β-catenin target genes before the MBT, and report that β-catenin primes target promoters for activation at the onset of zygotic gene expression. Before the MBT, β-catenin target promoters associate with C-terminal domain phosphorylated Pol II and histone modifications that correlate with an active transcriptional state, such as histone H3 acetylated at lysine 9 and 14 (AcH3), and trimethylated at lysine 4 (H3K4me3), independently of their level of mRNA expression. Deposition of H3K4me3, in particular, requires both pre-MBT β-catenin and Pol II function. In these early embryos, β-catenin interacts with a methyltransferase activity directed at Histone H3 arginine 8, which we ascribe to the arginine methyltransferase Prmt2. β-catenin recruits Prmt2 to target promoters before the MBT and this is necessary and sufficient to drive dorsal specification. We therefore provide the first direct evidence for a complex pre-transcriptional mechanism at work in early embryos to pre-set patterns of gene expression, and provide an initial analysis of chromatin architecture during this critical period of development.

## 3.2: Results

The maternal Wnt/β-catenin pathway in *Xenopus* (and zebrafish) specifies dorsal cell fates before the MBT, under conditions of global transcriptional repression. Two classes of dorsal genes are expressed in response to maternal β-catenin (Yang et al., 2002b). For the first class of genes, exemplified by *siamois* and *xnr3*, mRNA expression is first detected at the MBT (Figure 3.1A). The second class of genes, exemplified by *xnr5* and *xnr6*, is transcribed as early as the 256-cell stage, bypassing pre-MBT global transcriptional repression (Figure 3.1A).

Maternal β-catenin is required for dorsal specification and to activate dorsally restricted genes expressed both before and after the MBT, but it is not yet clear which of these targets are required for dorsal development. *Siamois* was originally identified by its ability to induce complete secondary axes and to rescue dorsal development in ventralized embryos (Lemaire et al., 1995), whereas combined loss of *siamois* and the closely related gene *twin* blocks dorsal development (Ishibashi et al., 2008), indicating that *siamois* and *twin* play essential and instructive roles in the induction of dorsal fate. However, these experiments were performed in the presence of maternal β-catenin, leaving open the possibility that dorsal development depends on additional targets of maternal Wnt/β-catenin signaling. If the primary function of pre-MBT β-catenin activity is to induce *siamois (*and *twin)* expression, then *siamois* over-expression should be

Figure 3.1


A
4 cells
16 cells
64 cells
256 cells
1000 cells
MBT
Stage 9
Stage 9+
Siamois
Xnr3
Xnr5
Xnr6
a>
b>
Prmt2
ODC
ODC (-RT)
B
i
Control
ii
β-MO
iii
β-MO + Siamois mRNA
iv
β-MO + β-catenin mRNA
C
Non-Injected
+ΔNTcf3-GR
No Dex
4 cells
32 cells
:Dex
Siamois
EF1-α
D
ChIP
Serum
AcH3
β-catenin
0.1% Input
1% Input
Siamois
Xnr3
Xnr5
Myf5
Mlc2
1000-cell stage

## Figure 3.1: β-catenin regulation of Wnt target genes before the MBT.

(A) An RT-PCR time course of early embryonic stages demonstrates the onset of expression for maternal β-catenin target genes (*Siamois*, *Xnr3*, *Xnr5*, and *Xnr6*). Maternally expressed *Ornithine decarboxylase* (*Odc*) and *Prmt2* are shown as controls for loading. *Odc* (-RT) indicates no reverse transcriptase, as a control for genomic DNA contamination.

(B) Siamois expression fully rescues β-catenin depleted embryos. Embryos (N=30) were injected with the β-catenin morpholino (ii) and either 2pg *siamois* (iii) or 300pg *β-catenin* (iv) mRNAs. Control (i) and injected tadpoles were scored for development of dorsal tissues. Rescue was observed in >95% of mRNA-injected embryos.

(C) Wnt/β-catenin activity must be inhibited before the 32-cell stage (stage 6) to prevent *Siamois* mRNA expression after the MBT. Embryos were injected into two dorsal blastomeres at the 4-cell stage with 500pg ΔNTcf3-GR mRNA. Wnt/β-catenin activity was inhibited by addition of dexamethasone (Dex) to the culture medium at the indicated stages. *Siamois* mRNA expression was measured by RT-PCR at stage 10 and compared to the loading control *EF1α*.

(D) β-catenin binds organizer gene promoters before the MBT. Pre-MBT occupancy of maternal β-catenin target promoters (*Siamois, Xnr3, and Xnr5*), or a zygotic β-catenin target promoter (*Myf5*) by either acetylated histone H3 (AcH3) or β-catenin was observed by ChIP on 1000-cell stage embryos. *Myosin light chain 2* (*Mlc2*): negative control locus. “Input” indicates chromatin prior to ChIP.

sufficient to rescue dorsal development in β-catenin-depleted embryos. To test this, β-catenin was depleted by injection of a morpholino oligonucleotide (MO), which completely blocked dorsal development (Figure 3.1B, panel *ii*), as described previously (Heasman et al., 2000); expression of *siamois* in these embryos rescued dorsal developmental to the same extent as β-catenin itself (Figure 3.1B, panels *iii* and *iv*). Therefore, establishment of zygotic *siamois* expression represents the essential patterning event driven by the maternal Wnt/β-catenin pathway.

It is paradoxical, then, to consider how a transcription factor such as β-catenin functions to pattern the embryonic axes during a period of global transcriptional repression. One possibility is that, while β-catenin is present throughout cleavage stages, it only activates target genes such as *siamois* and *xnr3* at the MBT. This seems unlikely, as we have shown previously that dorsal specification by β-catenin is complete by the end of the 32-cell stage (Yang et al., 2002b). However, those experiments did not directly address the requirement for early β-catenin function in the induction of *siamois* transcription at the MBT. We have therefore tested the sensitivity of *siamois* expression to inhibition of β-catenin activity before the MBT. Using a dexamethasone-inducible dominant-negative TCF3 (ΔNTcf3-GR), we find that, while inhibition of β-catenin at the 4-cell stage significantly reduces *siamois* expression after the MBT, inhibition at the 32-cell stage has little or no effect (Figure 3.1C), suggesting that the 32-cell

stage embryo has already received a signal from β-catenin to activate the expression of *siamois* after the MBT.

### *3.2.1: β-catenin establishes poised chromatin architecture at target promoters before the midblastula transition.*

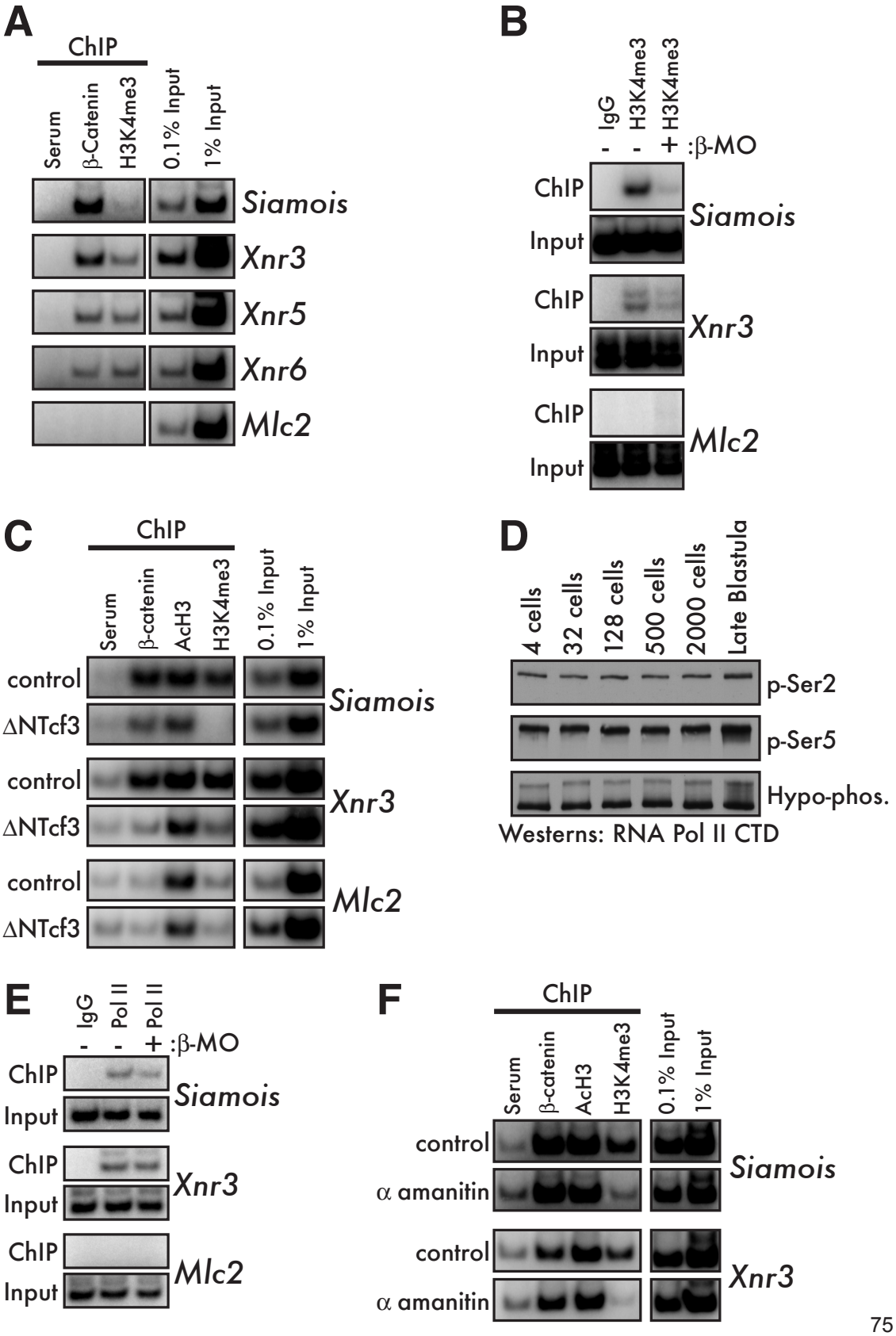
We therefore hypothesized that β-catenin binds to target promoters during this early time and establishes a heritable "mark" that is later interpreted at the MBT as a signal to transcribe the associated loci. To test this, we performed chromatin immunoprecipitation (ChIP) assays (Blythe et al., 2009) for β-catenin in pre-MBT embryos (Figure 3.1D). As expected, β-catenin binds promoters of target genes that are transcribed before the MBT. More importantly, β-catenin also binds—before the MBT—to promoters of late-responding maternal Wnt target genes, despite a lack of mRNA accumulation for *siamois* and *xnr3*. In contrast, before the MBT, β-catenin does not bind a cluster of Tcf/Lef binding sites flanking the zygotic Wnt target gene *myf5* (Yang et al., 2002a), although it binds these sites after the MBT (Figure 3.1D and data not shown). Interestingly, the promoters of all genes tested—maternal and zygotic β-catenin target genes and a negative control locus, *myosin light chain 2* (*mlc2*)—associate with acetylated H3 (AcH3) before the MBT. This latter observation was unexpected and suggests that, before the MBT, the chromatin surrounding inactive promoters nevertheless bears typical euchromatic landmarks. Most importantly, despite conditions of global transcriptional repression and the rapid cleavage divisions of

the early embryo, β-catenin has access to and binds the promoters of genes critical for dorsal specification.

In addition to AcH3 (Figure 3.1D) and AcH4 (data not shown), β-catenin target promoters associate before the MBT with modified histones that correlate with active transcription: Histone H3 trimethyl-lysine 4 (H3K4me3) (Figure 3.2A) and H3 dimethyl-lysine 4 (data not shown). Importantly, β-catenin is necessary for H3K4 methylation, as depletion of β-catenin reduces H3K4 di- and tri-methylation at the *siamois* and *xnr3* promoters (Figure 3.2B and data not shown). In addition, inhibition of β-catenin activity by ΔNTcf3 reduces β-catenin binding to the *siamois* and *xnr3* promoters and blocks H3K4 trimethylation (Figure 3.2C). In contrast, ΔNTcf3 has less of an effect on AcH3 at the *siamois* and *xnr3* promoters, suggesting that pre-MBT acetylation at *siamois* and *xnr3* is established, at least in part, independently of β-catenin.

Pre-MBT β-catenin activity is sensitive to inhibition of RNA Pol II (Yang et al., 2002b). Pol II is also required for β-catenin-mediated H3K4 methylation in *Drosophila* (Parker et al., 2008). We therefore investigated whether Pol II is required to establish active chromatin architecture at β-catenin target promoters before MBT. Transcriptionally initiating Pol II is typically phosphorylated at serine 5 within the C-terminal domain (CTD) heptapeptide repeats, whereas phospho-serine 2 in the Pol II CTD correlates with transcriptional elongation (Sims et al., 2004). Although embryos are transcriptionally repressed prior to the MBT, both

Figure 3.2


A
ChIP
Serum
β-Catenin
H3K4me3
0.1% Input
1% Input
Siamois
Xnr3
Xnr5
Xnr6
Mlc2
B
- IgG
- H3K4me3
+ :β-MO
ChIP
Input
Siamois
Xnr3
Mlc2
C
ChIP
Serum
β-catenin
AcH3
H3K4me3
0.1% Input
1% Input
control
ΔNTcf3
Siamois
Xnr3
Mlc2
D
4 cells
32 cells
128 cells
500 cells
2000 cells
Late Blastula
p-Ser2
p-Ser5
Hypo-phos.
Westerns: RNA Pol II CTD
E
- IgG
- Pol II
+ Pol II
+ :β-MO
ChIP
Input
Siamois
Xnr3
Mlc2
F
ChIP
Serum
β-catenin
AcH3
H3K4me3
0.1% Input
1% Input
control
α amanitin
Siamois
Xnr3

## Figure 3.2: β-catenin establishes active chromatin architecture at target promoters before the MBT.

(A) The promoters of β-catenin target genes associate with histone H3 trimethylated at K4 (H3K4me3). Promoter occupancy by either β-catenin or H3K4me3 was observed by ChIP on 1000-cell stage embryos.

(B) β-catenin is necessary for H3K4me3 accumulation at target promoters. 1000-cell stage control and β-catenin depleted (β-MO) embryos were subjected to ChIP for H3K4me3. All sibling β-MO embryos were completely ventralized at later stages (not shown).

(C) H3K4me3, but not AcH3, requires β-catenin function. MBT-stage control (upper panel for each gene) and ΔNTcf3 mRNA-injected (500pg, lower panels) embryos were subjected to ChIP for β-catenin, AcH3, and H3K4me3.

(D) Initiating and elongating forms of RNA Polymerase II are present in pre-MBT embryos. Whole embryo lysates were separated by SDS-PAGE and western blots were performed using antibodies to phosphoserine 2, phosphoserine 5, and hypo-phosphorylated isoforms of the RNA Polymerase II C-terminal heptapeptide repeats.

(E) Initiating RNA Pol II binds the *siamois* and *xnr3* promoters before MBT, independently of β-catenin. 1000-cell stage control and β-catenin depleted (β-MO) embryos were subjected to ChIP for RNA Pol II (p-Ser5). All sibling β-MO embryos were completely ventralized at later stages (not shown).

(F) RNA Pol II function is necessary for H3K4 trimethylation at β-catenin target promoters. MBT-stage control (upper panels) and α-amanitin injected (10ng, lower panels) embryos were subjected to ChIP for β-catenin, AcH3, and H3K4me3.

initiating (p-Ser5) and elongating (p-Ser2) forms of Pol II are present in the embryo throughout pre-MBT stages (Figure 3.2D), suggesting that a subset of Pol II is phosphorylated in a manner that correlates with active transcription. This differs from a previous report of limited Pol II p-Ser5 and p-Ser2 before the MBT (Palancade et al., 2001). However, the experiments presented here were performed using whole cell lysates and additional phosphatase inhibitors, which may account for the increased detection of phospho-Pol II before the MBT. Furthermore, H3K4 methylation also correlates with occupancy of initiating Pol II (Ng et al., 2003b). Interestingly, before the MBT, Pol II (p-Ser5) also occupies the promoters of *siamois* and *xnr3*, although this interaction is independent of β-catenin (Figure 3.2E). This result raises the possibility that in addition to β-catenin, functional Pol II is required to establish methylated H3K4 at target promoters. To test this, we measured MBT-stage methylation of H3K4 at β-catenin targets following treatment with the Pol II inhibitor α-amanitin (Figure 3.2F). Inhibition of Pol II greatly reduces H3K4me3 at the *siamois* and *xnr3* promoters, but does not affect binding of β-catenin or H3 acetylation.

Collectively, these results indicate that β-catenin and Pol II collaborate to establish H3K4 methylation at β-catenin target promoters before the MBT. These promoters additionally bear several marks of transcriptional activation—acetyl-Histone H3/H4 and Pol II (p-Ser5)—that are established independently of β-catenin. These observations support a model in which β-catenin directs the

establishment of poised chromatin architecture at target gene loci before the MBT.

Transcriptionally poised loci are typically marked by initiated, yet stalled RNA Pol II (p-Ser5), H3K9/14 acetylation, and H3K4me3 in the absence of active mRNA expression (Guenther et al., 2007). In pre-MBT embryos, the lack of *siamois* and *xnr3* mRNA could reflect the establishment of a *bona fide* poised state at these genes; alternatively, these genes could be maintained in a silent state due to global transcriptional silencing in the pre-MBT embryo. In general, global transcriptional silencing in pre-MBT embryos stems from rapid chromatin assembly at genes that suppresses the function of the basal transcriptional apparatus (Prioleau et al., 1994). Additionally, constraints on gene specific trans-activators further suppress precocious zygotic gene expression before the MBT (Almouzni and Wolffe, 1995). Because we observe binding of β-catenin and Pol II (pSer-5) at these promoters, this suggests that the lack of mRNA expression for *siamois* and *xnr3* is not due to impaired assembly of the transcriptional apparatus, nor on an inability of β-catenin to access promoters. Additionally, other β-catenin target genes (*xnr5* and *xnr6*) also bind β-catenin, accumulate H3K4me3, and are transcribed before the MBT (Figure 3.1A, 3.2A), which indicates that the mechanisms that prevent *siamois* and *xnr3* expression regulate events downstream of Pol II recruitment and transcriptional initiation. Alternatively, the otherwise "active" *siamois* and *xnr3* loci could nonetheless be suppressed by the lack of an interphase during the pre-MBT cell cycle, as is seen for a subset of loci in pre-MBT *Xenopus* and *Drosophila* embryos (Edgar et al.,

1986; Kimelman et al., 1987). Therefore, we tested this by treating embryos with the translational inhibitor cycloheximide (CHX), which arrests the pre-MBT cell cycle in a G2-like state within one cell cycle after treatment (Miake-Lye et al., 1983). Compared to control embryos, CHX-arrested embryos do not precociously express *siamois* at stage 8 (Figure 3.3A). Additionally, CHX treatment does not impair the expression of the pre-MBT gene *xnr6* at stage 8, nor does it affect the onset of expression of the post-MBT zygotic genes *siamois* and *vent2*, although the embryos are clearly arrested with sub-MBT cell numbers (Figure 3.3B, compare stage 8.5 control and +CHX embryos). Therefore, the chromatin architecture at the *siamois* and *xnr3* loci indicates that these loci are transcriptionally poised before the MBT, which is maintained transcriptionally silent independently of the pre-MBT cell cycle.

