The Dependence of Global Chromatin Architecture on the Histone Chaperone DAXX

by

Lindsy Michelle Rapkin

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Biochemistry
University of Toronto

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2015

Abstract

An essential component of gene regulation is the organization of eukaryotic genomes into functionally and spatially discrete domains. One such domain is the chromocentre, which is a cluster of pericentric heterochromatin enriched in methylated histone H3 (H3K9me3), major satellite repeat DNA, and the histone variant H3.3 specifically deposited by the death-domain-associated protein (DAXX) chaperone. While studies relying on biochemical-based approaches have provided insight into the mechanistic details of the DAXX-dependent chromatin assembly pathway, they have not yielded a comprehensive model of the consequences of this deposition on global chromatin organization. Using electron spectroscopic imaging (ESI), I show that in the absence of DAXX, there is a breakdown in chromocentre structure and a loss of morphological distinction between the repressive histone modification H3K9me3 and chromatin compaction. In addition, I demonstrate that the structural integrity of the nucleolus requires DAXX-dependent heterochromatin organization as its loss causes an increased number of "mini" nucleoli and the dispersal of ribosomal DNA genes. As well, I investigated the role of DAXX in the C2C12 muscle differentiation model and found that concomitant with the formation of terminally differentiated myotubes is the relocalization of DAXX from promyelocytic leukemia nuclear body (PML NBs) to constitutive heterochromatin domains. Taken together, these data reveal that DAXX is a regulator of heterochromatin organization and for the first time, shows the direct consequences of its loss on the global nuclear landscape.
I dedicate this thesis H and W.
Acknowledgments

After a wonderfully insightful conversation with Professor Joseph Goren at the University of Calgary, I applied to graduate school at the University of Toronto and, following his advice, sent Dr. David Bazett-Jones an email asking if I can join his lab. I interviewed with David and he described this amazing tyrammide-based project that he was so excited about. The project sounded fantastic and I couldn't wait to start working on it. After a rotation, I committed to David's lab and found out shortly thereafter that another student was going to be working on the tyrammide project! I decided to continue in the lab anyway because I knew that any project emerging from David's lab would be interesting and relevant. Best decision I could have made. And so began my work on DAXX and everything that came with it...

David, thank you for accepting me into your lab. Thank you for being an inspiring mentor. Thank you for pushing me when I couldn't self-motivate. Thank you for allowing me access to your insight and for shaping me as a scientist. I feel so completely honored and privileged to have had the opportunity to study under your supervision. Most importantly, thank you for being supportive of my personal choices. Knowing that I didn't have to choose between graduate school and a family made the entire experience that much more incredible.

I would like to thank my final supervisory committee members. Despite not being there from the start, Drs. Johanna Rommens and Alex Palazzo, you were both extremely helpful and I appreciate all of your advice.

An acknowledgement section would not be complete without thanking all of the members, both past and present, of the Bazett-Jones lab. Drs. Graham Dellaire, Chris Eskiw, Reagan Ching, and Greg Block, together with Ingo Lou, you made the first few years the lab exciting. Your love of science was obvious and quite motivating. Dr. Rozalia Nisman, thank you for being a good friend and amazing role model. Dr. Hesam Dehghani; it was such a privilege working with you. You are an inspiration, always full of new and amazing ideas. You left quite a void when you moved home. Dr. Liron Even-Faitelson, it was so great having you join the lab. You are a good friend and always full of insight, both scientifically and personally. It was fun watching our kids become friends. Dr. David Anchel, I, much to your chagrin, consider you a friend. It was strangely comforting finishing our degrees together, knowing you were going through the same
motions as I was. I think I speak for everyone who has worked with you that we are all excited to see your future accomplishments. Ren Li, you are so skilled and talented, it is incredible. Thank you for all your help. My project would never have come to life without your expertise. Dr. Kashif Ahmed, thank you, well, for everything. You are so kind, patient, and helpful. I don't know how I would have finished without your advice, technical help, insight, ideas, and support. Dr. Eden Fussner-Dupas, thank you for being such a dear friend. The lab was always so much fun with you around. You are an amazing scientist and person and there is no way I would have accomplished what I have without your support and encouragement.

I sincerely thank my family, including my mom, Barry, Robyn, Darcy, Mike and Benji. Thank you for your unwavering support. Thank you for always lending a hand with the kids and for helping me through my personal crises. I also thank Big Bubby Sylvia and my late grandfather, Zaidy Harvey. They shaped the family and thus shaped me. I love you all.

My deepest thanks to my fiancé, Dr. Eric C. Swanson. Thank you for encouraging me and forcing me to keep moving forward. I can't wait for our future together. I love you, xL.

Finally, a special thanks to my two favorite people in the world: Harper and Waylon. Harper, you are so mature and insightful, it's scary. Your wit and dry humor is incredible. Thank you for reminding me that life really is absolutely fantastic. You are such a special girl. Waylon, thank you for being such a wonderful little guy. You are hilarious and smart. You have this amazing ability to brighten the lives of everyone around you. Thank you for doing the same for me. Love you both, Mama.
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Chapter 1

1 Introduction

1.1 Nuclear organization and the formation of functional domains

The nucleus of mammalian cells is a highly organized organelle that is compartmentalized into functionally and spatially discrete structures called nuclear bodies. The best way to envisage the complexity within the nucleus is by labeling cells with antibodies against structure-specific proteins or the use of stains, such as DAPI, that label nucleic acid. In Figure 1.1, I performed an immunofluorescence experiment in a mouse embryonic fibroblast (MEF) cell line to highlight nuclear organization. Antibodies against B23 (green) were used to mark nucleoli, the sites of ribosome biogenesis, DAXX (red) to mark promyelocytic leukemia nuclear bodies (PML NBs), and ATRX (blue) to show the pericentric heterochromatin domains. What is most striking about this image, and nuclear organization in general, is the clear distinction between the various nuclear bodies and the obvious respect for molecular boundaries that components of each domain appear to respect.
Figure 1.1: Nuclear organization.

Mouse embryonic fibroblast cell nucleus immunolabeled with DAXX (red), ATRX (blue), and B23 (green). The image clearly demonstrates the remarkable compartmentalization of the nucleus with DAXX at PML NBs, ATRX at chromocentres, and B23 at nucleoli.
While some nuclear bodies are chromatin-based structures (i.e., chromocentres and nucleoli), others are protein-based structures that make contacts with the surrounding chromatin (i.e., PML NB). What is the reason for a compartmentalized nucleus and the formation of nuclear bodies? The establishment of chromatin-based structures is an essential component of genome organization critical for gene function. For example, in 1930, Muller reported a variegated white eye phenotype after X-ray-induced mutations (Muller, 1930). He observed that as the location of the white gene changed from euchromatic or expressed regions of the chromosome to locations adjacent to the silenced pericentric heterochromatin domains, the size, shape, and location of white regions within the eye varied. This is one of the earliest reports of the role of chromosome structure in gene regulation and challenged the view at the time that chromosomes were merely vehicles to appropriately segregate genes during mitosis (Girton and Johansen, 2008). The role of nuclear bodies, however, is not solely restricted to gene regulation. Nuclear bodies, both chromatin-based and protein-based, create microenvironments within the nucleus where specialized functions and biological processes can occur (Misteli, 2007). The largest and most prominent chromatin-associated nuclear body is the nucleolus. Nucleoli are the sites of ribosomal RNA (rRNA) synthesis and biogenesis. They form around nucleolar organizing regions (NORs) which are ribosomal DNA- (rDNA) containing regions of specific chromosomes (Boisvert et al., 2007). It is there that rDNA genes are transcribed and rRNA subunits are modified and together with ribosomal proteins, assembled into functional ribosomes. Even though they may not be chromatin-based, protein-based nuclear structures may be tightly associated with the surrounding chromatin. In large part due to the proteins that accumulate there, PML NBs are implicated in a variety of cellular processes including apoptosis, tumor suppression, and DNA repair (Maul et al., 2000). While the precise functions of PML NBs remain elusive, they appear to make contacts with specific regions chromosomal regions (Ching et al., 2013; Ching et al., 2005) suggesting that they are involved in gene regulation. Furthermore, the structural integrity of PML NBs is dependent on the contacts it makes with the surrounding chromatin. For example, Eskiw et al (2004), treated U2OS cells with DNaseI and reported that PML NBs destabilized and fractionated into small PML protein-containing structures.
In the following sections, I will provide an overview of chromatin domains, nucleoli, and PML NBs, the nuclear bodies relevant to this thesis. I will introduce the concept of chromatin landscapes which are the molecular basis for chromatin-based nuclear bodies. Following those sections, I will introduce a protein that associates with several nuclear bodies and a player in the field of chromatin biology, the death domain-associated protein (DAXX), and will discuss its recently ascribed function as a histone chaperone. Finally, I will provide an overview of how chromatin landscapes and nuclear bodies are visualized with a focus on imaging-based techniques, specifically electron spectroscopic imaging (ESI).

1.1.1 Heterochromatin and euchromatin

DNA does not exist in its 'naked' form within the nucleus. Rather it is assembled into chromatin made of repeating units called nucleosomes. Nucleosomes consist of 147 base pairs of DNA wrapped around an octameric complex consisting of two of each of the core histone proteins H2A, H2B, H3, and H4 (Kornberg, 1974). This gives rise to the 10 nm fiber that was historically referred to as the "beads-on-a-string" morphology (Olins and Olins, 1974; Woodcock et al., 1976a; Woodcock et al., 1976b). Not only does the structure of the 10 nm fiber accomplish the neutralization of the inherently negative DNA molecule by the positively charged histones, it provides the first level of chromatin compaction (Fussner et al., 2011a). Chromatin is then further compacted into higher-order structures to ensure and regulate gene expression patterns. Chromatin can be classified into two main classes: euchromatin and heterochromatin. These terms were coined in 1928 by Emil Heitz who established this cytological classification based on differentially staining chromosomal regions (Heitz, 1928). He described euchromatin as lightly staining regions of the genome that dispersed during mitosis. In contrast, heterochromatin was densely-stained regions that remain compacted during the cell cycle. He later extended this definition to include the transcriptional potential of the different chromatin states and hypothesized that euchromatin represents active regions of the genome, whereas heterochromatin is passive or repressed (Heitz, 1929). As molecular biology techniques improved, a more comprehensive definition of heterochromatin and euchromatin was possible and it now includes a molecular distinction based on the association of each type of chromatin with a particular set of histone tail post translational modifications. Euchromatin, which accounts for a large proportion of the genome, is enriched in trimethylated H3K4, H3K36, and H3K79 in contrast to
heterochromatin that is associated with high levels of trimethylated H3K9, H3K27, and H4K20 (Kouzarides, 2007). Histone tail modifications accomplish two important regulatory goals: the first is the disruption of local chromatin or its "opening". The second is the recruitment of non-histone proteins and complexes in order to carry out and regulate biological processes including transcription, DNA replication, and repair.

1.1.2 Facultative versus constitutive heterochromatin

There are two types of heterochromatin: constitutive and facultative. Facultative heterochromatin forms in specific euchromatin regions and is composed of condensed, transcriptionally repressed, and silenced DNA. Unlike constitutive heterochromatin, however, facultative heterochromatin is dynamic and has the potential to convert between heterochromatin and euchromatin within the following contexts: (1) developmental states and cell cycle stages, (2) changes in nuclear localization, or (3) parental/heritable gene expression patterns (i.e. monoallelic) (Trojer and Reinberg, 2007). For example, Cai et al. showed that SATB1 functioned in a temporal manner to tether its target genes to a cage-like network that served as a platform to recruit chromatin-remodeling factors that effectively regulate tissue-specific gene expression (Cai et al., 2003).