### *3.2.2: β-catenin associates with a Histone H3 (R8) methyltransferase activity in early* Xenopus *embryos.*

We next sought to characterize the nature of β-catenin activity in cleavage-stage embryos. β-catenin interacts with a number of factors involved in transcriptional control and chromatin remodeling (Barker et al., 2001; Hecht et al., 2000; Koh et al., 2002; Sierra et al., 2006; Takemaru and Moon, 2000), but whether any of these proteins represent obligate β-catenin cofactors or instead interact with β-catenin in a tissue or context-dependent manner is unclear. We

Figure 3.3

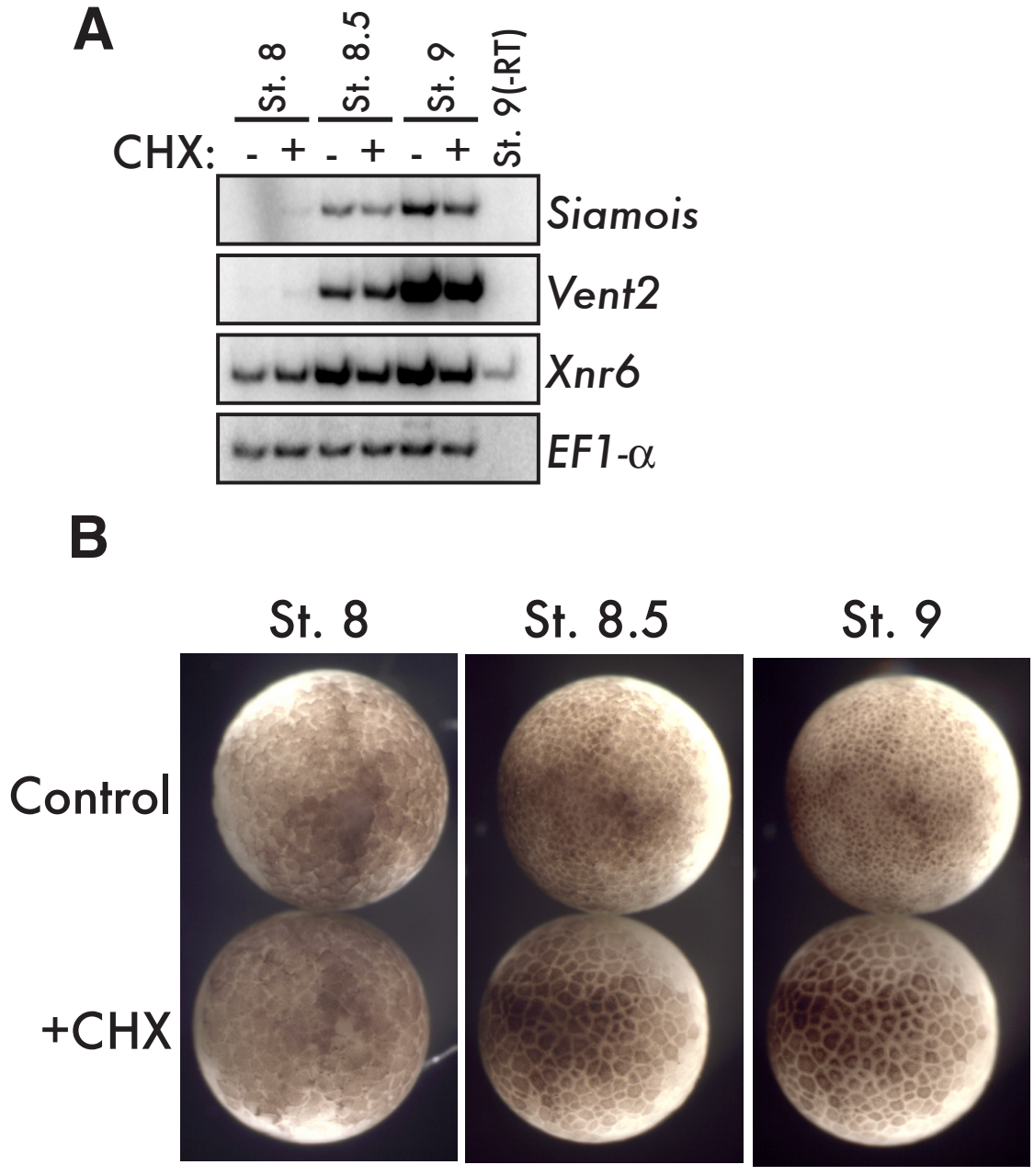

A
St. 8
St. 8.5
St. 9
St. 9(-RT)
CHX: - + - + - +
Siamois
Vent2
Xnr6
EF1-α
B
St. 8
St. 8.5
St. 9
Control
+CHX

**Figure 3.3: Pre-MBT expression of *siamois* is not suppressed by the lack of an interphase.**

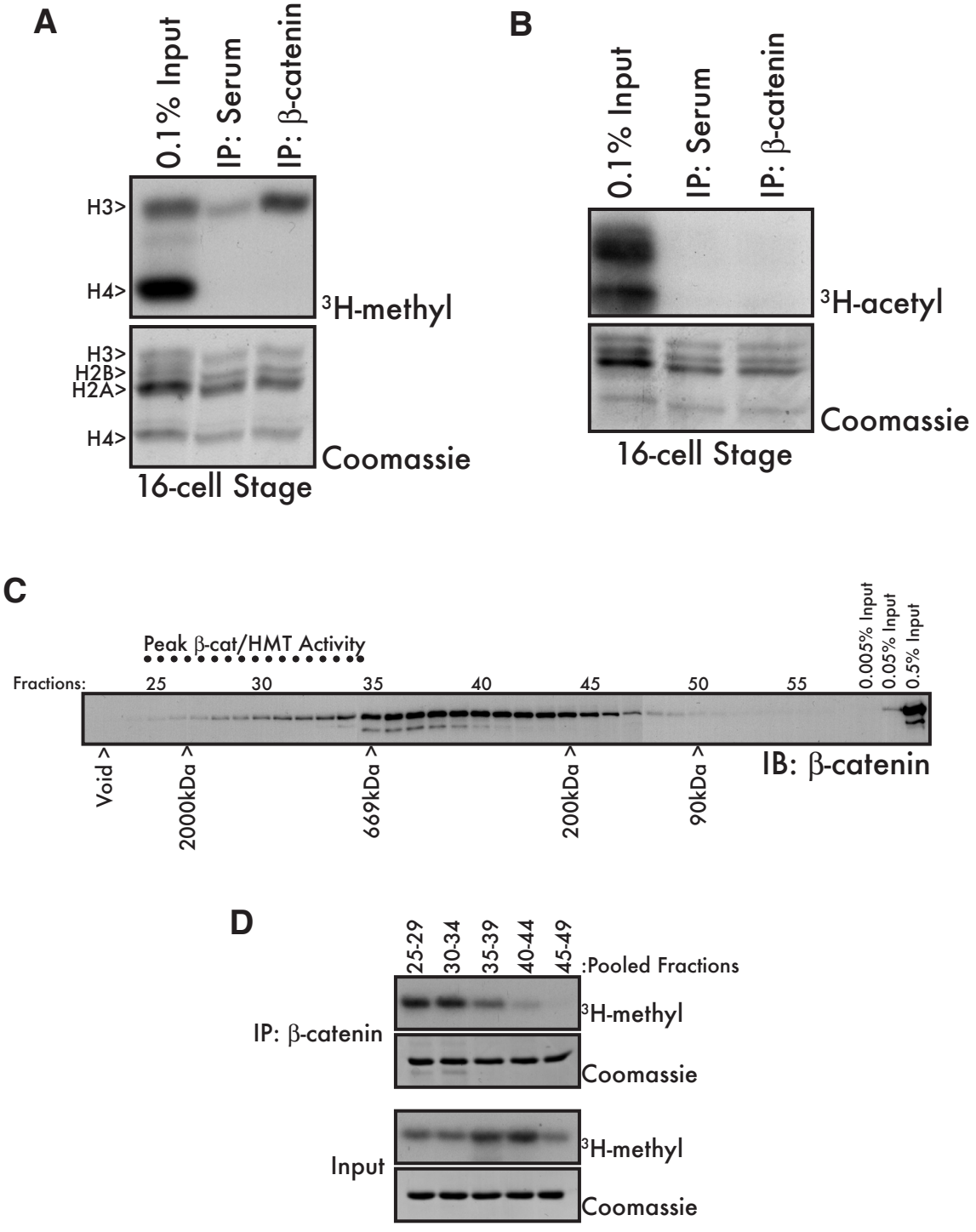
(A) Embryos were treated with cycloheximide (CHX, 200μg/ul) in the culture medium beginning at the 256/500-cell stage (late Stage 7.5). Control and CHX-treated embryos were collected one hour before, at, or one hour after the MBT (Stage 8, 8.5, and 9, respectively) for RT-PCR expression analysis of the indicated markers.

(B) Representative images of control and CHX-treated embryos were collected at the stages used for marker gene analysis. Stage 8 embryos (0.5 hr post treatment) already show cell cycle arrest, and no cell divisions take place following entry into the 1000-cell stage (Stage 8).

hypothesized that, during dorsal specification, β-catenin interacts with a chromatin-modifying activity that functions to establish active chromatin architecture at target promoters.

To identify β-catenin-associated chromatin modifying activities in 8- to 32-cell stage embryos, we immunoprecipitated β-catenin and performed *in vitro* histone acetyl- or methyl-transferase (HAT or HMT) assays (Figure 3.4). During the period when β-catenin activates the expression of dorsal determinants (prior to the 32-cell stage), β-catenin interacts with a HMT activity that specifically methylates Histone H3 but not H4 (Figure 3.4A). Under these assay conditions, we were unable to detect an associated HAT activity (Figure 3.4B). β-catenin is predicted to interact with several functionally different macromolecular complexes (Gottardi and Gumbiner, 2001). To determine whether the β-catenin associated HMT (β-cat/HMT) activity associates with a subset of β-catenin complexes, we performed size exclusion chromatography followed by β-catenin IP/HMT assays on pooled fractions. β-catenin complexes have a broad molecular weight (MW) distribution in early embryos (Figure 3.4C, peak MW ~500kD), consistent with results from other experimental systems (Papkoff et al., 1996; Stewart and Nelson, 1997). The β-cat/HMT activity, however, is restricted to the highest MW fractions (Figure 3.4D, average MW~1.5MDa). The β-cat/HMT activity peak is also distinct from the peak HMT activity in the input pooled fractions (average MW ~350kD). Therefore, the β-cat/HMT resides in a high-MW complex.

Figure 3.4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0.1% Input
IP: Serum
IP: β-catenin
H3>
H4>
3H-methyl
H3>
H2B>
H2A>
H4>
Coomassie
16-cell Stage
B
0.1% Input
IP: Serum
IP: β-catenin
3H-acetyl
Coomassie
16-cell Stage
C
Peak β-cat/HMT Activity
Fractions:
25
30
35
40
45
50
55
0.005% Input
0.05% Input
0.5% Input
IB: β-catenin
Void >
2000kDa >
669kDa >
200kDa >
90kDa >
D
25-29
30-34
35-39
40-44
45-49
:Pooled Fractions
IP: β-catenin
3H-methyl
Coomassie
Input
3H-methyl
Coomassie

## Figure 3.4: β-catenin associates with a histone methyltransferase activity before the MBT.

(A) β-catenin from 16-cell stage embryos specifically associates with a histone H3 methyltransferase (HMT). β-catenin was immunoprecipitated from 16-cell embryos and histone methyltransferase (HMT) activity was visualized by fluorography (1 day exposure) for incorporation of [$^3$H] methyl groups into calf thymus histones (top panel). Equal loading of histones is shown by coomassie staining (lower panel). "Input" represents HMT activity in embryo lystes, with activity toward both H3 and H4.

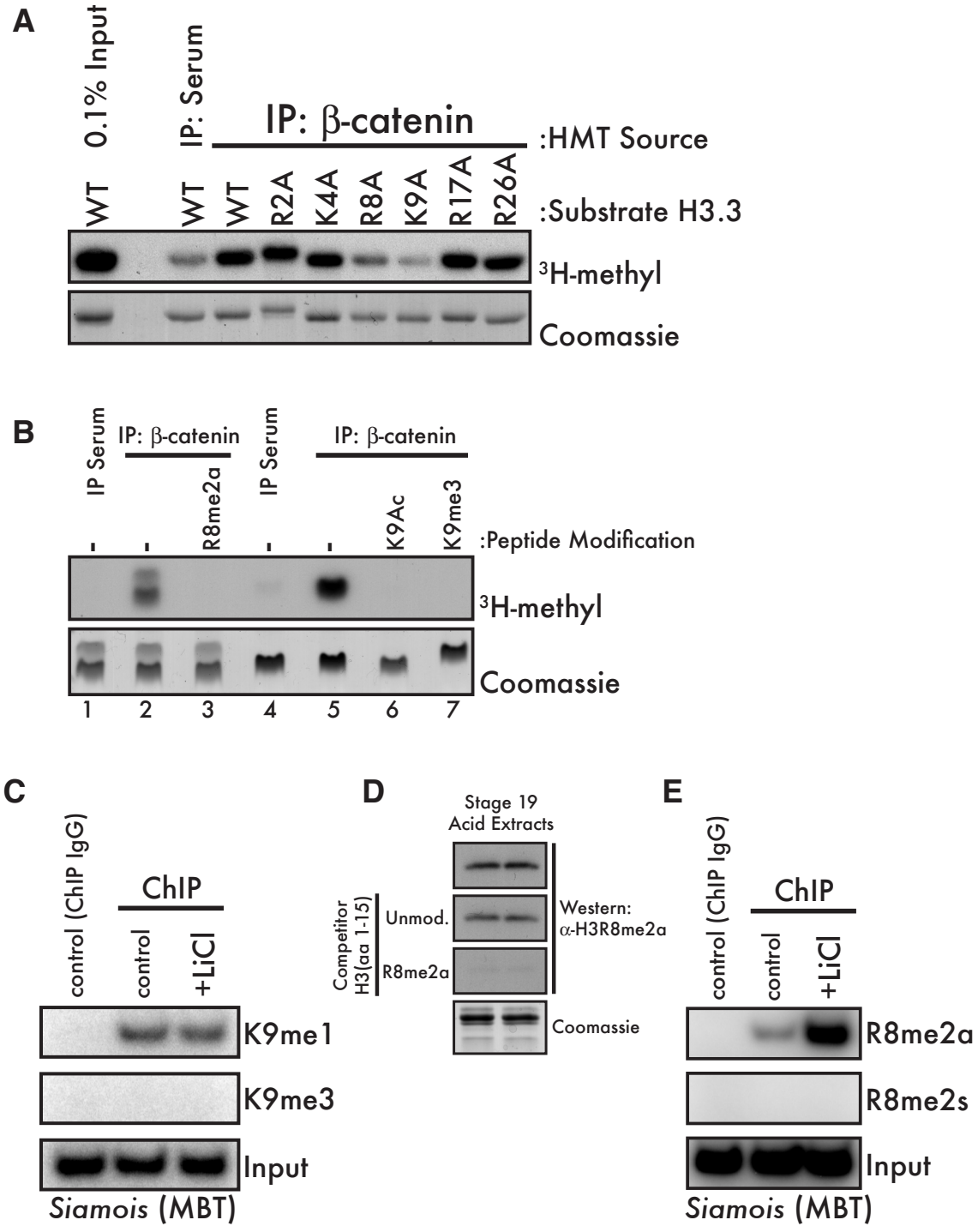
(B) β-catenin does not interact with a histone acetyltransferase activity in 16-cell stage embryos. Conditions were similar as described for panel A, except that [$^3$H] acetyl CoA was substituted for [$^3$H] SAM. The fluorography image is a 12-day exposure. The input embryo lysates have apparent HAT activity directed against each of the four histone substrates.

(C and D) HMT activity associates with only a fraction of embryonic β-catenin. 8- to 32-cell stage embryo lysates were separated by S400-HR size exclusion chromatography (SEC) and the size distribution of β-catenin was determined by western blot (B). The image in (B) is a composite of three images all taken at the same exposure. β-catenin positive fractions were pooled as indicated, and immunoprecipitated as in A. β-catenin-associated HMT activity (C, top panels) was measured relative to activity in the pooled fractions prior to IP (C, lower panels). The average molecular weight of β-cat/HMT (1500kDa) was estimated relative to the elution profile of standards. The fraction predicted to contain monomeric β-catenin (90kD, fraction 50) is indicated.

To identify the residue on histone H3 targeted by the β-cat/HMT complex, we performed β-catenin IP/HMT assays using as the substrate recombinant H3.3 (rH3.3) with alanine point mutations at candidate target residues (Figure 3.5A) on the H3 N-terminal tail previously shown to be methylated: arginines (R) 2, 8, 17, 26 and lysines (K) 4 and 9 (Bedford and Clarke, 2009; Kouzarides, 2007). As with H3, the β-cat/HMT significantly methylates rH3.3(WT) over background. Importantly, mutation of K4 has no effect on H3 methylation in this assay, suggesting that in early *Xenopus* embryos, β-catenin indirectly establishes H3K4 methylation at target promoters (Figure 3.2). These observations also rule out R2, 17, and 26 as major methyl acceptor sites for the β-cat/HMT. In contrast, mutation of either R8 or K9 prevents H3 methylation by the β-cat/HMT. To confirm this observation, we performed β-catenin IP/HMT assays using Histone H3 peptides pre-modified at either R8 (asymmetric dimethyl) or K9 (acetyl and trimethyl). The β-cat/HMT methylates the unmodified H3 (1-15) peptide (Figure 3.5B, lane 2) to a similar level as full-length H3 (data not shown). However, modification of either R8 or K9 prevents methylation by the β-cat/HMT (Figure 3.5B, lanes 3, 6, and 7). Thus, in addition to targeting either position R8 or K9, the β-cat/HMT is also sensitive to the modification status of these residues.