Evidence for the spatial control of gene expression came from a study showing that one component of the human β-globin locus control region could suppress silencing or maintain the expression of a transgene by keeping it a physical distance from the repressive centromeric heterochromatin environments (Francastel et al., 1999). The best-studied example of facultative heterochromatin and a paradigm for monoallelic gene expression is the inactive X chromosome of female mammalian cells. To account for the dosage compensation of the X chromosome between XX females and XY males, one of the X chromosomes is selectively inactivated (Chow and Heard, 2009). Inactivation is initiated from the X inactivation center by the accumulation of transcripts from the X-linked gene Xist (Brockdorff et al., 1991; Brown et al., 1991). The 17 kb and 15 kb transcripts from human and mouse, respectively, are localized to the inactive X chromosome and are essential for silencing (Brockdorff et al., 1992; Brown et al., 1992; Wutz et al., 2002). The underlying chromatin of the inactive X chromosome is enriched in the histone variant macroH2A (Costanzi and Pehrson, 1998), associated with repressive histone modifications (i.e. H3K27me3 and H3K9me3), and poorly associated with euchromatin marks (i.e. H3K4me3) (Chow and Heard, 2009).
1.1.2.1 Telomeric heterochromatin

In contrast to facultative heterochromatin, which forms in euchromatin domains to repress gene transcription, constitutive heterochromatin forms at repetitive elements of the genomes such as centromeres and telomeres. Not only does constitutive heterochromatin function in the transcriptional silencing of repetitive regions of the genome, but the imposed structure is essential for chromosome function and the maintenance of genome integrity (Beisel and Paro, 2011). Telomeres are nucleoprotein structures that protect the ends of linear chromosomes from replicative-dependent chromosome end shortening and from being recognized as double strand breaks (Palm and de Lange, 2008). Abnormal regulation of telomere maintenance is not only a hallmark of cellular aging, but telomere dysfunction compromises the proliferative capacity of highly proliferative organs and contributes to the accumulation of cancer-associated chromosomal structural defects (Lee et al., 1998; Maser and DePinho, 2002). Mammalian telomere DNA is made up of short tandem TTAGGG repeats that can form higher-order chromatin structures such as t-loops and G-quadruplex arrangements (Bochman et al., 2012; Doksani et al., 2013; Griffith et al., 1999). Several heterochromatin-associated proteins and chromatin modifications are associated with telomeres including the heterochromatin associated protein 1 (HP1) and repressive histone modification H3K9me3(Garcia-Cao et al., 2004; Perrini et al., 2004; Peters et al., 2001). In mouse embryonic stem (ES) cells, telomeric chromatin is further distinguished by its association with the histone variant H3.3 (Goldberg et al., 2010). Despite the classical view that constitutive heterochromatin domains are non-transcriptionally permissive environments, telomere DNA is transcribed by RNA polymerase II giving rise to UUAGGG-repeat containing non-coding RNAs (TERRAs) (Azzalin et al., 2007; Schoeftner and Blasco, 2008). TERRA RNA is a structural component of telomeres and is required to maintain its stability and heterochromatic state (Deng et al., 2009).

1.1.2.2 Centromeric heterochromatin

The centromere is a specialized region of chromatin that ensures the faithful segregation of chromosomes during cell division (Choo, 1997). Kinetochores are multiprotein complexes that form on the centromere and act as anchor points for the spindle apparatus that facilitates separating chromosome during mitosis (Allshire and Karpen, 2008). Centromere dysfunction can lead to segregation errors and chromosome instability, events often observed in cancer (Rasnick and Duesberg, 1999). Unlike telomeres that show repeat sequence homology between species,
the DNA composition of centromeres is wildly variable (Gent and Dawe, 2012). Despite the absence of common centromeric sequences, there are two distinguishing features of centromeric chromatin: 1) the deposition of the H3 histone variant CenH3, and 2) the constitutive heterochromatin nature of the underlying long arrays of tandem repetitive elements (Bouzinba-Segard et al., 2006). In the mouse genome, there are two cytologically distinct classes of centromeric repeats; major satellites that are associated with pericentromeric regions and are enriched in H3K9me3 and HP1, and minor satellites that associate with CenH3-enriched chromatin (Guenatri et al., 2004; Joseph et al., 1989; Wong and Rattner, 1988). In addition to its association with CenH3, the histone variant H2AZ has been shown to be deposited at mouse centromeres (Greaves et al., 2007). Not only do the centromere-specific histone variant associations provide an epigenetic marker for centromere chromatin, they are also required for centromere function. For example, Howman et al. (2000) used gene targeting to disrupt the Cenpa (CenH3) gene and found that CenH3 null embryos had chromosome segregation defects, failed to form structurally normal kinetochores, and displayed lethality between E3.5 to 8.5. Like telomeres, centromeres are associated with functional RNAs which have been shown to be an integral component of CenH3-associated chromatin, regulate chromosome segregation and sister chromatid cohesion in mitosis, and potentiate the activity of the chromosomal passenger complex protein Aurora B kinase (Bouzinba-Segard et al., 2006; Ferri et al., 2009; Wong et al., 2007). Furthermore, the accumulation of small minor satellite transcripts alters the epigenetic profile and localization of heterochromatic proteins associated with centromeric chromatin suggesting they play a key role in centromere architecture and function (Bouzinba-Segard et al., 2006).