H3K9 methylation is generally associated with heterochromatin and transcriptional repression (Kouzarides, 2007), and is therefore an unlikely target residue for the β-cat/HMT. Several ChIP experiments failed to detect di- or tri-

Figure 3.5


A
0.1% Input
IP: Serum
IP: β-catenin
:HMT Source
WT
WT
WT
R2A
K4A
R8A
K9A
R17A
R26A
:Substrate H3.3
³H-methyl
Coomassie
B
IP Serum
IP: β-catenin
IP Serum
IP: β-catenin
-
-
R8me2a
-
-
K9Ac
K9me3
:Peptide Modification
³H-methyl
Coomassie
1 2 3 4 5 6 7
C
control (ChIP IgG)
ChIP
control
+LiCl
K9me1
K9me3
Input
Siamois (MBT)
D
Stage 19
Acid Extracts
Competitor H3(aa 1-15)
Unmod.
R8me2a
Western: α-H3R8me2a
Coomassie
E
control (ChIP IgG)
ChIP
control
+LiCl
R8me2a
R8me2s
Input
Siamois (MBT)

## Figure 3.5: The β-catenin associated methyltransferase targets R8 on the Histone H3 tail.

(A) β-cat/HMT activity requires R8 and K9 on the histone H3 tail. β-catenin IP/HMT assays were performed on either wild type (WT) recombinant H3.3 or H3.3 with the indicated point mutations.

(B) The β-cat/HMT methylates a site within the first 15 residues of the H3 tail and is sensitive to modification of both R8 and K9. β-catenin IP/HMT assays were performed on peptides corresponding to unmodified H3 (aa 1-15, lanes 1&2), asymmetrically dimethylated R8 (aa 1-15, lane 3), unmodified H3 (aa 1-21, lanes 4&5), acetylated K9 (aa 1-20, lane 6) and trimethylated K9 (aa 1-24, lane 7).

(C) Activation of β-catenin has no effect on H3K9 methylation at the *Siamois* promoter. MBT-stage control and lithium chloride treated (LiCl, 300mM for 10 minutes, 1 hour prior to harvest) embryos were subjected to ChIP for either mono- or tri-methylated H3K9 (K9me1 and K9me3 respectively) using native, non-crosslinked chromatin.

(D) An anti-H3R8me2a antibody recognizes histone H3 in stage 19 *Xenopus* acid extracts by western blot (upper 3 panels). A 200-fold molar excess of the R8me2a H3 peptide (aa 1-15) blocked immunoreactivity (”R8me2a”; 3$^{rd}$ panel), whereas the unmodified H3 peptide (“Unmod.”; 2$^{nd}$ panel) did not significantly reduce immunoreactivity, demonstrating antibody specificity for the R8me2a modification.

(E) Activation of β-catenin increases asymmetric H3R8 dimethylation at the *Siamois* promoter. ChIP was performed as described in panel C, using instead either asymmetrically- or symmetrically-dimethylated H3R8 (H3R8me2a or H3R8me2s, respectively).

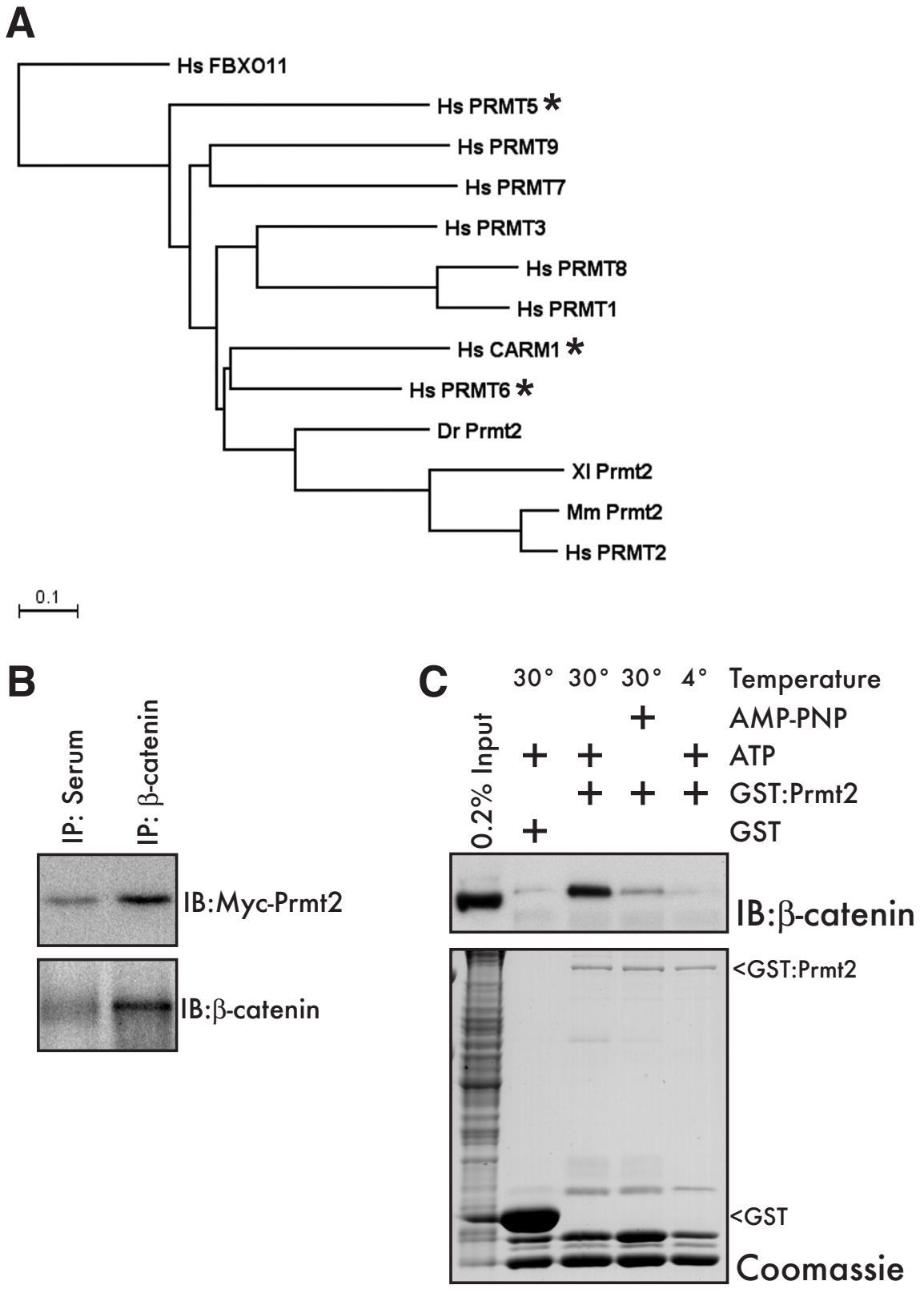
methylated H3K9 at the *siamois* and *xnr3* promoters between the 1000-cell stage and MBT, and H3K9me1 levels were not sensitive to changes in β-catenin activity (Figure 3.5C and data not shown). On the other hand, the effect of H3R8 methylation on transcriptional control is poorly understood. To test whether β-catenin activity regulates H3R8 methylation at target promoters, we generated an antibody to asymmetrically dimethylated H3R8 (H3R8me2a). The H3R8me2a antiserum was depleted of antibodies to unmodified H3 and affinity purified with the R8me2a peptide. The purified antibody specifically detects R8me2a-modified H3 by dot blot (data not shown) and by competitive western blot (Figure 3.5D), which also confirms that the H3R8me2a modification occurs *in vivo*. Subsequently, we measured H3R8 methylation at the *siamois* promoter by ChIP. H3R8me2a associates with the *siamois* promoter at the MBT, and symmetric H3R8 dimethylation (H3R8me2s) is not detected (Figure 3.5E). To test whether association of H3R8me2a correlates with β-catenin activity, we exposed pre-MBT embryos to a pulse of lithium chloride (LiCl), which stabilizes β-catenin throughout the embryo, resulting in a radially expanded domain of dorsal progenitors (Kao et al., 1986). One hour after the LiCl pulse, H3R8me2a increased dramatically at the *siamois* and *xnr3* promoters (Figure 3.5E and data not shown), indicating that H3R8 methylation correlates positively with β-catenin activity. From these results, we conclude that the β-cat/HMT asymmetrically dimethylates H3R8 and is sensitive to the modification state of H3K9. Our results

also indicate that β-catenin interacts with a type I (asymmetric) arginine histone methyltransferase in early *Xenopus* embryos.

### *3.2.3: β-catenin recruits the arginine methyltransferase Prmt2 to target loci*

We undertook a candidate-based approach to identify the β-catenin-associated arginine methyltransferase. Of the ten members of the protein arginine methyltransferase (Prmt) family (Figure 3.6A), three have been shown to methylate histone H3 *in vitro*: Carm1, Prmt5, and Prmt6 (asterisks in Figure 3.6A) (Guccione et al., 2007; Hyllus et al., 2007; Pal et al., 2004; Schurter et al., 2001). Of these, only the type II, symmetric methyltransferase Prmt5 has been shown to target specifically R8. We tested candidates for interaction with β-catenin by expressing myc-tagged Prmts in *Xenopus* embryos and IP-ing β-catenin. By this approach, none of the Prmts known to methylate H3 (Carm1, Prmt5, and Prmt6) co-IP-ed with β-catenin (data not shown). However, Prmt2, which is most closely related to Carm1 and Prmt6, co-IPs with β-catenin in blastula stage embryos (Figure 3.6B). We also confirmed that Prmt2 is maternally expressed (Figure 3.1A). Although recombinant β-catenin and Prmt2 do not interact directly in vitro (data not shown), GST-tagged Prmt2 interacts with β-catenin in *Xenopus* embryo lysates in a temperature and ATP-dependent manner (Figure 3.6C). Thus, β-catenin and Prmt2 interact in early *Xenopus* embryos and require an unknown catalytic activity to assemble in a complex.

Figure 3.6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Hs FBXO11
Hs PRMT5 *
Hs PRMT9
Hs PRMT7
Hs PRMT3
Hs PRMT8
Hs PRMT1
Hs CARM1 *
Hs PRMT6 *
Dr Prmt2
Xl Prmt2
Mm Prmt2
Hs PRMT2
0.1
B
IP: Serum
IP: β-catenin
IB:Myc-Prmt2
IB:β-catenin
C
30°
30°
30°
4°
Temperature
AMP-PNP
ATP
GST:Prmt2
GST
0.2% Input
IB:β-catenin
<GST:Prmt2
<GST
Coomassie

**Figure 3.6: β-catenin interacts with the arginine methyltransferase Prmt2.**

(A) Phylogenetic analysis of the Prmt family (Clustal, Gonnet Matrix) demonstrating the relationship between human (Hs) PRMTs 1-9(4q31) and FBXO11 with Prmt2 from mouse (Mm), *Xenopus laevis* (Xl), and zebrafish (Dr). Asterisks indicate PRMTs that are known to methylate histone H3 in vitro.

(B) Prmt2 interacts with β-catenin in early *Xenopus* embryos. Myc-tagged mouse Prmt2 (500pg) was expressed in *Xenopus* embryos, and embryo lysates were immunoprecipitated with anti-β-catenin or pre-immune serum and subjected to western blot with either anti-myc (upper panel) or anti-β-catenin antibodies.

(C) Prmt2 and β-catenin interact in an ATP-dependent manner. Embryo lysates (16-cell) were incubated for 1 hour with GST-Prmt2 beads at 4° or 30°C and with ATP or the nonhydrolyzable ATP analog AMP-PNP. Bound proteins were eluted and β-catenin was visualized by western blot (upper panel). GST beads alone were used as a negative control and the relative amounts of bait proteins in each lane were visualized by coomassie staining (bottom, arrowheads). “Input” indicates 16-cell embryo lysate.

By sequence, Prmt2 is most closely related to the type I methyltransferases Carm1 and Prmt6 (Figure 3.6A), but its substrate preference has not been determined because recombinant Prmt2 does not have activity *in vitro* (Scott et al., 1998). Also, to our knowledge, HMT activity associated with endogenous Prmt2 has not been reported. Interestingly, endogenous Prmt2 IP-ed from mouse embryonic stem cells methylates histone H3 (Figure 3.7A). Likewise, myc-Prmt2 expressed in *Xenopus* embryos methylates histone H3, and this activity requires H3R8 (Figure 3.7B). In these experiments, Prmt2 IPs contain HMT activity towards histone H3, reflecting either the activity of Prmt2 itself or the activity of another HMT that co-IPs with Prmt2. Further investigation will be required to determine the factors that regulate endogenous Prmt2 catalysis.

Importantly, Prmt2 binds the *siamois* promoter in pre-MBT embryos (Figure 3.7C) as determined by ChIP. Since Prmt2 and β-catenin interact, we also tested whether depletion of β-catenin could reduce Prmt2 binding at this promoter. Indeed, β-catenin knockdown results in reduced Prmt2 occupancy at the *siamois* promoter, suggesting that β-catenin recruits Prmt2 to target promoters. We therefore conclude that Prmt2 represents the HMT activity that associates with β-catenin in early *Xenopus* embryos based on the observations that β-catenin interacts with Prmt2, Prmt2 has a HMT activity directed at H3 that is sensitive to the R8A mutation, and β-catenin recruits Prmt2 to target genes during the pre-MBT period.

Figure 3.7

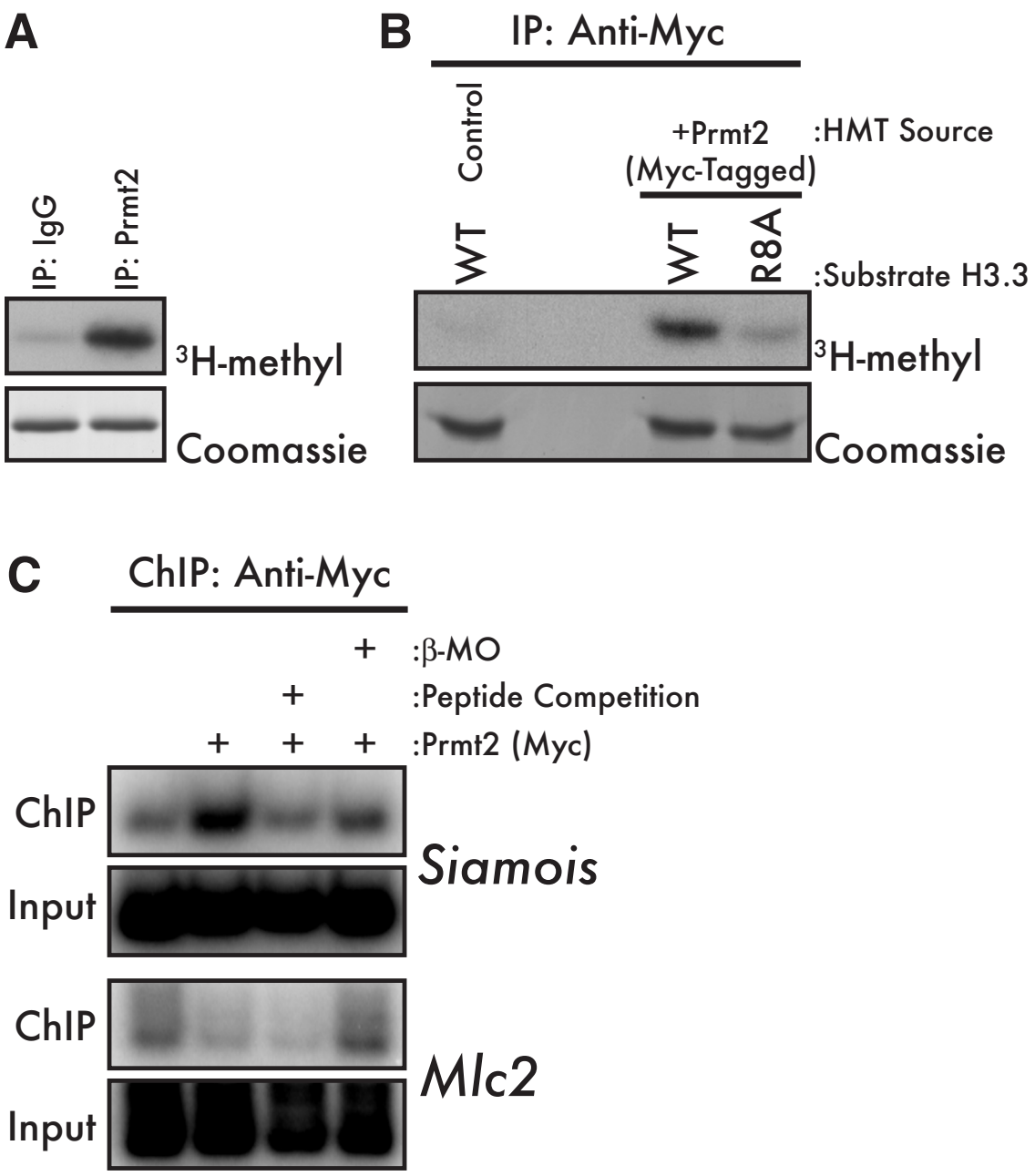

A
IP: IgG
IP: Prmt2
3H-methyl
Coomassie
B
IP: Anti-Myc
Control
+Prmt2
(Myc-Tagged)
:HMT Source
WT
WT
R8A
:Substrate H3.3
3H-methyl
Coomassie
C
ChIP: Anti-Myc
+ :β-MO
+ :Peptide Competition
+ + + :Prmt2 (Myc)
ChIP
Input
Siamois
ChIP
Input
Mlc2

**Figure 3.7: Prmt2 methylates histone H3 and is recruited by β-catenin to the *Siamois* promoter before the MBT.**

(A) Endogenous Prmt2 methylates histone H3. Endogenous Prmt2 from a mouse embryonic stem cell culture was immunoprecipitated and subjected to an IP/HMT assay using recombinant H3.3 as a substrate. Rabbit IgG was used as a negative control.

(B) Histone H3 R8 is necessary for Prmt2 HMT activity. Myc-Prmt2 was immunoprecipitated from 32- to 128-cell Xenopus embryos expressing Myc-tagged Prmt2 and HMT activity was measured using wild-type (WT) or R8A histone H3.3 as substrates. Non-injected embryos served as negative controls.

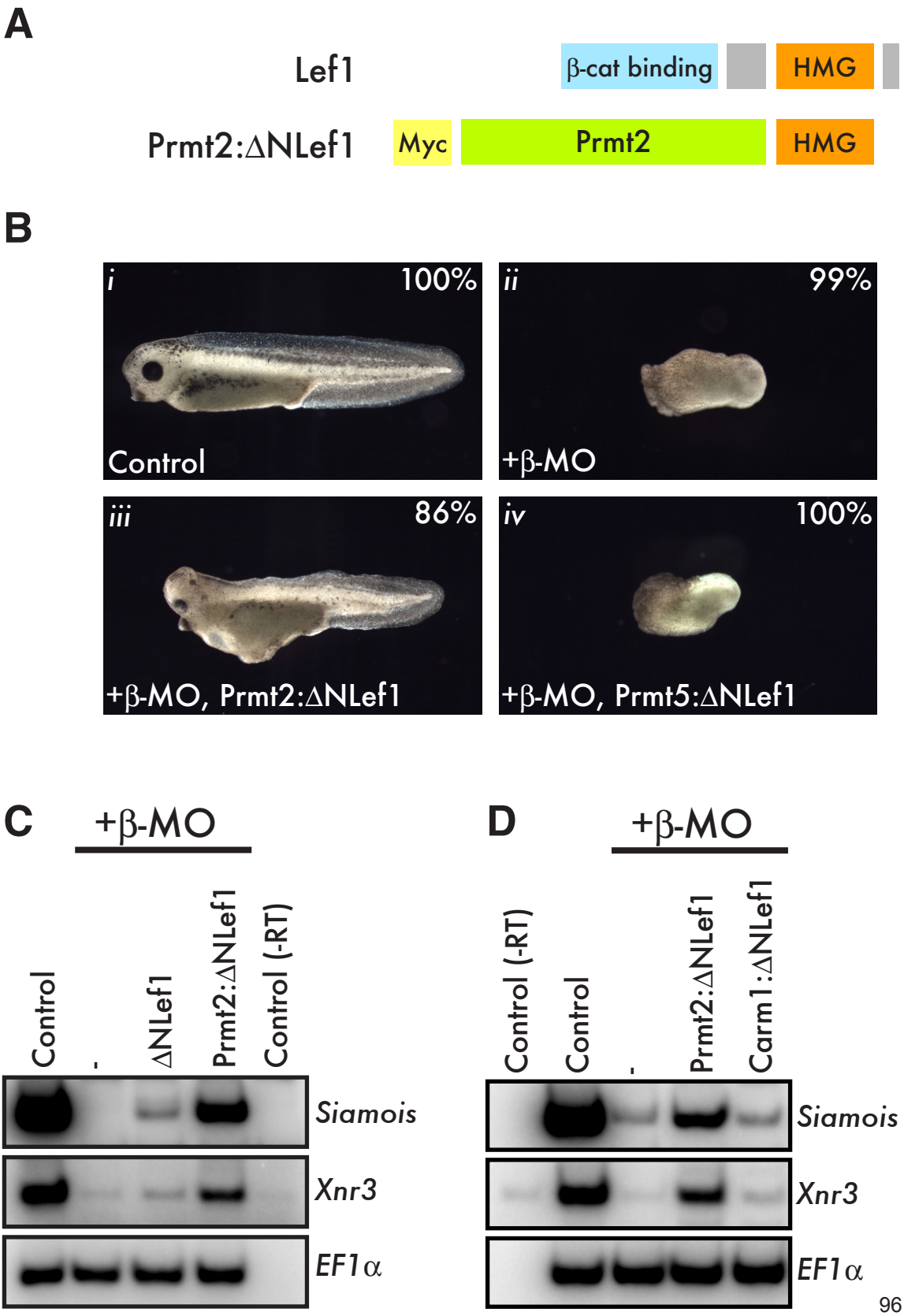
(C) β-catenin-dependent interaction of Prmt2 with the *Siamois* promoter. Wild type or β-MO injected, 1000-cell embryos expressing Myc-Prmt2 were subjected to ChIP using the anti-Myc-tag antibody. Non-injected embryos served as a negative control. In addition, the specificity of ChIP was confirmed by including a 200-fold excess of the Myc-peptide (lane 3) in the IP.

*3.2.4: Directing Prmt2 to β-catenin target promoters is sufficient to drive dorsal specification in the absence of β-catenin*

We determined whether Prmt2 activity at β-catenin target genes is sufficient to specify dorsal cell fates. β-catenin interacts with chromatin via the Tcf/Lef family of DNA-binding factors (Behrens et al., 1996; Molenaar et al., 1996), and previous investigations have exploited this interaction to target factors of interest to Tcf/Lef binding sites and test their effects on target gene expression (Vleminckx et al., 1999). Therefore, we generated chimeric proteins (Figure 3.8A) between Prmt2 and the DNA binding domain of Lef-1 (ΔNLef1), reasoning that we could direct Prmt2 to target genes and evaluate its effect on dorsal specification. Chimeras between ΔNLef1 and Carm1, Prmt5, or Prmt6 were also generated as controls. All chimeric proteins were expressed to similar levels in blastula stage embryos (data not shown).

Targeting Prmt2 to β-catenin target genes is sufficient to rescue dorsal specification in β-catenin depleted embryos (Figure 3.8B). Embryos depleted for β-catenin develop with a typical "ventralized" phenotype (Figure 3.8B ii; also figure 1B ii), whereas expression of the Prmt2:ΔNLef1 chimera in β-catenin-depleted embryos restores the full range of dorsal and anterior structures (Figure 3.8B iii), remarkably similar to control embryos (Figure 7B i), albeit typically with a single eye (86% rescue, N=172). Importantly, expression of Prmt2:ΔNLef1 also rescues organizer gene expression to a greater extent than the ΔNLef1 DNA

Figure 3.8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Lef1
β-cat binding
HMG
Prmt2:ΔNLef1
Myc
Prmt2
HMG
B
i
100%
Control
ii
99%
+β-MO
iii
86%
+β-MO, Prmt2:ΔNLef1
iv
100%
+β-MO, Prmt5:ΔNLef1
C
+β-MO
Control
-
ΔNLef1
Prmt2:ΔNLef1
Control (-RT)
Siamois
Xnr3
EF1α
D
+β-MO
Control (-RT)
Control
-
Prmt2:ΔNLef1
Carm1:ΔNLef1
Siamois
Xnr3
EF1α

**Figure 3.8: Directing Prmt2 to β-catenin target gene promoters is sufficient to drive dorsal specification.**

(A) Schematic of the Prmt2:ΔNLef1 chimeric construct. To direct Prmt2 to Tcf/Lef DNA binding sites, the DNA-binding HMG domain of mouse Lef1 was fused to the C-terminus of mouse 6xMyc-Prmt2.

(B) Targeting Prmt2 to Tcf/Lef binding sites is sufficient to rescue dorsal specification in β-catenin depleted embryos. Embryos were depleted for β-catenin (β-MO) and subsequently injected with 500pg of either Prmt2:ΔNLef1 (*iii*) or Prmt5:ΔNLef1 (*iv*) mRNA. Rescue of β-MO-induced ventralization (*ii*) was measured at tadpole stages. Note the rescue of the anterior-most, dorsally derived cement gland and eye in panel *iii*, compared to control, non-injected embryos (*i*). The percentages in the upper right corner of each panel indicate the frequency at which the phenotypes shown were observed.

(C) Prmt2:ΔNLef1 rescues expression of *Siamois* and *Xnr3* in β-catenin depleted embryos. Embryos were depleted for β-catenin (β-MO) and subsequently injected with Prmt2:ΔNLef1 or ΔNLef1 mRNA. Expression of *Siamois* and *Xnr3* was measured by RT-PCR at stage 10. *EF1α* expression is shown as a loading control.

(D) Carm1 does not rescue dorsal specification in β-catenin depleted embryos. Injections and RT-PCR were performed as described for panels B and C to compare the extent of rescue by Prmt2:ΔNLef1 and Carm1:ΔNLef1 (500pg, each mRNA).

binding domain alone, as determined by RT-PCR on gastrula-stage embryos (Figure 3.8C).

To test whether the Prmt2 rescue of β-catenin depletion is due to its potential catalytic activity, we mutated the S-adenosylmethionine (SAM) binding domain (Qi et al., 2002). In our hands, this mutation did not interfere with Prmt2 catalytic activity as determined in an IP/HMT assay, where mutant Prmt2 was co-expressed with wild type, endogenous Prmt2 (data not shown). Crystallographic and biochemical evidence suggest that Prmts function as obligate dimers or oligomers (Weiss et al., 2000). It is likely that our Prmt2 mutants retain HMT activity due to complex formation with endogenous, catalytically active Prmt2. As an alternative approach, we tested whether other Prmts, with distinct predicted Histone H3 target residues, were similarly able to rescue dorsal specification in β-catenin depleted embryos.

Prmt5 shares target residue specificity with Prmt2, but symmetrically dimethylates H3R8 (Pal et al., 2004), and the Prmt5:ΔNLef1 chimera is unable to rescue dorsal specification in β-catenin depleted embryos (Figure 3.8B iv—0% Rescue, N=31)). On the other hand, the robust transcriptional activator Carm1 asymmetrically methylates H3 R2, R17, and R26, with only a weak activity towards R8 (Chen et al., 2000; Schurter et al., 2001). Expression of the Carm1:ΔNLef1 chimera is toxic to embryos shortly after gastrulation, so phenotypic rescue could not be scored. However, at gastrula stages, the

Carm1:ΔNLef1 chimera does not rescue *siamois* and *xnr3* expression by RT-PCR, compared to the Prmt2 chimera (Figure 3.8D). Finally, Prmt6, which targets H3R2 (Guccione et al., 2007; Hyllus et al., 2007), also did not rescue dorsal specification (not shown, 0% rescue, N=30). Thus, of all the Prmts tested, only the recruitment of Prmt2 to β-catenin target promoters is sufficient to rescue dorsal specification, demonstrating the unique role of Prmt2 in the regulatory events that establish the transcriptional network driving dorsal development in *Xenopus* embryogenesis.

### *3.2.5: Prmt2 is necessary for dorsal specification and for conferring resistance to ΔNTcf3 before the MBT*[*]

Finally, we tested whether Prmt2 is necessary for the activation of β-catenin target gene expression at the MBT. We designed a morpholino oligonucleotide to block the translation of Prmt2 (Prmt2MO). Because Prmt2 is expressed maternally, zygotic injection of Prmt2MO will only prevent *de novo* translation of maternal Prmt2 mRNA, yet pre-existing maternal Prmt2 protein will persist and remain functional in early embryos. Therefore, we have tested the role of Prmt2 during dorsal specification by depleting oocytes of Prmt2 and generating maternal Prmt2-depleted embryos by the host-transfer method (Mir

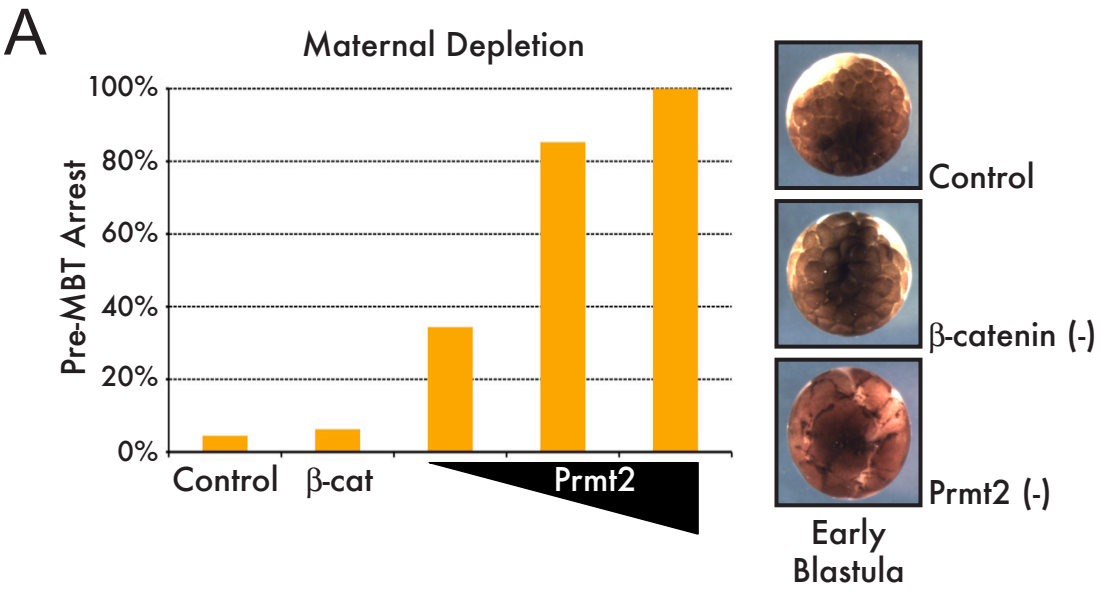
[*] The results in this section are to be considered preliminary as of the submission of this dissertation (September, 2009) and represent ongoing research being performed prior to the resubmission of this work for publication per the request of our reviewers.

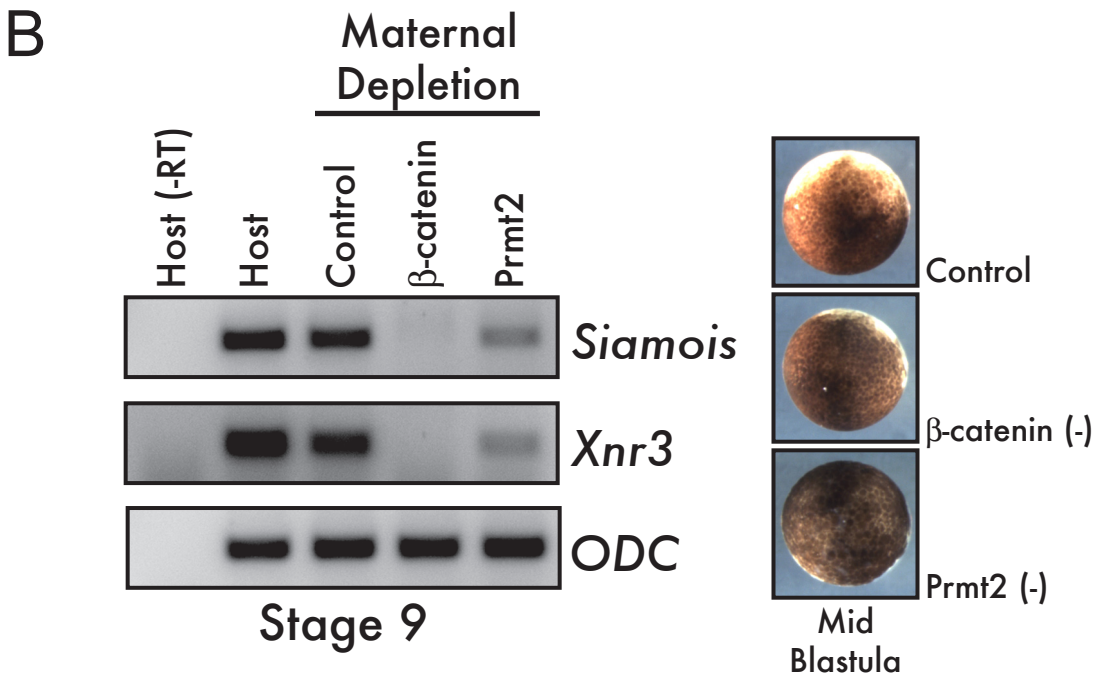
and Heasman, 2008). Maternal Prmt2-depleted embryos displayed Prmt2MO dose-dependent arrest of cleavage-stage development compared to control (non-injected) and β-catenin-depleted (β-MO) embryos (Figure 3.9A). This indicates that Prmt2 has broader roles in development beyond its contribution to dorsal specification. We measured the effect of Prmt2 depletion on *siamois* and *xnr3* expression in embryos receiving the lowest dose of Prmt2MO (10ng) as the majority of these embryos appeared morphologically normal at the MBT (Figure 3.9B, right side). Similarly to β-catenin depletion, depletion of maternal Prmt2 significantly reduces *siamois* and *xnr3* expression after the MBT (Figure 3.9B). Unfortunately, these embryos fail to survive past gastrula stages, thus we were unable to determine the morphological consequences of maternal Prmt2 depletion. Nonetheless, our results are consistent with a model where recruitment by β-catenin of the H3R8 HMT Prmt2 is necessary for the establishment of the dorsal gene expression program.

Zygotic depletion of Prmt2 results in highly penetrant lethality at the gastrula-stage (Figure 3.10A and B), so that evaluation of later morphological defects is also not feasible. Because this phenotype is rescued by injection of Prmt2 mRNA and is not seen with a control morpholino, we conclude that this lethal phenotype is specific and reflects additional critical functions for Prmt2, for example a more general role in the regulation of chromatin structure. Unlike the maternal depletion, zygotic depletion of Prmt2 does not significantly affect expression of β-catenin target genes in early gastrulae (Figure 3.10C lane 10, and data not shown). Nonetheless, we tested whether zygotic Prmt2 depletion

could enhance a weak β-catenin loss of function phenotype. Because the period of competency for embryos to become ventralized by ΔNTcf3-GR coincides with the interaction between β-catenin and Prmt2 (4 to 32-cell stage, Figure 3.1C and Figure 3.10C, lanes 3-5), we tested whether zygotic depletion of Prmt2 could enhance Wnt pathway inhibition by ΔNTcf3-GR at the 32-cell stage. Indeed, Prmt2 depletion enhances the weak inhibition of *siamois* expression by ΔNTcf3-GR activated at the 32-cell stage (lanes 6 & 7), and this is rescued by co-expression of Prmt2 mRNA (lanes 8 & 9). These results further support our observation that recruitment of Prmt2 is critical for the establishment of the dorsal developmental program. Since non-β-catenin bound Tcf3 functions primarily as a transcriptional repressor in early *Xenopus* embryogenesis (Houston et al., 2002) and that poised loci behave as insulator elements (Chopra et al., 2009), we propose a model where β-catenin's recruitment of Prmt2 to promoters establishes poised chromatin architecture that is refractory to transcriptional repression by ΔNTcf3 (or non-β-catenin-bound, wild type Tcf3). Future investigations will examine the mechanism whereby establishment of H3R8me2a is able to counteract ΔNTcf3-mediated transcriptional repression.

Figure 3.9


A
Maternal Depletion
100%
80%
60%
40%
20%
0%
Pre-MBT Arrest
Control
β-cat
Prmt2
Control
β-catenin (-)
Prmt2 (-)
Early
Blastula


B
Maternal
Depletion
Host (-RT)
Host
Control
β-catenin
Prmt2
Siamois
Xnr3
ODC
Stage 9
Control
β-catenin (-)
Prmt2 (-)
Mid
Blastula

## Figure 3.9: Maternal Depletion of Prmt2 Suppresses Dorsal Gene Expression.

(A) Maternal depletion of Prmt2 results in a dose-dependent pre-MBT cell cycle arrest phenotype. Maternal depletion by host transfer was performed with injection of 10ng β-MO or 10-, 20-, or 40ng Prmt2MO. Compared to control (non-injected) and β-MO injected embryos, Prmt2MO injected embryos arrest dose dependently during early development. Pictured at right are representative images of control, β-catenin depleted, and Prmt2 depleted (40ng) embryos. (B) Maternal Prmt2 depletion suppresses dorsal gene expression. Expression of *siamois* and *xnr3* was measured by RT-PCR in morphologically normal control, β-catenin depleted, and Prmt2 depleted (10ng) embryos (representative embryos pictured at right) compared to host embryos (not pictured). Similarly to β-catenin depletion, maternal Prmt2 depletion reduces *siamois* and *xnr3* expression after the MBT (stage 9). *ODC* is shown as a loading control.