1.1.2.3 Pericentromeric heterochromatin

In the mouse interphase nucleus, pericentric major satellite sequences of chromosomes cluster into cytologically detectable structures called chromocentres (Figure 1.2) (Hsu et al., 1971). Major satellites consist of a 234 bp repeat unit, comprise approximately 6 megabases of the genome, and replicate in the middle of S phase in contrast to the minor satellites of centromeres that replicate later in S-phase of the cell cycle (Choo, 1997; Guenatri et al., 2004). Pericentric chromatin contains the H3K9me3 epigenetic mark, and this modification in conjunction with an RNA component are required to concentrate HP1 to chromocentres (Maison et al., 2002; Peters
et al., 2001; Taddei et al., 2001). During mouse development, the organization of pericentric chromatin into chromocentres occurs at the 2-cell stage (Ahmed et al., 2010; Probst et al., 2007). Interestingly, rather than being enriched in H3K9me3 and HP1, paternal pericentric chromatin during the first cleavage stages are enriched in H3K27me3 and recruit members of the Polycomb repressive complex 1 (Puschendorf et al., 2008; Santos et al., 2005). By the 8-cell stage, however, the pericentric domains of the two parental genomes become equivalent (Merico et al., 2007; Puschendorf et al., 2008). Major satellite-containing chromatids are the last centromeric component to separate during mitosis, suggesting these repetitive sequences play a key role in regulating the attachment of sister chromatid during mitosis (Guenatri et al., 2004; Probst et al., 2009). Indeed, in cells lacking the Suv39h methyltransferases, the loss of H3K9me3 and HP1 at pericentromeres impairs sister chromatid cohesion at major satellites, likely explaining the missegregation phenotype observed in Suv39h knockout fibroblasts (Guenatri et al., 2004; Peters et al., 2001). Like both telomeric and centromeric chromatin, pericentromeres are transcribed and the resulting transcripts are of heterogeneous lengths and contain repetitions of the major satellite repeat (Lehnertz et al., 2003). The peak in expression of these functional RNAs coincide with the reorganization of pericentric chromatin into chromocentres at the 2-cell stage and the interference of this expression not only impairs the de novo establishment of paternal pericentromeric heterochromatin but results in developmental arrest, prior to chromocentre formation (Probst et al., 2010).
Figure 1.2: H3K9me3-enriched chromocentres.
(Left) MEF cell nucleus immunolabeled for H3K9me3. Discrete regions with H3K9me3-enrichment are chromocentres. Scale bar, 5 µm. (Right) High-resolution electron spectroscopic imaging (ESI) micrograph of a chromocentre in a MEF nucleus labeled with gold-conjugated H3K9me3 antibodies. The white dots (examples highlighted by arrowheads) are gold particles indicating H3K9me3-modified histones. The highly compact chromatin of the chromocentre is marked by the dashed outline. Chromatin is pseudo-colored yellow and protein-based structures cyan. Scale bar, 0.5 µm (For an overview of the principles of ESI see Section 1.4.4.1.)
1.1.3 Nucleolus

The most prominent and often largest nuclear structure is the nucleolus. At the end of mitosis, nucleoli form around tandem repeats of ribosomal DNA (rDNA) genes termed nucleolar organizer regions (NORs). The resulting subnuclear compartment concentrates the machinery required for generating functional ribosomes (Boisvert et al., 2007). The process of ribosome biogenesis requires the transcription of rDNA genes by RNA polymerase I (RNA pol I), cleavage of the precursor 47S transcript into the mature 28S, 18S, and 5.85S rRNAs, followed by post-transcriptional modifications, rRNA folding, the loading of ribosomal proteins, and the subsequent export of the ribosomal subunits into the cytoplasm (Fatica and Tollervey, 2002). The nucleolus is not a homogenous structure, rather it is divided into morphologically distinct subregions responsible for specific aspects of ribosome biogenesis (Boisvert et al., 2007). The three subregions are: fibrillar centres (FCs), dense fibrillar components (DFCs), and granular components (GCs). RNA pol I complexes are concentrated in the FC regions while the majority of rRNA transcription occurs at the border between the FCs and DFCs. rRNA transcripts are processed and modified largely in the DFC and ribosome assembly occurs in the GC region (Boisvert et al., 2007; Tschochner and Hurt, 2003). The nucleolus is surrounded by a shell of late replicating, highly compact heterochromatin consisting mainly of silenced rDNA genes as well as major and minor satellite sequences (Haaf and Schmid, 1991; Pluta et al., 1995). However, whole-genome sequencing studies revealed that AT-rich sequence elements, low gene density regions, and transcriptionally repressed genes can also associate with the perinucleolar compartment (Nemeth et al., 2010; van Koningsbruggen et al., 2010). Together, these data describe a fascinating structure where the most active site of cellular transcription is embedded within a repressive environment (Guetg and Santoro, 2012).

In mouse cells, rDNA repeat clusters are found within the centromeric regions of chromosomes 12, 15, 16, 18, and 19 (Dev et al., 1977; Kurihara et al., 1994). The tandem repeat nature of rRNA genes is necessary to ensure that the required high production levels of rRNA transcription are met. Most eukaryotes, however, contain more rRNA genes than are actually needed. As a result, only a fraction of these remain active and are used for rRNA synthesis, while the remaining copies are silenced by dosage-compensation mechanisms (Dammann et al., 1993; Santoro and Grummt, 2005). Experiments aimed at determining the histone modifications with
respect to the transcriptional activity of rRNA genes found that the nucleolar epigenetic profile is similar to the rest of the nucleus where silent rRNA repeats are enriched in the repressive H3K9me3 and H4K20me3 modifications whereas H4Ac and H3K4me2 are features of the active rRNA genes (Mayer et al., 2006; Santoro and Grummt, 2001; Santoro et al., 2002). Furthermore, active and silent repeats exist in distinct chromatin compartments where active genes correspond to transcriptionally permissive euchromatin whereas silenced genes correspond to densely packed heterochromatin that is refractory to transcription (Conconi et al., 1989). Interestingly, drug-based DNA-crosslinking experiments demonstrated that the chromatin structure of active and silent populations are maintained independent of transcription and are stably inherited through mitosis (Conconi et al., 1989).

While the silencing of rRNA genes is important in the regulation of rRNA synthesis and ribosome biogenesis, there is evidence supporting a role for rRNA silencing unrelated to transcription. Indeed, a yeast strain containing a reduced number of rRNA genes (43 less than the wild type strain) silences a comparable fraction (10-20%) of the rDNAs rather than dedicating all of the remaining genes to transcription, suggesting rDNA silencing plays a role independent of transcription (French et al., 2003; Merz et al., 2008). Inheritance of silent rDNA chromatin is regulated by the nucleosome remodeling complex (NoRC) comprising the TIP5 and SNF2h subunits (Guetg et al., 2012; Santoro and Grummt, 2005; Santoro et al., 2002; Zhou et al., 2002). In mid-late S phase, TIP5 binds to silent rRNA genes and recruits the DNA methyltransferases and histone modifying enzymes responsible for re-establishing the heterochromatin state of the rDNA loci (Li et al., 2005; Santoro et al., 2002; Zhou et al., 2002). Depletion of TIP5 results in genomic instability at pericentric heterochromatin and denies the formation of perinucleolar heterochromatin mediated by the H3K9me3 histone modification implicating rRNA silencing in nuclear/nucleolar chromatin organization (Guetg et al., 2010). Similarly, DNA methyltransferase I (Dnmt1) deficient cells have structurally disorganized nucleoli and altered rRNA gene nuclear localizations (Espada et al., 2007). Furthermore, knockdown and overexpression of the TIP5 interacting partner poly(ADP-ribose) polymerase I (PARP1) influences the chromatin structure of repetitive regions of the genome and rDNA heterochromatin (Guetg et al., 2012). Together, these data link rDNA silencing to the establishment of the heterochromatic state, and thus implicate the nucleolus as a key determinant in the maintenance of genome integrity.
1.1.4 PML bodies