Figure 3.10

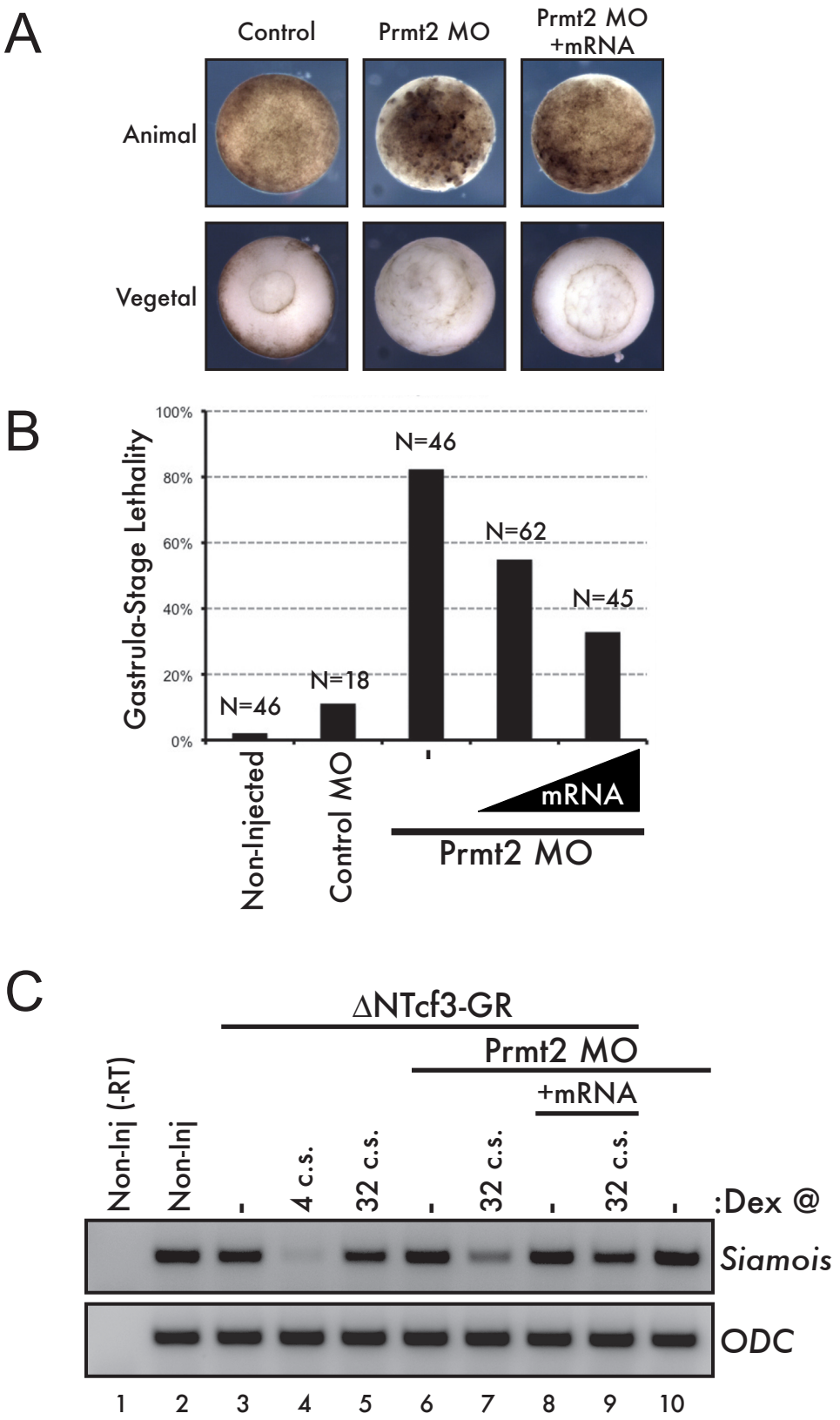

A
Control
Prmt2 MO
Prmt2 MO +mRNA
Animal
Vegetal
B
Gastrula-Stage Lethality
100%
80%
60%
40%
20%
0%
N=46
N=18
N=46
N=62
N=45
Non-Injected
Control MO
mRNA
Prmt2 MO
C
ΔNTcf3-GR
Prmt2 MO
+mRNA
Non-Inj (-RT)
Non-Inj
4 c.s.
32 c.s.
32 c.s.
32 c.s.
:Dex @
Siamois
ODC
1 2 3 4 5 6 7 8 9 10

**Figure 3.10: Zygotic Prmt2 depletion is gastrula-stage lethal, and enhances a weak β-catenin loss-of-function phenotype.**

(A) Zygotic Prmt2MO injection is gastrula-stage lethal. Representative animal (top) and vegetal (bottom) views of injected embryos demonstrates that, compared to controls (left), Prmt2MO injection (40ng, center) results in abnormal cellular morphology, cell lysis, and embryonic death at sibling stage 11-12. Co-expression of Prmt2 mRNA (500pg, right) rescues gastrula-stage lethality.

(B) Gastrula-stage lethality in Prmt2MO-injected embryos is specific for loss of Prmt2 function. The histogram quantifies gastrula stage lethality in non-injected (column 1), control morpholino- (column 2, 40ng), and Prmt2MO-injected (column 3, 40ng) embryos from three independent experiments. Greater than 80% of Prmt2MO-injected embryos fail to survive gastrulation, and this phenotype is rescued dose-dependently by Prmt2 mRNA (column 4: low dose, 100-200pg mRNA; column 5: high dose 400-600pg mRNA).

(C) Zygotic depletion of Prmt2 enhances a weak β-catenin loss of function phenotype. Control and Prmt2MO-injected (40ng) embryos were injected with either ΔNTcf-GR (500pg) or ΔNTcf-GR and Prmt2 mRNA (500pg each), treated with dexamethasone at either the late 4-cell stage (4 c.s.) or the late 32-cell stage (32 c.s.), and collected for marker analysis at stage 10. Compared to non-injected controls (lane 2) or no-dexamethasone controls (lanes 3, 6, & 8), *siamois* expression is strongly reduced by ΔNTcf-GR activation at the 4-cell stage but not at the 32-cell stage. Zygotic depletion of Prmt2 enhances repression of *siamois* expression by 32-cell stage activated ΔNTcf-GR (lane 7). Co-expression of Prmt2 mRNA suppresses the effect of zygotic Prmt2 depletion on ΔNTcf-GR function (lane 9). Zygotic depletion of Prmt2 alone has no effect on *siamois* expression at stage 10 (lane 10).

## 3.3: Discussion

This work identifies a complex pre-transcriptional mechanism underlying cell fate specification in early embryos, and also represents the first detailed analysis of chromatin architecture at endogenous loci prior to the MBT. We demonstrate that β-catenin functions to establish poised chromatin architecture at its target genes under conditions of global transcriptional repression. Additionally, β-catenin interacts with the H3R8 HMT Prmt2 between the 4- and 32-cell stages. Recruitment of Prmt2 to organizer gene promoters before the MBT is both necessary and sufficient to establish the dorsal gene expression program. The constellation of marks established at organizer gene promoters suggests a mechanism for transcriptional poising that may play a critical role in early embryogenesis. The following sections summarize major topics that will be discussed in greater depth in Chapter 4.

### *3.3.1: H3R8 Methylation and the Histone Code*

A remaining question is what role asymmetric H3R8 methylation plays in either the establishment or maintenance of active chromatin architecture, in particular H3K4 methylation. The Histone Code Hypothesis postulates that histone modifications function combinatorially to regulate events such as transcriptional activation (Strahl and Allis, 2000). Along these lines, H3R8

methylation could recruit downstream complexes containing an H3K4 HMT, resulting in the subsequent methylation of H3K4. Although factors that “read” H3R8me2a have yet to be identified, histone arginine methylation influences the patterns of histone tail modifications [reviewed in (Bedford and Clarke, 2009), and see below]. Alternatively, H3R8 methylation could inhibit the activity of a repressive factor, such as an H3K4 de-methylase, which would otherwise maintain promoter transcriptional repression. In support of this model, H3R8 methylation has been shown to inhibit de-methylation of K4 by LSD-1 (Forneris et al., 2006). In addition, we observe that β-catenin mediated H3R8 methylation is sensitive to the modification state of the neighboring residue, K9, as also observed for the symmetric H3R8 HMT Prmt5 (Pal et al., 2004). Similarly, Prmt1, whose methylation of Histone H4R3 potentiates downstream H4 acetylation, will not methylate a pre-acetylated H4 tail (Wang et al., 2001). Conversely, H3K9 methylation by the HMT G9a is inhibited by methylation of H3R8 (Rathert et al., 2008). These observations suggest that H3 R8 and K9 antagonism represents a critical regulatory node underlying the interpretation of the Histone Code, where asymmetric methylation of R8 activates and K9 represses gene expression. We speculate that antagonistic H3K9 methylation could thus represent a mechanism to restrict the expression domains of dorsal determinants in early embryos.

### *3.3.2: Pre-Setting Patterns of Gene Expression in the Embryo*

Transcriptional poising occurs in eukaryotes from yeast to mammals in a wide range of contexts (Bernstein et al., 2006; Guenther et al., 2007; Muse et al., 2007; Radonjic et al., 2005; Rougvie and Lis, 1988; Zeitlinger et al., 2007). A promoter is poised when it bears hallmarks of transcriptional activity in the absence of detectable mRNA expression, either due to polymerase pausing/stalling or the presence of counteracting chromatin modifications (Margaritis and Holstege, 2008). While pre-MBT *Xenopus* embryos are fully transcriptionally competent (Prioleau et al., 1994), several overlapping mechanisms dominantly suppress zygotic gene expression [reviewed in (Veenstra, 2002)]. For example, a plasmid with the minimal *myc* promoter will establish active patterns of DNase-hypersensitivity before the MBT and can interact with DNA-binding factors to establish a committed but repressed chromatin structure (Prioleau et al., 1995). Interfering with these repressive activities can reveal a suppressed pro-transcriptional activity. Depleting embryos of the DNA methyltransferase Dnmt1 causes the precocious expression of many genes, suggesting that these genes are poised for activation prior to the MBT, but are prevented from being transcribed due to the counteracting influence of Dnmt1 and DNA methylation (Stancheva and Meehan, 2000). Also, active chromatin architecture can be inherited epigenetically through the pre-MBT period and embryos generated from transplantation of transcriptionally active

nuclei will display pre-MBT expression of genes that were active in the original donor cells (Ng and Gurdon, 2005). This transcriptional memory is linked to chromatin modifications that correlate with active transcription, particularly the incorporation of the histone variant H3.3 (Ng and Gurdon, 2008). In sum, these observations demonstrate the competency of pre-MBT embryos to establish and maintain active-but-repressed (i.e. poised) chromatin.

This work extends these observations by demonstrating that transcriptional poising underlies pre-MBT dorso-ventral cell fate specification. Before the MBT, β-catenin interacts with target promoters, resulting in methylation of H3R8 and, in collaboration with Pol II, H3K4. H3K4me3, in particular, correlates with active gene expression (Santos-Rosa et al., 2002), but has also been observed at poised loci (Bernstein et al., 2006; Guenther et al., 2007). The Wnt pathway component Pygopus binds methylated H3K4 via its PHD domain (Fiedler et al., 2008). Pygopus is also absolutely required for dorsoventral specification (Belenkaya et al., 2002), further raising the possibility that Pygopus functions downstream of H3K4 methylation to activate dorsal gene expression at the MBT. Another common feature of transcriptionally poised loci is the occupancy of stalled Pol II, which we also observe at *siamois* and *xnr3* before the MBT (Figure 2E). The identity of the counteracting repressive influence at these loci is currently unknown, but our results are consistent with a model where regulated entry into the elongation phase of transcription accounts for pre-MBT gene repression at the *siamois* and *xnr3* loci.

The pre-MBT β-catenin target genes have a brief period of competency to become transcriptionally repressed by ΔNTcf3, which is sensitive to both Pol II inhibition and depletion of Prmt2 [(Yang et al., 2002b) and Figure 3.10C]. We conclude that this competency is linked to the establishment of poised chromatin architecture at β-catenin target genes. Since transcriptionally poised loci can behave as chromatin insulators (Chopra et al., 2009), we propose that the poised chromatin architecture of the *siamois* and *xnr3* loci serves the dual purpose of marking these genes for activation at the MBT, and for antagonizing the possible down-regulation of these genes by transcriptional repressors, such as non-liganded Tcf3.

### *3.3.3: Context-dependent chromatin modifying activities for β-catenin?*

Our work also indicates that, in early embryos, β-catenin associates with a large complex that contains HMT activity (Figure 3.4). Numerous studies have identified association of β-catenin with other chromatin modifying and remodeling factors. A recent genome-wide screen for β-catenin interactors in colorectal carcinoma cell lines (Major et al., 2008) confirmed several of these interactions and identified novel complex members. Notably, the Histone H4 arginine methyltransferase Prmt1 was identified as part of the extended β-catenin interaction network. Carm1 has also been shown to interact with β-catenin in the context of androgen receptor signaling (Koh et al., 2002). In pre-MBT embryos, β-catenin interacts with Prmt2, but not with Carm1 or Prmt1, suggesting that the

β-catenin/chromatin remodeling complex associates with different Prmt family members in a context-dependent manner. Additionally, in colon cancer cell lines, the β-catenin/chromatin remodeling complex contains the MLL family of H3K4 methyltransferases (Major et al., 2008; Sierra et al., 2006). Our analysis indicates that, in early *Xenopus* embryos, the primary HMT activity associated with β-catenin is directed at H3R8, but the possibility remains that β-catenin interacts with MLL family members in later developmental stages. A systematic comparison of these complexes from diverse tissue sources and developmental stages will be necessary to investigate this possibility further.

# Chapter 4: Conclusions and Future Directions

In this work, I have addressed how the Wnt/β-catenin signaling pathway functions before the MBT to specify dorsal cell fates. I have uncovered a mechanism of transcriptional poising that underlies the activation of critical dorsal determinants, which illustrates a multi-step process for the activation of gene expression where trans-activator function is uncoupled from the transcriptional response. Because a number of additional genes are induced immediately following the activation of zygotic gene expression at the MBT, this mechanism of transcriptional poising may extend to other gene regulatory networks that function in the early embryo. Furthermore, this work suggests that regulated entry into productive transcriptional elongation may serve as a mechanism for regulating the onset of zygotic gene expression at the MBT. In the following section, these conclusions will be explored in greater depth, and follow-up experiments will be suggested to expand this work in several new directions.

## 4.1 Accounting for pre-MBT β-catenin activity: Prmt2 function

Shortly following the activation of the Wnt/β-catenin pathway in future dorsal blastomeres, β-catenin functions to poise its target genes for activation at the MBT. β-catenin recruits a large complex to target gene promoters that includes the H3R8 methyltransferase Prmt2, which is both necessary and sufficient to activate dorsal gene expression after the MBT. In particular, Prmt2's

function at pre-MBT promoters appears to prevent transcriptional repression by non-β-catenin bound Tcf3, suggesting that H3R8 methylation counteracts an antagonistic (and not yet identified) activity associated with Tcf3. In addition to this mark, β-catenin target gene promoters are also bound by RNA Pol II, and are acetylated at H3K9/K14, and methylated at H3K4. Of these additional modifications, H3K4 methylation requires both RNA Pol II and β-catenin function in order to be established. Since I have no evidence that early (pre-32-cell stage) β-catenin complexes have any H3K4 HMT activity, I propose that H3K4 methylation is established at target gene promoters downstream of H3R8 methylation. However, my analysis has not determined whether H3K4 methylation is dependent on prior H3R8 methylation, or whether these modifications function independently of one another to promote gene activation downstream of β-catenin. Thus, it will be critical to evaluate whether targeting Prmt2 to β-catenin target gene promoters is sufficient to establish H3K4me3 before the MBT, as this will determine whether establishment of H3K4 methylation is indeed dependent on prior H3R8 methylation. Thus, three models to account for the role of H3R8 methylation at β-catenin target genes are presented below.

### *4.1.1 The H3R8me2 "reader"/ H3K4me3 "writer" hypothesis*

As discussed in Chapter 3, several models could account for H3R8-dependent H3K4 methylation, where methylated H3R8 could serve either to

recruit a H3K4 HMT, or to prevent removal of this mark by a H3K4 demethylase, or to prevent the establishment of repressive chromatin modifications. The first model stems from a prediction of the “Histone Code” hypothesis, where histone modifications function combinatorially to influence gene expression (Strahl and Allis, 2000). Along these lines, histone modifications serve as ligands that recruit additional chromatin remodeling complexes, such that a pre-existing modification is “read” in order to “write” additional modifications or to recruit transcriptional-regulatory factors. Therefore, it is possible that H3R8 methylation directly functions in the establishment of H3K4 methylation at promoters, by serving as a ligand for a H3K4 HMT complex. Identifying a candidate H3R8 “reader” and H3K4 “writer” complex could begin with a biochemical purification of complexes that preferentially bind H3R8me2-modified but not non-modified H3 tail peptides. These complexes could be screened for *in vitro* H3K4-dependent HMT activity, followed by large-scale purification and identification of co-purified proteins by mass spectroscopy. If preliminary experiments were to suggest that early embryos express factors that preferentially bind to H3R8-methylated H3 peptides, identification of these factors would be informative as well. While numerous factors bind H3 methylated at lysine residues (Ruthenburg et al., 2007), fewer methyl-arginine binding factors have so far been identified. In these few cases, the methyl-arginine binding factors contain Tudor domains (Côté and Richard, 2005; Vagin et al., 2009), but no interactions between arginine-methylated histone tails have been shown for these factors. Pursuing this model would thus likely identify a novel H3-directed methyl-arginine reader.

Furthermore, if the reader were also to bind a H3K4 HMT, this would support the hypothesis that H3R8 methylation can serve as a scaffold for the recruitment of additional chromatin modifying activities. The proposed role of H3R8 reading and H3K4 methylation in dorsal specification could be tested further by loss of function for these identified factors.