The mammalian cell nucleus contains many distinct protein-based nuclear bodies including promyelocytic leukaemia nuclear bodies (PML NBs). PML NBs are dynamic macromolecular structures ranging in size from 0.1-1.0 μm in diameter (Lallemand-Breitenbach and de The, 2010). Under normal growth conditions, 10 to 30 bodies can be found in the inter-chromatin space (Borden, 2008; Grande et al., 1996). The main constituent of PML NBs is the PML protein itself, a protein originally identified in cells from patients suffering from acute promyelocytic leukaemia (APL). In APL cells, the expression of the oncogenic PML-RARα fusion protein, the product of a reciprocal t(15;17) chromosomal translocation, results in the disruption of PML NBs (de The et al., 1990). Treatment of APL patients with all-trans retinoic acid (ATRA) or arsenic trioxide (As₂O₃) leads to the degradation of PML-RARα, the reformation of PML NBs, and often disease remission (Zhu et al., 2001).

To date, over 60 proteins have been reported to accumulate in PML NBs (Dellaire and Bazett-Jones, 2004) (Nuclear Protein Database http://npd.hgu.mrc.ac.uk). Since a consensus on PML NB function remains elusive, the identification of proteins that associate with PML NBs are used as a means to assign functions to PML NBs. As a result, they have been implicated in a variety of nuclear processes including transcription, apoptosis, senescence, and DNA-damage response pathways (Dellaire and Bazett-Jones, 2004). In the absence of a specific ascribed function, several models have been proposed to describe the functions of PML NBs. The first proposes that PML NBs act as storage sites that accumulate specific proteins and releases them when necessary. The second model describes PML NBs as a catalytic surface where proteins accumulate to be posttranslationally modified. A third model suggests that PML NBs are active sites of nuclear functions such as chromatin regulation (Bernardi and Pandolfi, 2007). Indeed, studies using ChIP, immuno-FISH, immuno-TRAP, and nano-dissection technologies have identified specific regions that associate with PML NBs (Chen et al., 2014; Ching et al., 2013; Gialitakis et al., 2010; Kumar et al., 2007; Shiels et al., 2001; Sun et al., 2003) supporting a role for PML NBs in the regulation of a unique set of genomic loci.
1.2 Establishing global chromatin environments

1.2.1 Histone modifications

The core histones are predominantly globular except for their N-terminal tails which protrude outside of the nucleosome core particle (Davey et al., 2002). These histone tails have not only been shown to interact with both nucleosomal and linker DNA but are considered essential to function in the formation of higher-order chromatin structures (Angelov et al., 2001; Mutskov et al., 1998). For example, de la Barre et al. showed, using a nucleosome-mediated competitive inhibition experiment in Xenopus egg extracts that the histone tail of H2B is essential for chromosome condensation (de la Barre et al., 2001) and the H3 tail is important for higher-order yeast heterochromatin structures (Sperling and Grunstein, 2009). What makes the tail region of histone so important in chromatin organization? A striking feature of histone tails is the large number of residues that are susceptible to at least eight types modifications (Table 1). To date, the two main functions of histone modifications are to disrupt contacts between nucleosomes as a means to make chromatin susceptible to biological processes and to recruit non-histone protein complexes often possessing catalytic activity to further modify chromatin (Kouzarides, 2007).

Often, a consequence of histone modifications are changes to higher-order chromatin structures. Nowhere is this more apparent than the effects of the acetylation modification that have the capacity to unfold chromatin by neutralizing the basic charge of lysine residues (Shogren-Knaak et al., 2006). It should be noted, however, that the dogma of one-modification-one-function is quite simplistic and a complicating factor is the abundance of crosstalk between modifications. Firstly, different modifications can occur on a given residue, albeit not at the same time. Therefore, there may be some form of antagonism between modifications. Secondly, the recruitment of a protein to its modified target locus can be disrupted by adjacent modifications. Thirdly, the presence of a modification at an enzyme's substrate recognition site could impair the catalytic activity of the enzyme. Finally, an enzyme could bind its target substrate more efficiently if a second modification is present (Kouzarides, 2007).
Table 1.1: Histone posttranslational modifications.

<table>
<thead>
<tr>
<th>Histone</th>
<th>Modified Site</th>
<th>Modification Pattern</th>
<th>Modifying Enzymes</th>
<th>Functions Regulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>L26</td>
<td>Methylation</td>
<td>Ezh2</td>
<td>Transcription silencing</td>
</tr>
<tr>
<td></td>
<td>S27</td>
<td>Phosphorylation</td>
<td>Unknown</td>
<td>Chromatin decondensation</td>
</tr>
<tr>
<td>H2A</td>
<td>K5</td>
<td>Acetylation</td>
<td>Tip60, p300/CBP</td>
<td>Transcription activation</td>
</tr>
<tr>
<td></td>
<td>S139</td>
<td>Phosphorylation</td>
<td>ATR, ATM, DNA-PK</td>
<td>DNA repair</td>
</tr>
<tr>
<td>H2B</td>
<td>K12</td>
<td>Acetylation</td>
<td>P300/CBP, ATF2</td>
<td>Transcription activation</td>
</tr>
<tr>
<td></td>
<td>K120</td>
<td>Ubiquitylation</td>
<td>UbcH6</td>
<td>Meiosis</td>
</tr>
<tr>
<td>H3</td>
<td>T3</td>
<td>Phosphorylation</td>
<td>Haspin</td>
<td>Mitosis</td>
</tr>
<tr>
<td></td>
<td>K4</td>
<td>Methylation</td>
<td>MLL, ALL-1</td>
<td>Transcription activation</td>
</tr>
<tr>
<td></td>
<td>K9</td>
<td>Methylation</td>
<td>Suv39h, G9a</td>
<td>Transcription repression</td>
</tr>
<tr>
<td></td>
<td>S10</td>
<td>Phosphorylation</td>
<td>AuroraB</td>
<td>Mitosis</td>
</tr>
<tr>
<td></td>
<td>K27</td>
<td>Methylation</td>
<td>Ezh2, G9a</td>
<td>Transcription repression, X-inactivation</td>
</tr>
<tr>
<td>H4</td>
<td>K16</td>
<td>Acetylation</td>
<td>Tip60</td>
<td>Chromatin condensation</td>
</tr>
<tr>
<td></td>
<td>K20</td>
<td>Methylation</td>
<td>Suv4-20h</td>
<td>Transcription repression, heterochromatin</td>
</tr>
</tbody>
</table>

Examples of different classes of histone modifications, their corresponding modifying enzymes, and associated functions. Other classes of modifications include methylation on arginine residues, sumoylation, ADP ribosylation, deimination (conversion of arginine to citrulline), and proline isomerization. L, leucine; S, serine; K, lysine; T, threonine.
On a larger scale, the functional consequences of histone posttranslational modifications are to partition the genome through the establishment of global chromatin environments and to assist in the execution of DNA-based biological processes by unraveling or changing local chromatin structures (Kouzarides, 2007). With respect to the former, a link exists between certain cellular processes and specific modifications. For example, within a few minutes of DNA damage, H2AX is phosphorylated (γ-H2AX) at the site of repair, followed by the colocalization of repair factors. Importantly, treating cells with the kinase inhibitor wortmannin prevented γ-H2AX formation and the subsequent recruitment of repair factors reinforcing the requirement for the histone modification in the DNA damage response (Fillingham et al., 2006; Paull et al., 2000). Phosphorylation has also been implicated in the condensation and decondensation of chromatin during the cell cycle. During mitosis, the phosphorylation of histone H3 on the serine residue at position 9 (H3S9) by the Aurora B kinase causes the dissociation of HP1 from H3K9me3-containing chromatin (Fischle et al., 2005). Ectopic serine phosphorylation of Drosophila histone H3 at position 10 (H3S10) is sufficient to induce a change in chromatin structure from a condensed heterochromatin-like state to a more open euchromatin-like state providing evidence that this modification at interphase can function as a regulator of higher-order chromatin structure (Deng et al., 2008). However, the classic example of histone modification and function is in the context of transcription such that the acetylation, methylation, phosphorylation, and ubiquitylation of a specific subset of residues have been implicated in activation in contrast to the methylation, ubiquitylation, and sumoylation associated with repression (Kouzarides, 2007).

Relevant to this thesis, however, is the use of histone posttranslational modifications to dictate and preserve different chromatin environments. The open nature of euchromatic DNA makes it more conducive to biological processes and typically, actively transcribed euchromatin contains high levels of acetylation and is enriched in trimethylated H3K4, H3K36, and H3K79. In contrast, heterochromatin is characterized by H3K9me3, H3K27, and H4K20 trimethylation and low levels of acetylation (Kouzarides, 2007). The model of specific active and silent marks was challenged when ChIP on ChIP experiments on mouse ES cells revealed the presence of both H3K27 and H3K4 methylation on developmental transcription factors that are expressed at low levels (Bernstein et al., 2005; Bernstein et al., 2006). After differentiation, these bivalent domains retained either the repressive H3K27me3 or the active H3K4me3 mark, but not both
suggesting that the bivalent modification keeps the transcription factors in a poised state, ready to respond to relevant differentiation cues.

Of all the enzymes responsible for modifying histones, the methyltransferases are the most specific. It has been shown that the Suv39h H3K9 histone methyltransferases (HTMases) maintain H3K9me3 at pericentric heterochromatin whereas the HTMase G9a regulates H3K9 methylation at euchromatic loci (Peters et al., 2003). In an elegant series of experiments, Peters et al. (2003) found that in the absence of Suv39h, pericentric heterochromatin was enriched in H3K27me3, rather than H3K9me3, indicating a plasticity between the repressive systems. Insight in the functional consequences of specific histone modifications stems from studies focusing on their respective enzymes. For example, the impaired viability, chromosome segregation defects, and increased risks of tumorigenesis in Suv39h-deficient mice assign a role for H3K9me3 in protecting genome stability during development (Peters et al., 2001). Similarly, cells lacking the H4K20 HTMase Suv4-20h2 display reduced global chromatin compaction and altered chromocentre organization during interphase, as well as chromosome segregation defects during mitosis implicating the H4K20me3 mark in the regulation of nuclear architecture and mitosis (Hahn et al., 2013).

1.2.2 Histone variants

During DNA replication, the amount of available histones needs to double in order to meet nucleosome demands and to properly package replicated DNA. To accomplish this, histones can either be recycled or newly synthesized. With respect to newly synthesized histones, a set of histone genes known as canonical or replicative histones are expressed in a manner that correlates strongly with S phase. There are, however, a set of histone genes that are expressed throughout the cell cycle and during quiescence and these give rise to the replacement histone variants (Loyola and Almouzni, 2007). Originally, it was thought that histone variants functioned as replacement histones in lieu of replicative ones: however the discovery of CenH3 (the centromere-specific H3 variant) and recent evidence implicating histone variants in biological functions provide an emerging view that replacement histones have specific features and functions (Loyola and Almouzni, 2007). Indeed, histone variants confer unique properties on chromatin through the recruitment of various protein complexes, and together they mediate nucleosome dynamics and biological processes such as gene expression and DNA repair.
(Elsasser et al., 2012; Horigome et al., 2014; Maze et al., 2014). Furthermore, through their selective deposition into specific regions of the genome, histone variants provide a key mechanism to distinguish and compartmentalize chromatin. While a variety of variants exist (summarized in Figure 1.3A), the focus of the following section will include those of histone H3, as they are the variants relevant to this thesis.
Figure 1.3: Histone variants.