### *4.1.2 The H3R8me2 "lock" hypothesis*

While the first model provides a direct mechanism for the establishment of H3K4 methylation in response to β-catenin dependent H3R8 methylation, it is also possible that H3K4 methylation is established independently of H3R8 methylation, which instead functions in a protective role. Thus, H3R8 methylation could prevent the activity of a H3K4 demethylase, thereby "locking-in" H3K4me3 at promoters and preserving this mark for the eventual activation of β-catenin target genes. H3R8 methylation prevents demethylation of H3K4 by LSD1 in cultured cells, but it is unknown whether this is also the case in an embryonic setting (Forneris et al., 2006). Interestingly, Tcf3 interacts with the co-repressor complex CtBP (Brannon et al., 1999), which has been shown by others to contain LSD1 [named NPAO in (Shi et al., 2003)]. Thus, it will be interesting to determine whether Tcf3 interacts with LSD1 in pre-MBT embryos, as this could nicely link this proposed mechanism for H3R8 methylation with the effects of Prmt2 depletion on transcriptional repression by ΔNTcf3 (Chapter 3, Figure 3.10). Preliminary studies could determine whether Tcf3 physically interacts with LSD1

or other H3K4 demethylases. Subsequent experiments could screen for MBT-stage binding of known H3K4 demethylases to the *siamois* and *xnr3* promoters either in ventral blastomeres or in β-catenin depleted embryos. Alternatively, candidate demethylases could be pre-screened for sensitivity to H3R8 methylation by *in vitro* demethylation assays (Shi et al., 2004; Whetstine et al., 2006). This model predicts that loss of function for this R8-sensitive demethylase would result in expanded dorsal gene expression, so *in vitro* results could be confirmed in an embryonic context as well. However, loss of function for H3K4 demethylases may likely demonstrate similar pleiotropy as seen with the depletion of Prmt2.

Notably, this second model does not directly account for the mechanism whereby H3K4 methylation is established at poised β-catenin target promoters. I have shown that, in addition to β-catenin, RNA Pol II phosphorylated at CTD serine 5 binds the *siamois* and *xnr3* promoters and that RNA Pol II function is also necessary for the deposition of H3K4me3. In budding yeast, the COMPASS complex, containing the Set1 HMT, catalyzes promoter H3K4 methylation (Krogan et al., 2002). In yeast, as in metazoans, H3K4me3 is largely restricted to the 5' end of genes. This 5' restriction of H3K4me3 reflects the interaction of COMPASS with initiating (CTD Ser5-phosphorylated) but not elongating (CTD Ser2-phosphorylated) RNA Pol II (Krogan et al., 2003; Ng et al., 2003b). COMPASS is recruited to RNA Pol II by the Paf1 protein complex, which is itself a critical factor for mediating the switch between initiation- and elongation-phases of transcription (Krogan et al., 2003; Shi et al., 1996). Besides recruiting

COMPASS to RNA Pol II, Paf1 further potentiates H3K4 methylation by recruiting the Rad6/Bre complex. Rad6 ubiquitylates H2B, and this is a prerequisite for promoter H3K4 methylation and transcriptional activation (Dover et al., 2002; Ng et al., 2003a; Wood et al., 2003). Importantly, in *Drosophila*, β-catenin has been shown to interact with the Paf1 complex, and this interaction is necessary for the full transcriptional output of the Wnt/β-catenin pathway (Mosimann et al., 2006). Were β-catenin to recruit Paf1 to poised loci before the MBT, Paf1 could serve as a means to bridge directly β-catenin with the RNA Pol II complex. β-catenin could present stalled RNA Pol II with the Paf1 complex, which would subsequently promote both H2B ubiquitylation and H3K4 methylation. Future work will address whether β-catenin recruits Paf1 to poised loci, and whether these promoters are also marked by H2B ubiquitylation. Since Paf1 also mediates the transition from transcriptional initiation to elongation, it will be intriguing to determine whether β-catenin recruits Paf1 to poised loci, which are presumed to be stalled between initiation and elongation.

Alternatively, I have not ruled out that β-catenin itself eventually recruits a H3K4 HMT following its initial binding to promoters. Indeed, β-catenin has been shown to interact with metazoan homologs of the yeast Set1 protein, the MLL family of Histone H3K4 HMTs, thereby directing H3K4 methylation to target gene promoters (Major et al., 2008; Sierra et al., 2006). In *Xenopus* embryos, I do not detect any β-catenin associated H3K4-directed HMT activity prior to the 32-cell stage of development. Thus, to develop this hypothesis, it is necessary to

consider a model where chromatin-remodeling complexes are interchanged on the C-terminal transactivation domain (CTA) of β-catenin.

While experimental evidence for shuttling of factors on the β-catenin CTA is lacking, this hypothesis has been suggested elsewhere (Mosimann et al., 2006). This hypothesis is appealing because a large number of factors that interact directly with overlapping portions of the β-catenin CTA have been identified in several experimental systems, and it is difficult to account for how they could all interact simultaneously. In certain examples, it is clear that they do not: the β-catenin CTA has been shown to interact with the p300/CBP histone acetyltransferases (HATs) (Hecht et al., 2000; Takemaru and Moon, 2000), yet little or no β-catenin associated HAT activity is detected in either pre-MBT *Xenopus* embryos (Chapter 3), or in a colon carcinoma cell line (Sierra et al., 2006). This suggests that β-catenin could interact with any number of its potential partners in a context-dependent manner, and furthermore that these factors could switch during either the early and late phases of the transcriptional cycle, or over the course of embryonic development.

I have developed a method for isolating a subpopulation of cellular β-catenin that contains chromatin-modifying activity. The possibility of factor switching on the β-catenin CTA could be addressed by comparing this complex across different timepoints in development, for example to determine if the CTA ever gains HAT activity, or switches between a H3R8- and H3K4- HMT activity. Interestingly, Prmt2 and β-catenin interact in an ATP-dependent manner (Chapter 3), suggesting that β-catenin's acquisition of an H3R8-directed HMT

activity is a regulated process. This complex assembly assay provides a starting point for further investigation of the factors that could regulate the characteristics of β-catenin chromatin modifying activities. Additional experiments could test for the requirement of serine/threonine or tyrosine kinase activites for Prmt2 and β-catenin to interact by adding specific phosphatases or kinase inhibitors to the *in vitro* complex assembly reactions. If kinase activity is necessary for complex assembly, identification of target proteins could begin by including [$^{32}$P]-γATP in the complex assembly reactions, which would radiolabel target phosphorylated proteins within the complex, and facilitate their eventual identification. Additionally, based on the *in vitro* inhibitor / phosphatase experiments, it may be possible to hypothesize which class of kinase is responsible for mediating complex assembly. In this case, a role for such a kinase during dorsal specification could be tested *in vivo* by loss of function analysis. Alternatively, an ATP-dependent chaperone activity could be required to assemble the β-catenin/Prmt2 complex. If this were the case, a biochemical approach could be taken to identify the putative chaperone, beginning with the fractionation of the cell lysate followed by mass spectroscopy on fractions capable of mediating the complex assembly reaction.

### *4.1.3 The H3R8 / H3K9 competition hypothesis*

Finally, Prmt2-mediated H3R8 methylation could prevent establishment of transcriptionally repressive chromatin modifications–such as H3K9 methylation–

at β-catenin-bound promoters. H3K9 methylation is a critical component of constitutive and facultative heterochromatin (Shilatifard, 2006). H3K9-methylated nucleosomes serve as binding sites for the HP1 family of transcriptional repressors, thereby functioning as a fundamental building block for gene silencing. I observe that β-catenin mediated H3R8 methylation is sensitive to the modification status of the neighboring residue (K9) (Chapter 3), which has also been observed for the symmetric H3R8 HMT Prmt5 (Pal et al., 2004). Conversely, H3R8 methylation inhibits the methylation of H3K9 by the HMT G9A (Rathert et al., 2008). Therefore, the antagonism between modifications at H3R8 and K9 observed in other model systems suggests a similar regulatory mechanism could underlie β-catenin's activity in early *Xenopus* embryos. In this model, promoters not marked by methylated H3R8 would be subject to H3K9 methylation and packaging into heterochromatin, thereby silencing their expression and providing a mechanism to prevent misexpression of these genes during subsequent iterations of the Wnt/β-catenin pathway.

This model provides an attractive explanation for the embryo's competency to activate dorsal gene expression in response to the Wnt/β-catenin pathway. Before the MBT, the entire embryo is competent to activate the dorsal gene expression program in response to the Wnt/β-catenin pathway, but the embryo undergoes a change in competency to respond to this pathway at the MBT (Darken and Wilson, 2001; Hamilton et al., 2001; Kao et al., 1986; Yamaguchi and Shinagawa, 1989). This is also evident in the embryo's endogenous gene expression patterns, as dorsal determinants activated by

maternal Wnt signaling are not expressed in the ventrolateral compartment of the embryo upon activation of zygotic Wnt8. The competence of the embryo to activate dorsal gene expression may be reflected in the following: before the MBT, in the absence of β-catenin activity, promoters are nonetheless marked by H3K9/K14 acetylation, and bind CTD Ser5-phosphorylated Pol II, which suggests—at the very least—open chromatin architecture that is capable of binding (and eventually being activated by) β-catenin (Chapter 3, see Figure 3.2). It would be interesting to investigate whether the promoters that fail to bind β-catenin but are nonetheless maintained in an open state before the MBT become transcriptionally silenced or if they persist in an open conformation after the MBT. Similarly, the lack of H3K9-me2 and -me3 at promoters and the ability of β-catenin to establish H3R8me2 until at least an hour before the MBT could reflect this competency as well (Chapter 3). Unfortunately, I have not yet determined whether these properties of β-catenin target gene promoters change after the MBT. However, on the basis of this model, I predict that –after the MBT– non-utilized maternal β-catenin target gene promoters will lose their open conformation and become packaged in transcriptionally silent heterochromatin that is refractory to H3R8 methylation. Future work will address whether this chromatin-based mechanism underlies the competency for the embryo to activate the dorsal gene expression program.

This model also stipulates that an additional factor be recruited to β-catenin target genes after the MBT in order to silence them by methylating H3K9. Two candidates for this post-MBT repressor are BMP-activated Smad1/5 and

non-β-catenin bound Tcf3. Smad1 has been shown to interact with the H3K9 HMT Suv39H1 in certain contexts (Frontelo et al., 2004), although this result has not been confirmed in *Xenopus*. The BMP pathway, however, is the major ventralizing pathway in post-MBT embryos. Notably, the BMP pathway is activated in embryos at the MBT, at the time when the embryo loses competence to activate dorsal gene expression in response to the Wnt/β-catenin pathway (Lee et al., 2001). However, preventing Smad1/5 phosphorylation by depleting embryos of all three major BMP ligands (BMP2 -4, and -7) does not result in a strong dorsalized, maternal β-catenin gain of function phenotype, as would be expected if Smad1 was solely necessary for regulating the competence of embryos to respond to the Wnt/β-catenin pathway (Reversade et al., 2005). On the other hand, while Tcf3 has not (yet) been demonstrated to interact with a H3K9 methyltransferase, depletion of maternal Tcf3 dorsalizes embryos, indicating that the primary function of maternal Tcf3 is to prevent ectopic activation of dorsal gene expression (Houston et al., 2002). Indeed, Tcf3-depleted embryos activate *Siamois* and *Xnr3* expression in the ectodermal animal cap after the MBT, which is not expected to have substantial Wnt/β-catenin activity even after the MBT (Houston et al., 2002). In light of my model, this could be explained by the failure to close the open chromatin structure of these genes, resulting in their incidental and opportunistic expression throughout the embryo. These observations are supported by studies on the *Siamois* promoter, where deletion of Tcf3 binding sites results not in the failure to activate expression on the dorsal side of the embryo, but rather ectopic ventral

expression (Brannon et al., 1997). Non-liganded Tcf3 does interact with co-repressor complexes that are displaced from chromatin following β-catenin binding (Brannon et al., 1999; Roose et al., 1998). Since H3K9 HMTs have been shown to be an integral part of certain co-repressor complexes (Schultz et al., 2002; Vaute et al., 2002), it is possible that the interaction of Tcf3 with a H3K9 HMT has simply gone unnoticed.

If the BMP pathway is responsible for modulating the competence of the embryo to respond to the Wnt/β-catenin pathway, then I predict that embryos lacking BMP pathway activity will become dorsalized following activation of the Wnt pathway even after the MBT. While BMP2/4/7-depleted embryos are not severely dorsalized, it is possible that these embryos are also impaired in zygotic Wnt8 expression, which would remove the main source of ventral zygotic β-catenin from the embryo (Reversade et al., 2005). These experiments could be repeated, with additional treatment of embryos with lithium chloride (LiCl) before and after MBT. Treatment before MBT will dorsalize embryos, whereas control embryos are not dorsalized by post-MBT treatment. If I impair the switch in competency to activate dorsal gene expression by depleting BMP2/4/7, then it is expected that post-MBT LiCl treatment will result in dorsalized embryos. It is also possible that targeting an H3K9 demethylase to Tcf/Lef binding sites could extend the competency of the embryo to activate dorsal gene expression in response to LiCl treatment, regardless of the recruiting factor. Since it is already known that depletion of Tcf3 results in ectopic dorsal gene expression (Houston et al., 2002), I could determine whether Tcf3 interacts with a H3K9 HMT activity

after the MBT. If this were to be the case, then the associated HMT could be identified by mass spectroscopy or by screening candidate factors, followed by loss of function analysis to determine whether Tcf3-mediated H3K9 methylation underlies the embryo's competency to activate dorsal gene expression in response to the Wnt/β-catenin pathway.

### *4.1.4 Concluding remarks: the function of H3R8 methylation at β-catenin target genes before the MBT.*

I have outlined three distinct models to account for the function of pre-MBT Prmt2-mediated H3R8 methylation at β-catenin target gene promoters. In summary, H3R8 methylation could serve either as an intermediate modification that recruits a H3K4 HMT, thus directly contributing to poised chromatin architecture, or by protecting poised loci from counteracting repressive factors. These models are not mutually exclusive: for example, it is not difficult to imagine that H3R8 methylation could both contribute to H3K4 methylation and eventually prevent its removal later in development. As such, pursuing the preliminary experiments outlined for each model will likely elucidate the role of this histone modification during dorsal specification, and will also establish a model for the chromatin-based regulation of cell fate specification during early embryogenesis. Importantly, the models in which H3R8 methylation protects promoters from inactivation raise the possibility that that the competence to respond to developmental signals is regulated at the level of chromatin. These models also

underscore the apparent importance of H3K4 methylation in establishment of poised chromatin architecture. β-catenin activity is required for establishment of H3K4 methylation at target gene promoters, functioning either directly or indirectly (as described above). In the following section, I will address β-catenin's role in the establishment of poised chromatin architecture, and consider the functional implications of promoter H3K4 methylation in pre-MBT embryos.

### 4.2 Poised chromatin architecture

Transcriptional poising represents a widespread mechanism of post-initiation control of gene expression. Transcriptionally poised loci are bound by RNA Pol II phosphorylated at CTD serine 5, and are marked by histone modifications that correlate with transcriptional initiation, H3K9/K14 acetylation and H3K4 trimethylation (Guenther et al., 2007; Margaritis and Holstege, 2008). As such, these loci are thought to have undergone successful pre-initiation complex formation, and the preliminary steps of transcriptional initiation. Poised RNA Pol II is typically stalled downstream of the promoter, at a checkpoint between the initiation to elongation phases of transcription (Saunders et al., 2006). Alternatively, RNA Pol II can be retained at the promoter, unable to progress beyond the "promoter clearance" phase of transcriptional initiation (Saunders et al., 2006). Finally, recent evidence suggests that poised loci can also undergo misregulated transcription throughout the entire gene, producing unstable, non-spliced transcripts due to a failure to recruit RNA processing

factors associated with the entry into the productive elongation phase of transcription (Hargreaves et al., 2009). In the case of pre-MBT poised loci, I observe CTD Ser5 phosphorylation of RNA Pol II, which suggests that the polymerase complex has undergone promoter escape and is either stalled or engaged in misregulated transcription. In the context of poised gene regulation, gene-specific trans-activators (such as c-Myc, HSF, and NFκB) typically function by recruiting elongation factors to otherwise poised loci, thereby releasing RNA Pol II from promoter-proximal stalling (Eberhardy and Farnham, 2001, 2002; Hargreaves et al., 2009; Lis and Wu, 1993). One significant observation that arises from my work is that β-catenin does not appear to function to release RNA Pol II from stalling at poised promoters; rather, β-catenin functions to establish fully poised chromatin architecture before the MBT.

In the absence of β-catenin, dorsal target gene promoters have certain elements of poised chromatin architecture –stalled RNA Pol II and H3K9/K14 acetylation– but lack H3K4 trimethylation. Thus, in the absence of β-catenin, these promoters could be said to be “incompletely poised”. As discussed above (Chapter 4.1.3), this incompletely poised chromatin architecture indicates that these promoters are maintained in an open conformation, and maintenance of this basal, open state could possibly underlie the embryo’s competence to activate the expression of these genes.

Upon activation, β-catenin binds to these open promoters and, in collaboration with RNA Pol II, introduces the final component of poised chromatin architecture, H3K4 trimethylation. As discussed above (Section 4.1), H3K4

trimethylation could be established at these promoters either directly or indirectly by β-catenin. Unfortunately, little is known about the role of H3K4 trimethylation in the context of transcriptional poising. This mark is characteristically established at sites of transcriptional initiation, yet H3K4 methylation also coordinates the recruitment of 3' RNA processing machinery (reference), indicating a role for this modification throughout the transcriptional cycle. In the case of poised loci, it is not clear if acquisition of H3K4 trimethylation simply coincides with the extent that RNA Pol II has entered into the transcription cycle, or whether this mark plays an active role in the regulated maintenance of transcriptional poising. Along these lines, binding of β-catenin could advance RNA Pol II one step further in the initiation process, coincidentally establishing H3K4 methylation in the process. Alternatively, binding of β-catenin and establishment of H3K4 methylation could establish a landmark at poised promoters to facilitate their release into productive elongation. This raises the question of what mechanism maintains stalling before the MBT, and which factors function to release the poised polymerase at the MBT. In the following section I will discuss a generalized model for the maintenance of poised RNA Pol II and consider how β-catenin function contributes to this state.

### *4.2.1 β-catenin, H3K4me3, and ISWI: a potential regulatory node in poised chromatin architecture?*

The sequence of events for the establishment, maintenance, and activation of transcriptionally poised loci has been characterized in the most detail for the *Drosophila* heat shock promoters. One critical factor for establishing poised chromatin architecture is the ATP-dependent chromatin remodeler ISWI (Tsukiyama), which is initially recruited by pioneering factors in order to open chromatin structure and promote pre-initiation complex formation. Notably, ISWI has additional roles throughout the transcriptional cycle, both mediating pausing/stalling of RNA Pol II at the transition between initiation and elongation, and coordinating 3' processing events later in elongation [reviewed in (Sims et al., 2004)]. It is conceivable that ISWI complexes are transferred from one recruitment site to another as ISWI carries out these distinct functions. Importantly, ISWI could be recruited by H3K4me3 to newly initiated RNA Pol II (Santos-Rosa et al., 2003; Wysocka et al., 2006). Based on my observation that β-catenin is necessary for H3K4me3 at poised promoters, and observations from others that ISWI interacts both physically and genetically with β-catenin (Liu et al., 2008; Sierra et al., 2006), I propose that establishment of H3K4me3 by β-catenin could serve as a molecular switch to recruit factors, such as ISWI, that directly regulate RNA Pol II stalling. In the following section, I will present a

synthesis of observations from several model systems both to discuss poised chromatin architecture, and to demonstrate the basis for this new hypothesis.