(A) Table of well-studied H2A and H3 variants, their histone chaperones, and location of deposition. (B) Protein sequence alignment of the canonical (H3.1, H3.2) and replacement (H3.3) H3 variants. H3.3 differs from H3.1 by 5 amino acids, and those changes are shown in turquoise. The position of residues associated with modifications correlating with active transcription are shown in purple (K4 and K36) and those correlating with repressed chromatin in yellow (K9 and K27). The AAIG motif unique to variant H3.3 is outlined in gray. (Adapted from (Campos and Reinberg, 2010))
An important feature of histone variants is their ability to partition the genome. A classic example are centromeres that rely on features independent of the underlying DNA sequences, such as the enrichment of the centromere-specific histone variant CenH3. CenH3 replaces histone H3 in a fraction of centromeric nucleosomes to effectively organize and distinguish them as discrete chromatin domains (Allshire and Karpen, 2008; Earnshaw and Rothfield, 1985; Sullivan et al., 2011). Using extended human chromatin fibers, Dunleavy et al. (2011) showed that while both H3.1 and H3.3 are deposited at centromeric chromatin in S phase, H3.3 specifically is reduced in G1 leading to the proposition of a replacement model that describes H3.3 as a placeholder at centromeres that is eventually replaced with CenH3 at G1. This model is consistent with studies showing that the deposition of CenH3 at centromeres by the phosphorylated form of the CenH3-specific chaperone Holliday junction recognition protein (HJURP) is restricted to a window between late telophase and early G1 in mammals (Dunleavy et al., 2009; Foltz et al., 2009; Muller and Almouzni, 2013; Muller et al., 2014). Interestingly, overexpression of CenH3 leads to the histone chaperone DAXX-mediated, rather than HJURP-mediated deposition of CenH3 outside of centromeric chromatin (Choi et al., 2011; Gascoigne et al., 2011; Lacoste et al., 2014). These observations, together with the finding that CenH3 overexpression promotes genetic instability and aneuploidy in pRb-depleted cells (Amato et al., 2009) suggest that CenH3 and HJURP may not only play a role in chromatin dynamics, but may also contribute to human disease. Indeed, upregulation and mistargeting of CenH3 has been found in human primary colorectal cancer (Tonomaga et al., 2003) and hepatocellular carcinoma (Li et al., 2011) and increased levels of HJURP associate with a poor prognostic outcome in breast cancer (Hu et al., 2010).

The noncentromeric histone variants can be grouped into two classes: canonical or replicative-dependent (RD) histones including H3.1 and H3.2 and replacement or replicative-independent (RI) histones such as H3.3. The deposition of H3.1 is mediated by the chaperone chromatin assembly factor-1 (CAF-1) and the de novo deposition of H3.1 occurs mainly during S phase and coincides with actively replicating regions of the genome (Ray-Gallet et al., 2011; Tagami et al., 2004). Interestingly, when replicative H3.1 incorporation is impaired through CAF-1 depletion, the H3.3-specific chaperone histone regulator A (HIRA) can facilitate the introduction of H3.3 at replication sites (Ray-Gallet et al., 2011). Despite the incorporation of H3.3 into replication sites, this salvage pathway is not sufficient to compensate for the loss of H3.1-dependent chromatin
integrity as evidenced by the developmental arrest at the 16-cell stage and the heterochromatin organization defect phenotype of CAF-1 null embryos (Houlard et al., 2006). Consistent with the emerging roles for histone chaperones as potential drivers for cancer initiation and /or progression, CAF-1 overexpression: (1) associates with histological grade breast, cervical, endometrial, and renal cell carcinomas (Polo et al., 2010), (2) characterizes aggressive malignant salivary gland tumors (Staibano et al., 2011), and (3) correlates with poor prognosis postatic cancers (Staibano et al., 2009).

In animals, the replacement histone variant H3.3 differs from the canonical H3.1 by only five amino acids (Figure 1.3B). In spite of the high sequence similarities, these residues account for the remarkably distinctive properties and deposition profiles of the variants. While H3.1 purifies with CAF-1, the isolation of H3.3 revealed the presence of HIRA (Tagami et al., 2004). Genome-wide studies showed the enrichment of H3.3 at promoters and in the body of active genes and this enrichment pattern was abrogated in the absence of HIRA suggesting a requirement for HIRA in H3.3 deposition at these genomic loci (Goldberg et al., 2010; Pchelintsev et al., 2013). Interestingly, the HIRA-dependent deposition of H3.3 promotes the establishment of bivalent chromatin domains in ES cells and in the absence of H3.3, the reduced levels of H3K27me3 at the promoters of developmentally regulated genes alters the developmental potential of ES cells and misregulates bivalent gene expression upon differentiation (Banaszynski et al., 2013). Targeted deletion of the Hira gene results in embryonic lethality by E10 with the embryos being described as balls of disorganized tissue and unrecognizable structures (Roberts et al., 2002). The phenotype observed in HIRA null embryos is similar to that of the other H3.3-specific chaperone DAXX, that exhibits embryonic lethality by day 9.5 (Michaelson et al., 1999).

The majority of studies investigating the deposition patterns of histone variants rely on tagged-expression analysis. To assess the high resolution genome-wide localization of H3.3 under endogenous conditions, Goldberg et al. (2010) used zinc-finger technology to engineer mouse ES lines carrying one wild type allele and one tagged H3.3 allele and using these cell lines, reported the dependence and independence of H3.3 enrichment profiles on HIRA. While HIRA was required for the deposition of H3.3 at active and repressed genes in ES cells, the transcription factor binding site and telomere enrichment were HIRA-independent. Using mass spectrometry, they identified DAXX and ATRX as histone chaperones responsible for the specific enrichment
of H3.3 at telomeres. Subsequently DAXX and ATRX were implicated in a chromatin assembly pathway responsible for depositing H3.3 at pericentric heterochromatin in MEFs (Drane et al., 2010). As alluded to in the previous paragraph, the targeted deletion of DAXX results in significant apoptosis at E7.5 and a lack of discernible tissues and organs causing embryonic lethality by E9.5 (Michaelson et al., 1999). Further solidifying the contribution of histone variants and chaperones to the formation of human cancers, H3.3 K27M and G34R/V mutations have been associated with pediatric glioblastomas (Lewis et al., 2013; Schwartzentruber et al., 2012; Wu et al., 2012) and pancreatic neuroendocrine tumors containing DAXX and/or ATRX mutations displayed abnormal telomeres (Heaphy et al., 2011; Jiao et al., 2011).