The establishment of poised chromatin architecture at the *Drosophila hsp70* locus begins with the binding of GAGA factor [Trithorax-like and perhaps others (Biggin and Tjian, 1988; Farkas et al., 1994; Granok et al., 1995)], which opens chromatin structure and allows pre-initiation complex factors to bind the promoter (Shopland et al., 1995). GAGA factor recruits the chromatin-remodeling complex NURF (which contains the ATP-dependent remodeler ISWI) to promoters thereby opening chromatin and establishing of DNase hypersensitive sites at poised loci (Lu et al., 1992; Tsukiyama et al., 1995; Tsukiyama and Wu, 1995). Additionally, GAGA factor interacts with a subunit of TFIID, Taf3, and could thereby nucleate preinitiation complex assembly directly (Chopra et al., 2008). This role extends beyond the regulation of the *hsp70* locus, as recent genome-wide study in *Drosophila* found GAGA factor associated with a majority of poised loci (Lee et al., 2008). GAGA factor also remains associated with chromatin throughout the cell cycle, and therefore stably marks poised loci through subsequent cell divisions (O'Brien et al., 1995; Raff et al., 1994). Beyond its role in establishing poised chromatin architecture, GAGA factor is critical for restricting the spreading of heterochromatin, as GAGA factor mutants enhance position effect variegation (Farkas et al., 1994). Alternatively, the role of GAGA factor in establishing boundaries between silent and active chromatin could stem from its role in transcriptional poising, as poised loci also function as insulator elements (Chopra et al., 2009). Thus, GAGA factor pioneers the establishment of

open chromatin structure at poised loci via recruitment of NURF/ISWI, which facilitates the formation of a paused or stalled polymerase complex.

Despite extensive characterization of GAGA factor in *Drosophila*, there is no clear vertebrate homolog for GAGA factor/Trithorax-like on the basis of sequence conservation. Nevertheless, the role of GAGA factors in establishing a poised state is likely conserved as the *c-myc* promoter in mouse, human, and (most importantly) *Xenopus* also contains GAGA-binding sites necessary for formation of DNase-hypersensitivity and to establish transcriptional poising (Asselin et al., 1989; Krumm et al., 1992; Miller et al., 1989; Prioleau et al., 1995; Pyrc et al., 1992). Notably, the *siamois* promoter also contains at least one putative GAGA-binding site (bases 88-95 in Genbank AF016226), although its requirement for *siamois* expression has not been tested. Since, in the absence of β-catenin, the *siamois* and *xnr3* promoters are nonetheless maintained in an "incompletely poised" state, it is likely that a GAGA-like pioneer factor functions upstream of β-catenin to open chromatin (via recruitment of NURF) and to nucleate preinitiation complex formation.

In yeast, ISWI (Isw1p) regulates the transition between the initiation and elongation phases in transcription, serving to regulate both polymerase stalling and transcriptional elongation via distinct complex subunits (Morillon et al., 2003). These multiple functions of Isw1p likely require unique recruitment mechanisms to target the promoter or both the initiating and elongating RNA Pol II complexes. The yeast Isw1b complex, which consists of Isw1p and two additional proteins, Ioc2p and Ioc4p, mediates promoter proximal pausing. Besides serving to

establish open chromatin structure via ATP-dependent nucleosome remodeling, the Isw1b complex also interacts directly with RNA Pol II CTD kinase complex TFIIH (via Ioc2p), thereby mediating RNA Pol II CTD phosphorylation at serine 5 (Morillon et al., 2003). Yeast mutant for the Ioc2p subunit fail to phosphorylate serine 5 of the CTD and prematurely enter the elongation phase of transcription by bypassing promoter proximal stalling. This observation underscores the critical function of CTD serine 5 phosphorylation to coordinate the transition from transcriptional initiation to elongation. Interestingly, the other Isw1b complex subunit Ioc4 regulates the elongation phase of transcription, indicating that the Isw1b complex plays several roles over the course of the transcription cycle (Morillon et al., 2003). While the *Drosophila* NURF and yeast Isw1b complexes are molecularly distinct, both complexes contain the highly conserved ATP-dependent chromatin-remodeling subunit ISWI, and are proposed to regulate the stalling checkpoint between initiation and elongation. Thus, in the case of poised loci, NURF/ISWI could have at least two phases of activity. In the first phase, NURF is recruited by GAGA factor in order to open chromatin structure and mediate CTD serine 5 phosphorylation by recruiting TFIIH. In the second phase, NURF is transferred to the initiated RNA Pol II complex to mediate stalling. NURF may therefore require an additional recruitment mechanism for its transfer from the GAGA factor to the newly initiated RNA Pol II complex.

Phosphorylation of the RNA Pol II CTD at serine 5 is obligatory for transcriptional poising (Schwartz et al., 2003) and recruits several complexes required for transcriptional regulation including H3K4 methyltransferases and

critical mediators of transcriptional stalling NELF and DSIF (see below). As discussed above (Section 4.1.2) the Paf1 complex mediates the interaction between serine 5 phosphorylated RNA Pol II and the H3K4 HMT complex, COMPASS, and this function is conserved for the MLL H3K4 HMT complex. Additionally, as described above (Section 4.1.2) β-catenin could function to present the Paf1 complex to newly initiated (CTD serine 5 phosphorylated) RNA Pol II, or could recruit the MLL complex directly in order to establish H3K4me3 at poised promoters. This establishment of H3K4me3 at promoters could serve as a tether for the NURF complex at promoters independently of GAGA factor, as the NURF complex subunit BPTF selectively binds H3K4me3 through its plant homeodomain (PHD) motif (Wysocka et al., 2006). Recruitment of ISWI complexes to H3K4me3 is conserved, as yeast Isw1b is also recruited to this mark (Santos-Rosa et al., 2003), perhaps via the PHD motif in Isw1b subunit Ioc2. Since β-catenin also interacts with ISWI itself (Sierra et al., 2006), β-catenin could alternatively recruit NURF to the stalled polymerase complex by transferring it to H3K4 methylated nucleosomes. Consequently, establishment of H3K4me3 at promoters could transfer NURF from pioneering factors to newly initiated, CTD serine 5 phosphorylated RNA Pol II. Therefore, I propose that by establishment of H3K4me3 at poised promoters, β-catenin functions to promote the transfer of NURF/ISWI from a GAGA-like pioneer factor to newly initiated RNA Pol II complexes in order to regulate RNA Pol II stalling.

RNA Pol II stalling is mediated by the dual action of the negative elongation factor (NELF) (Andrulis et al., 2000; Muse et al., 2007; Wu et al.,

2003), and by the positioning of nucleosomes that hinder further progression along the DNA template [reviewed in (Gilmour, 2009)]. NELF mediates stalling by forming a complex with CTD serine 5-phosphorylated RNA Pol II and the positive elongation factor complex DSIF. When bound by NELF, DSIF is unable to promote transcriptional elongation. The positive elongation factor complex p-TEFb (Cdk9/CyclinT) relieves NELF-mediated stalling by phosphorylating DSIF, RNA Pol II CTD serine 2, and NELF, which results in the dissociation of NELF from the complex and entry of RNA Pol II into productive elongation [reviewed in (Sims et al., 2004)]. In the case of the *Drosophila* heat shock promoters, recruitment of p-TEFb is sufficient to release the stalled polymerase (Lis et al., 2000). However, the sufficiency of p-TEFb to release stalled RNA Pol II does not rule out nucleosome positioning from playing a role in RNA Pol II stalling. In yeast, the Spt4 subunit of DSIF genetically interacts with ISWI to suppress RNA Pol II stalling (Morillon et al., 2003). This suggests that, upon derepression by p-TEFb, DSIF could stimulate entry into transcriptional elongation by activating NURF-dependent chromatin remodeling downstream of the stalled polymerase complex. Thus, interaction with H3K4me3 could bring NURF into proximity with newly initiated RNA Pol II to serve a dual function: to mediate the stalling checkpoint, and to facilitate the eventual release of RNA Pol II into productive elongation.

Due to its role in establishing open chromatin architecture, and potential roles in halting RNA Pol II at the stalling checkpoint, and eventually promoting the entry into transcriptional elongation, the NURF/ISWI chromatin-remodeling

complex could serve as a central hub for coordinating the establishment, maintenance, and transcriptional activation of poised chromatin architecture. I propose that establishment of H3K4me3 is essential for bridging the early, pioneering phase of NURF function to its later, transcriptional-regulatory function. To follow up on this hypothesis, initial experiments could determine whether NURF subunits interact with pre-MBT poised promoters, and whether this interaction is β-catenin dependent. While NURF is initially recruited to poised promoters by the pioneering GAGA factor, it is unknown how NURF is subsequently transferred to the stalled RNA Pol II complex. In the case of pre-MBT poised promoters, it is conceivable that β-catenin binding and H3K4 trimethylation serves as a switch to recruit NURF to the initiated RNA Pol II complex. This hypothesis predicts that loss of function for maternal ISWI will severely impair dorsal specification. Additionally, loss of function for maternal BPTF, the H3K4me3-binding subunit of NURF, could allow for a dissection of the proposed dual phases of NURF function during transcriptional poising. Pursuing this hypothesis would likely elaborate on the multiple observed roles of yeast ISWI complexes during the transcriptional cycle, and test the model that H3K4me3 serves as a critical switch that regulates transcriptional poising.

### 4.3 Transcriptional Poising and Cell Fate Determination

My work indicates that transcriptional poising underlies dorsal specification during *Xenopus* embryogenesis. In particular, the critical dorsal determinant, β-

catenin contributes to the establishment of poised chromatin architecture before the MBT, thereby marking genes for activation at the MBT. In general, this reflects a multi-step process of transcriptional activation, where initial pioneering factors open chromatin and promote preinitiation complex formation, and sequence-specific trans-activators are subsequently recruited either to establish transcriptional poising (e.g. pre-MBT β-catenin), or to release poised loci into productive elongation [e.g. c-Myc, NFκB, HSF (Eberhardy and Farnham, 2001, 2002; Hargreaves et al., 2009; Lis and Wu, 1993)]. I speculate that the initial, pioneered and open chromatin structure reflects the competency of an embryo to activate particular gene regulatory networks, and furthermore suggest that factors such as β-catenin direct cell fate specification by "locking in" this permissive chromatin structure. Essential to this model is the prediction that while factors like β-catenin direct cell fate specification by poising genes for activation, additional factors are necessary for releasing poised polymerase into productive elongation. In addition, I also speculate that the loss of competency to activate certain gene regulatory networks is accompanied by the closing of the pioneered, open chromatin structure. By establishing transcriptional poising, β-catenin serves a dual purpose: to promote the expression of target genes in response to secondary activating signals, and to protect these genes from transcriptional silencing caused by antagonistic regulatory mechanisms.

In light of my results, does transcriptional poising represent a major mechanism underlying zygotic genome activation at the MBT? As I have only focused on the output of a single gene regulatory network at the MBT, this

question remains open. Poising, however, is an attractive mechanism to explain the rapid induction of zygotic gene expression at the MBT, and extending this observation beyond the Wnt signaling pathway is certainly possible with current experimental approaches. The most common approach to identifying poised loci is to perform ChIP for RNA Pol II followed by deep sequencing of enriched genomic DNA [ChIP-seq, e.g., (Guenther et al., 2007; Muse et al., 2007; Zeitlinger et al., 2007)]. In the case of early *Xenopus* embryos, this approach could be impractical for several reasons, one being the limit of sensitivity for the ChIP-seq procedure, and the other being the lack of a whole genome sequence. To circumvent the lack of a *Xenopus laevis* genomic sequence, I could perform the experiment in *Xenopus tropicalis* or Zebrafish, however this will not alleviate the potential inherent limitation in ChIP-seq sensitivity. As an alternative approach, I could exploit the observation of Lis and colleagues (Lee et al., 1992; Rougvie and Lis, 1988) that in nuclear run-on experiments poised loci will produce transcripts in the presence of the detergent sarkosyl. Nuclei from pre-MBT embryos could be collected and subjected to nuclear run-on experiments in the absence and presence of sarkosyl, and poised loci would be determined by identifying sarkosyl-dependent transcripts by deep sequencing. While this procedure may also be limited by sensitivity, there is less of a limit on input material for the collection of nuclei as there is on the number of embryos that can easily be used in a ChIP experiment, so it would be more practical to scale-up a nuclear run-on experiment. The nuclear run-on approach would have the added benefit of identifying loci that are transcribed before the MBT, as they would be

transcribed in the absence of sarkosyl. Again, the lack of a genome sequence for *laevis* could be limiting in this case as well, so experiments could be performed in alternative model systems.

### *4.3.1 p-TEFb before the MBT, a clue from primordial germ cells?*

I propose that the establishment of a poised state promotes the rapid induction of target gene expression once the embryo reaches MBT. This additionally suggests that at least a subset of immediate-early zygotic genes are maintained silent during the pre-MBT period by regulated entry into productive elongation. This raises the additional question of how positive elongation factors such as p-TEFb are regulated before the MBT, and by what mechanism they are recruited to poised promoters upon activation of the zygotic genome. Notably, the *Drosophila* MBT regulator Smaug regulates the expression of p-TEFb subunits (Cdk9/CyclinT) (Benoit et al., 2009), supporting the idea that positive elongation factors are limiting in the pre-MBT embryo. In addition, p-TEFb is a critical regulatory target in the transcriptionally quiescent primordial germ cells of both *Drosophila* and *C. elegans* (Hanyu-Nakamura et al., 2008; Martinho et al., 2004; Zhang et al., 2003). P-TEFb activates transcriptional elongation by phosphorylating NELF, DSIF and RNA Pol II CTD serine 2, thus relieving NELF-mediated RNA Pol II stalling [reviewed in (Sims et al., 2004)]. P-TEFb is inhibited in primordial germ cells in *C. elegans* by PIE-1 (Ghosh and Seydoux, 2008; Zhang et al., 2003) and in *Drosophila* by the unrelated Polar Granule Component

(Pgc) protein (Hanyu-Nakamura et al., 2008; Martinho et al., 2004). Early evidence suggests that Pgc prevents p-TEFb recruitment to chromatin by sequestration, possibly in the cytoplasm (Hanyu-Nakamura et al., 2008). Unfortunately, little has been done on p-TEFb regulation in *Xenopus*. In light of the mechanisms described for p-TEFb regulation in transcriptionally quiescent primordial germ cells, it would be informative to determine, before the MBT, whether Cdk9 is catalytically active, or whether Cdk9 is sequestered in the cytosol. Since we have observed pre-MBT transcription of the *xnr5* and *xnr6* genes that is sensitive to the p-TEFb inhibitor DRB, this suggests that p-TEFb is not globally inactivated in early *Xenopus* embryos (Yang et al., 2002b). Interestingly, while pre-MBT embryos are mostly transcriptionally quiescent, a whole embryo lysate is capable of performing *in vitro* transcription (Prioleau et al., 1994; Toyoda and Wolffe, 1992). This suggests that interfering with the subcellular organization of the embryo is sufficient to circumvent pre-MBT transcriptional quiescence, perhaps due to sequestration of critical elongation factors, such as p-TEFb, in the cytoplasm. Finally, it is very likely that transcriptional poising underlies the apparent transcriptional silence of *Drosophila* and *C. elegans* primordial germ cells, although this has yet to be formally demonstrated. The functional conservation of regulatory mechanisms for the inhibition or p-TEFb and RNA Pol II elongation, coupled with the observation that RNA Pol II CTD serine 5 (but not serine 2) phosphorylation is present in primordial germ cells from each species (Seydoux and Dunn, 1997) strongly

suggests that genomic silencing in fly and worm germ cells is mediated by post-initiation regulatory mechanisms.

### *4.3.2 Poising and Gene Regulatory Networks*

In the context of gene regulatory networks, transcriptional poising adds an additional dimension to the interpretation of regulatory circuits. Typically, the successive programs of differential gene expression that comprise cell fate specification and differentiation are explained by linear relationships between transcription activators and their genomic targets. In this case, the establishment of a particular lineage would follow a genetic sequence of A→B→C, where early factors (A) represent “master regulators” of a developmental program which activate the expression of intermediate factors (B) that ultimately induce the expression of cell-type specific genes, characteristic of a fully differentiated lineage (C). Since transcriptional poising represents an uncoupling of trans-activator recruitment and a genomic transcriptional response, this suggests that the relationship between genes in a regulatory network need not be linear. While the activity of master regulators could still promote the expression of the intermediate factors, it is possible that master regulators could also function to promote the expression of cell-type specific genes (C) by poising them for activation by the intermediate factors (B). This is particularly attractive if one considers that intermediate factors could have several genomic targets (such that genes C and D have binding sites for B, yet B→C not D), and thus poising by

master regulators could ensure the expression of the proper target. This would be observed as the recycling of a signaling pathway or master regulator throughout a gene regulatory network, indicated by the constant genetic requirement for a factor to establish a lineage. This could be represented by A→B, (A+B)→C. In this case, it would not be known whether A's role in activating C in concert with B is due to a physical requirement for A at the C promoter, or to transcriptional poising by A earlier in development.

Experimentally, this distinction could be parsed out by examining the temporal requirement for a recycled master regulator over the course of differentiation. This is similar to my analysis of β-catenin function during dorsal specification using a hormone inducible dominant-negative TCF, where I find that β-catenin functions early, but not late in the establishment of dorsal gene expression (Chapter 3). Interestingly, β-catenin may play a similar role during mammalian hematopoiesis. While β-catenin has been shown to function during T- and B-cell development (Reya et al., 2000; van de Wetering et al., 2002; Xu et al., 2003), and during the self-renewal of hematopoietic stem cells (Reya et al., 2003), β-catenin must be deleted early, prior to lineage specification, in order to perturb hematopoiesis (Zhao et al., 2007). Deletion of β-catenin after the establishment of the lineage does not impair ongoing hematopoiesis (Cobas et al., 2004; Koch et al., 2008). This suggests that the early, embryonic function of β-catenin is sufficient to mediate β-catenin-dependent cell fate decisions throughout the lifespan of the animal, as hematopoiesis is not affected by conditional knock out of β-catenin in adults. Hypothesizing that β-catenin poises

promoters of hematopoietic lineage determinants could help account for the differences in the temporal requirements for β-catenin during blood development. To address this hypothesis, β-catenin promoter occupancy in hematopoietic stem cells could be examined by ChIP-seq, to determine whether β-catenin associates with both “intermediate factor” and “cell-type specific gene” promoters. Were β-catenin to interact with the promoters of cell-type specific gene promoters in advance of their expression, this would support a role for β-catenin in poising these genes for eventual activation. Follow-up work could determine whether, in fact, poised chromatin architecture is established at these genes in an “early” β-catenin dependent manner, by comparing RNA Pol II distribution in primary hematopoietic stem cells collected from control, “early-deleted” β-catenin, and “late-deleted” β-catenin mice. Discovering additional evidence of transcriptional poising as a critical regulatory mechanism during cell fate specification would lead to numerous new avenues of investigation towards the elucidation of chromatin-based mechanisms for developmental patterning.

# Appendix: Materials and Methods

### *Embryo collection and microinjection*

Embryo collection, manipulation, and microinjection was performed according to standard protocols (Sive et al., 2000). The β-catenin morpholino (Heasman et al., 2000) was heated to 65°C for 5 minutes and kept at room temperature prior to microinjection (20ng per blastomere at the 2-cell stage). For β-catenin morpholino rescue experiments, the morpholino was injected separately (at the 2-cell stage) from the rescuing construct (2 "dorsal" cells at the late 2 to 4-cell stage) to prevent precipitation of either component. We note that it was necessary to inject Prmt-ΔNLef1 mRNAs into the proximal sub-equatorial zone (4:00 position) to achieve rescue. Injection of Prmt-ΔNLef1 fusions was toxic to embryos when injected above the equatorial zone. The Prmt2 morpholino (5'- TGTCATTCCGTTCTGTATCTCTCCC -3') was reconstituted in $H_2O$ and stored at room temperature (as per the manufacturer's instructions) and working solutions were prepared in 0.1x MMR (final). Heating the Prmt2MO prior to injection was not necessary. For zygotic Prmt2 depletion, room temperature 1-cell stage embryos were injected once, equatorially, with 40ng Prmt2MO. Rescuing injections were performed separately, injecting mouse Myc-Prmt2 mRNA into both cells of a 2-cell embryo equatorially. The indicated amounts of mRNA specify the total amount injected per embryo.

The maternal Prmt2 depletion was performed essentially as described (Mir and Heasman, 2008). Manually defolliculated oocytes (>100 per group) were injected with 10, 20, or 40ng Prmt2MO or 10ng β-catenin MO and cultured for 24

hours prior to maturation with 2μM progesterone for 8-10 hours. Matured injected oocytes (and non-injected controls) were transferred to host females and collected at 1-hour intervals in high salt embryo collection medium (Mir and Heasman, 2008) and *in vitro* fertilized *en masse*.

Pre-MBT embryos were rigorously staged by timing all cell divisions (formation of cleavage furrows) between the 2-cell and 64-cell stage that occurred at room temperature, followed by calculation of the average cell cycle period (which varied from 22 to 26 minutes per cycle, depending on the batch of embryos). From these calculations, later pre-MBT stages were accurately predicted.

***Plasmids, in vitro transcription, and recombinant protein purification***

All plasmid constructs were generated in the vector pCS2+MT (Rupp et al., 1994) except where indicated. I.M.A.G.E. Consortium [LLNL] cDNA clones were obtained from Open Biosystems (Lennon et al., 1996). mRNA was synthesized by *in vitro* transcription using the SP6 mMessage mMachine kit (Ambion). All recombinant proteins were expressed and purified from BL21 Codon+RIL (Stratagene). Log-phase cells were grown in LB broth at 37°C and induced with 1mM IPTG for 3 hours prior to harvest. Purification of recombinant H3.3 was performed as previously reported (Himpel et al., 1999). Point mutations were generated with the Quikchange Site Directed Mutagenesis Kit (Stratagene). GST and GST:Prmt2 were purified under native conditions and retained on the glutathione sepharose beads for later use.

β-catenin-GFP (pCS2+): Full-length *Xenopus* β-catenin was PCR amplified and subcloned between the BamHI and EcoRI sites upstream of EGFP in the vector pCS2+ EGFP.

Siamois (pCS2+) was reported in (Kessler, 1997)

ΔNTcf3-GR (pCS2+): was reported in (Yang et al., 2002b) as ΔβTGR.

ΔNLef1: the HMG domain from mouse Lef1 (NM_010703.3 base pairs 1792 to 2176) was amplified from Lef1-ER pcDNA3 (Aoki et al., 1999) adding 5' XbaI and 3' NheI sites using Forward 5'- CGA GCC TCT AGA ACC TCA GGT CAA ACA GGA GCA C -3'; Reverse 5'- TAT AGT GCT AGC TCA GAT GTA GGC AGC TGT CA -3'. The resulting insert was subcloned into the XbaI site of pCS2+MT in frame with the 6x Myc Tag insert. This plasmid was used for the subcloning of Prmt candidates between the Myc and ΔNLef1 inserts.

Myc-Prmt2: the mouse Prmt2 coding sequence, lacking the start and stop codons, was amplified from IMAGE clone 40107558 adding 5' EcoRI and 3' XhoI sites using Forward 5'- GGA CTT GAA TTC AGA GGC ACC AGG AGA AGG TCC -3'; Reverse 5'- TAG GGA CTC GAG CCT CCA GAG AGG AAA GAC C -3'. The resultant insert was subcloned into either pCS2+MT or pCS2+MT ΔNLef1.

Myc-Carm1: the mouse Carm1 coding sequence, lacking the start and stop codons, was amplified from IMAGE clone 4935077, adding 5' and 3' EcoRI sites using Forward 5'- GGA CTT GAA TTC AGC AGC GGC GGC AGC GAC GGC GGT G-3'; Reverse 5'- GGC CTT GAA TTC AAA CTC CCA TAG TGC ATG GTG TTG GTC -3'. The resulting insert was subcloned into either pCS2+MT, or pCS2+MT ΔNLef1.

Myc-Prmt5: the mouse Prmt5 coding sequence, lacking the start and stop codons, was amplified from IMAGE clone 5321370 adding a 5' EcoRI site and a 3' XhoI site using Forward 5'- GGA CTT GAA TTC AGC GGC GAT GGC AGT CGG A -3'; Reverse 5'- TAG AGG CTC GAG GAG GCC TTA GGT ATA GGA GCG -3'. The resulting insert was subcloned into either pCS2+MT or pCS2+MT ΔNLef1.

Myc-Prmt6: the mouse Prmt6 coding sequence, lacking the start and stop codons, was amplified from IMAGE clone 5067159 adding a 5' EcoRI site and a 3' XbaI site using Forward 5'- GGA CTT GAA TTC ATC GCT GAG CAA GAA AAG AA -3'; Reverse 5'- ATG TGG TCT AGA GGG TCC TCC ACA GCA AAG TC -3'. The resultant insert was subcloned into either pCS2+ or pCS2+MT ΔNLef1.

HA-H3.3-$His_6$ (pET29): *Xenopus* H3.3 was amplified from IMAGE 4683607 adding a 5' BamHI sequence, a start codon, and a HA tag; and a 3' XhoI site using Forward 5'- AAA GGA TCC ATG TAC CCA TAC GAT GTG CCA GAT TAC GCT GCT CGT ACA AAG CAG AC -3'; Reverse 5'- AAA CTC GAG TTA AGC ACG TTC CCC ACG -3'. The resultant insert was subcloned into pCS2+. To subclone into pET29, HA-H3.3 was amplified from pCS2+ HA-H3.3 changing the 5' restriction site to NdeI using Forward 5'- AAA AAA CAT ATG TAC CCA TAC GAT GTG -3'; Reverse 5'- AAA CTC GAG AGC ACG TTC CCC ACG -3'. The resultant insert was subcloned into pET29b. Mutagenesis primers available upon request.

GST:Prmt2 (pGEX5): The Prmt2 insert from Prmt2 pCS2+MT was excised with EcoRI and XhoI and subcloned into pGEX5x3, downstream and in-frame with the GST tag. The pGEX5x3 vector alone was used to synthesize recombinant GST.

### *Sequencing of Xenopus Prmt2*

Searching both full-length cDNA and EST databases, we were unable to identify a distinct *laevis* Prmt2 transcript, but were able to identify Prmt2 homologs in both *Xenopus tropicalis* and *Danio rerio*. The *tropicalis* mRNA sequence was used to identify a singlet *laevis* EST 3' sequence deposited in the NCBI trace archives (EC276656). This EST corresponds to an I.M.A.G.E. clone (8532662), which we obtained and sequenced. This clone represents a full-length cDNA for *Xenopus laevis* Prmt2b (see below, RT-PCR), containing the entire predicted open reading frame and 5' UTR. (The full-length sequence will be submitted to Genbank).

### *RT-PCR*

RNA extraction, first strand synthesis, and PCR were performed as previously reported (Yang et al., 2002b). Purified RNA (5 embryos/sample) was DNase treated and re-purified by RNeasy (Qiagen). cDNAs were random primed from approximately 2.5μg (2/5ths embryo equivalent) of total RNA, and radiolabeled PCR was performed using the Promega GoTaq Flexi polymerase. The *Xenopus laevis* Prmt2 primer set was forward 5'- TTG CCA AAC CCC AGC CAG ACT A -3'; reverse 5'- AGT TCC AGG TGC CCT TGT TCT TCT -3'. This

primer set amplifies two products from early *Xenopus* embryos. The upper band corresponds to Prmt2a (298bp) as the parental *tropicalis* allele has additional sequence within the predicted amplicon. The lower band corresponds to the predicted RT-PCR product, Prmt2b (227bp). All other RT-PCR primer sets were as reported in (Yang et al., 2002b).

***Chromatin Immunoprecipitation***

Chromatin immunoprecipitation assays were performed exactly as described in (Blythe et al. 2009) with the exception of the experiments pictured in Figure 3.5C and E, which was performed on non-crosslinked chromatin, and omitting phosphatase inhibitors from the lysis buffer. Under these conditions, limited sonication produced sheared chromatin of 500bp average size.

Nested radiolabeled PCR was performed to amplify ChIPped DNA using the following primer sets. The outer *Siamois* and inner *Mlc2* primer sets were first reported in (Park et al., 2005).

Siamois: Outer Forward 5'- GAA GTC TTG CCA ACT TCT CTC A -3'; Outer Reverse 5'- GTC CTT TGA TGA TTC TGA TGA C -3'; Inner Forward 5'- CCA ACT TCT CTC ACT CAG TC -3'; Inner Reverse 5'- TTT CCC TTG ATC TTG CCC -3'.

Xnr3: Outer Forward 5'- ATA GCT TTA ATG TGC CAC AAT CTA C -3'; Outer Reverse 5'- GTA CAG TCT TGG GAG TTC CCT G -3'; Inner Forward 5'- CAT AAA GGC AAA TGG TTT CTG C -3'; Inner Reverse 5'- TTA TAC TGG GAT GGA CAG AGG C -3'.

Xnr5: Outer Forward 5'- GTA ATA GTG AGA GGT GCC AGT TG -3'; Outer Reverse 5'- CAG GTG ACA GGT TCC CTA ATC -3'; Inner Forward 5'- GGT GCC AGT TGC CCA AGC -3'; Inner Reverse 5'- TGG AAC CAA GGA GAA AAT CC -3'.

Xnr6: Outer Forward 5'- TCT GAG GTG TGA GGT ATA TGA AAG G -3'; Outer Reverse 5'- TGG GGC TCT TGA AAA CTG AAA TG -3'; Inner Forward 5'- GGT AGA TGA AAG GCT GAC AGG TGT G -3'; Inner Reverse 5'- GGC TGT TGA AAA CTG AAA TGA AGC -3'.

Mlc2: Outer Forward 5'- TGG GAT ATT TTA CTG AAC ACA ATG -3'; Outer Reverse 5'- CGT CCT GTG CCA CCT AAT G -3'; Inner Forward 5'- GAA TGT TAG CCC TTG TGC TCT T -3'; Inner Reverse 5'- GGA AAG TTC TCT TGA TCA TTT TA -3'.

Myf5: Outer Forward 5'- GCC ATA AGC CTC CTG AAC G -3'; Outer Reverse 5'- GCT GAA GAA GCC ATT GGT TTC -3'; Inner Forward 5'- CGT GTA TGT GTC TCT GGG TAG C -3'; Inner Reverse 5'- CAT TGG TTT CTG TTT GGA CTC C -3'.

Anti-H3R8me2a was generated by Cocalico Biologicals (Reamstown, PA) by immunizing rabbits with the H3 (1-15) R8me2a peptide and affinity purified as described in the text. The antibody limit of detection was ~1pmol H3R8me2a (1-15) by dot blot (not shown). Anti-β-catenin was described in (Blythe et al. 2009). Anti-H3acK9/14 (06-599) and -H3K4me3 (07-473) were purchased from Millipore. Anti-Pol II p-Ser5 (39233) was purchased from Active Motif. Anti-H3R8me2s was a kind gift of Said Sif (Ohio State University) (Pal et al., 2004).

Note: the anti-H3K4me3 antibody is no longer available from Millipore, but similar results were obtained with antibodies from both Abcam (ab8580) and Active Motif (39159).

### *Immunological Techniques*

With the exception of the experiment in Figure 3.2D, embryos were homogenized in HKM Buffer (20mM Hepes pH7.9, 100mM KCl, 0.2mM EDTA, 12.5mM MgCl2, 10% Glycerol, 0.5mM DTT) and suppliemented with Protease Inhibitor Cocktail (Sigma P8340) and Phosphatase Inhibitor Cocktail I and II (Sigma P2850 and P5726). Homogenates were centrifuged for 10 minutes at 4°C. Supernatants were collected and adjusted to 150mM KCl and 0.2% NP-40. Lysates were filtered through a 0.45mm cellulose acetate syringe filter (Millipore) to clear particulate material. Immunoprecipitations were performed with 1mg affinity purified or 5ml whole antiserum, and a corresponding amount of control antiserum. Antibody incubations were overnight at 4°C with mixing. The following morning, samples were centrifuged at 14,000xg for 10 minutes to pellet any precipitated materials and supernatants were transferred to a new tube prior to the addition of 50μl recombinant protein-G agarose (Invitrogen 15920-010). IPs were washed 5x in HNMZ buffer (50mM Hepes pH7.9, 150mM NaCl, 12.5mM $MgCl_2$, 0.5mM EDTA, 40uM $ZnSO_4$, 0.5% Tween-20, 10% Glycerol) (Sierra et al., 2006). Nitrocellulose was used for western blot transfer and immunoblotting was performed with primary antibody dilutions of 1:2000 into phosphate buffered saline, 0.1% Tween-20, 5%BSA (Fraction V). 1:5000 dilutions of HRP-linked

secondary antibody were used, substituting nonfat milk (Carnation) for BSA in the buffer. Westerns were developed using ECL (GE Healthcare).

GST Pulldown experiments were performed by first pre-clearing a 100 embryo equivalent of a 16-cell stage lysate with glutathione sepharose 4B (GE Healthcare). This partially cleared an abundant endogenous glutathione binding factor from lysates (residual binding is seen at the bottom of the lower panel of Figure 5C). Pre-cleared lysates were incubated with either GST- or GST:Prmt2-glutathione beads for 1 hour as described in the text. Bound complexes were washed five times in HNMZ buffer as described above prior to analysis by SDS-PAGE and western blot.

For the experiment in Figure 3.2D, whole embryo lysates were made in RIPA buffer (50mM Tris-HCl, pH 7.4, 0.1% SDS, 1% NP-40, 0.25% Na-Deoxycholate, 150mM NaCl, 1mM EDTA, 0.5mM DTT, and Sigma Protease and Phosphatase Inhibitors). Antibodies to the Pol II CTD (H5, H14, and 8WG16) were purchased from Covance. H5 and H14 were unable to detect Pol II in lambda-phosphatase-treated extracts, confirming the specificity of these antibodies for phospho-Pol II (data not shown). Omitting phosphatase inhibitors from the lysis buffer eliminated H5 immunoreactivity, and reduced H14 immunoreactivity (not shown).

### *IP/Histone Acetyl- and Methyl-transferase Activity Assays*

β-catenin was immunoprecipitated from a 100-embryo HKM lysate (volume 1ml) using 5μl anti-β-catenin serum. Myc-Prmt2 was immunoprecipitated

from a 50-embryo HKM lysate using 1μg affinity purified anti-Myc antibody (Sigma C3956). Endogenous Prmt2 was immunoprecipitated from a semiconfluent mouse embryonic stem cell culture (including feeder cells) lysed in HKM buffer (+0.2% NP-40, 150mM KCl final, one 10cm dish per IP) using 5μl of anti-Prmt2 antibody (Aviva Systems Biology ARP40196_T100). IPs were washed as described above, and precipitated immune complexes were adjusted to 50mM Tris-HCl (pH8.8), 0.5mM DTT, 1:100-diluted Protease Inhibitor Cocktail, HMT substrate and 1uCi [methyl $^{3}$H]-S-Adenosyl Methionine (Perkin Elmer NET155H) or 1uCi [acetyl $^{3}$H]-CoA (Perkin Elmer NET290) in a final volume of 20μl. Either 5μg Calf Thymus Histones (Sigma H9250), or 2.5μg recombinant H3.3 was used as a substrate in each reaction. HMT Assay reactions were incubated at 30°C for 1 hour and were stopped by addition of 20μl 2x SDS-PAGE sample buffer and boiling for 10 minutes. Reaction products (50%) were separated by 12% SDS-PAGE and coomassie stained to visualize total protein. Stained gels were digitally scanned prior to 30' incubation in Amplify Fluorographic Reagent (GE Healthcare NAMP-100). Amplified gels were dried and exposed to film at -80°C for 1 to 3 days prior to developing.

IP/HMT assays on peptide substrates were performed as described above except 5μg Histone Tail Peptide was used per reaction, products were separated by 16.5% Tris-Tricine electrophoresis, and gels were fixed for 1 hour in 5% glutaraldehyde prior to coomassie staining. Peptide synthesis of unmodified and R8me2a H3 (1-15) was performed by the Proteomics Resource Center of the Rockefeller University. Additional modified peptides were purchased from

AnaSpec: Unmodified H3 1-21 (#61701), H3 K9 acetyl 1-20 (#63680), and H3 K9me3 1-24 (#63679).

***Size exclusion chromatography***

A 400μl of a 1 embryo/μl HKM lysate (adjusted to 150mM KCl, 0.2% NP-40 and filtered; see above) was loaded onto a pre-calibrated, 15x1.5cm Sephacryl S-400 HR column (GE Healthcare, separation range 20 to 8000kDa) and developed in (50mM Tris-HCl pH7.4, 150mM KCl), collecting 400μl fractions. A portion of each fraction was analyzed by western blot for β-catenin, and the remainder was pooled and subjected to IP/HMT analysis. Relative molecular weights were calculated according to the elution profiles of molecular weight standards: (Blue Dextran ~2000kDa), (Thyroglobulin 669kDa), (β-amylase 200kD), and (Alcohol dehydrogenase 150kD). Using embryo extracts, the void volume was observed to be 33% CV (Fraction 22) by measuring A280.

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