Much insight into the role of histone variants during development has been gained from loss-of-function studies in Drosophila and mice. Flies lacking H3.3 exhibit strong phenotypes including infertility, reduced viability, meiotic defects, and widespread transcriptional defects (Sakai et al., 2009). Similarly, the knockout of H3.3 in mice lead to impaired embryo growth, chromosomal bridge formation, and severe karyotypic abnormalities (Bush et al., 2013). As well, the loss of H3.3 resulted in an increase in the number and intensity of DAPI foci suggestive of a pericentric heterochromatin phenotype. In an elegant series of experiments aimed at deciphering the roles of the differential incorporation of histone variants in the reorganization of chromatin post fertilization, Santenard et al. (2010) injected mouse zygotes with wild type H3.3-GFP and the H3.3K4R-GFP and H3.3K27R mutant constructs and followed development to the blastocyst stage. While the wild type and H3.3K4R mutants reached the blastocyst stage in similar ratios, embryos expressing H3.3K27R had reduced rates of development. Furthermore, H3.3K27R caused a global reduction in H3K27me3 leading to altered pericentric heterochromatin transcription, chromosome segregation defects, and mislocalized HP1 implicating K27 as a critical residue of H3.3 in development. H3 variant deposition during development is not static, rather it is a dynamic process that involves the coordinated deposition of several variants. For example, soon after fertilization, H3.3 is removed from the female pronucleus and it has been hypothesized that the erasure of the epigenetic mark carried by maternal H3.3 is required to establish the new pattern of gene expression in the zygote (Akiyama et al., 2011). Traditionally, H3.1 and H3.3 are thought to be associated with heterochromatin and euchromatin, respectively (Hake and Allis, 2006). Using FLAG-tagged H3.1 and H3.3 constructs, Akiyama et al. (2011) demonstrated that in the two-cell stage, H3.3 labeled throughout the entire chromatin region, in
contrast to H3.1 that did not accumulate all. At the blastocyst stage, however, intense H3.1 staining was seen in both euchromatin and heterochromatin while H3.3 was restricted to the euchromatic fraction. This, together with their findings that the depletion of CAF-1 changed the deposition pattern of H3 variants, impaired heterochromatin formation during late preimplantation stages, and altered the epigenetic profile of the preimplantation development paint a picture where the dynamic equilibrium of H3.1/H3.3 variants are required to establish developmentally-conducive chromatin landscapes (Akiyama et al., 2011).

1.2.3 Selective modification of histone variants

While improvements in genome-wide analysis techniques have allowed for high resolution visualizations of epigenetic landscapes including histone posttranslational modifications patterns and histone variant deposition profiles, a consensus on their inter-dependence and coordinated regulation remains elusive. Hake and Allis (2006) proposed the histone H3 barcode hypothesis which states that selective incorporation of specific variants could be a principle determinant for establishing particular post translational modifications. In this model, H3.3 would index transcriptionally active regions whereas H3.1 and H3.2 would be enriched in constitutive and facultative heterochromatin, respectively and a prediction is that certain modifications will be determined strictly by the choice in histone variant. Biochemistry studies on purified H3.1- and H3.3-containing complexes, however, revealed the presence of both activation and repressive marks (Hake et al., 2006) suggesting that histone variants are not the sole determining factor in guiding histone modification patterns. Indeed, non-incorporated H3/H4 histone dimers show enrichment in H4K4 and H4K16 acetylation and an absence of methylation on H3K9me3, H3K4, H3K27, H3K36, and H3K79, and show little distinction for the specific variant (Loyola et al., 2006). Interestingly, Loyola et al. (2006) did observe differing patterns of H3K9me1, H3K9ac, and H3K14ac in nonnucleosomal H3.1 and H3.3 suggesting that although variant usage does indeed play a role in specifying histone modification patterns, a more likely model of how cells appropriately establish epigenetic landscapes involves the coordination of histone variants and local environments (Loyola et al., 2006).

An emerging field gaining exciting momentum is the study of modified residues and their corresponding readers/writers that exhibit variant specificity in DNA-templated processes. Despite traditionally being implicated in gene activation, Hake et al. (2005) demonstrated a
stage-specific phosphorylation of H3.3 Ser-31 in late prometaphase and metaphase in contrast to the phosphorylation of H3.1S10 and H3.1S28 in prophase. Additionally, a speckled pattern of H3.3S31 phosphorylation was present on chromosomes aligned at the metaphase plate, implying that a subset of genomic loci were being targeted for this modification and argue for a functional consequence of this H3.3-specific modification. Reinforcing the importance of critical residues within a specified variant was the identification of H3.3K27M mutations in high grade pediatric glioma tumours (Schwartzentruber et al., 2012). Because of the critical function of H3K27 methylation in establishing repressive chromatin landscapes, Chan et al. (2013) investigated the effects of the H3.3K27M mutation on the epigenetic landscape of diffuse intrinsic pontine glioma (DIPG) cancer cell lines and found global reductions in H3K27me3 and a significant local gain of H3K27me and Ezh2 at genes associated with cancer pathways. These results highlight that mutations in unique residues of a specific variant can impose deleterious consequences on a cell and may, such as the case for H3.3K27M, drive tumorigenesis.

Key to understanding critical residues is uncovering the enzymes and chromatin assembly pathways responsible for establishing and regulating the specific modifications. In a recent and elegant paper, Jacob et al. (2014) identified ARABIDOPSIS TRITHORAX-RELATED PROTEIN 5 AND 6 (ATXR5/6) as H3K27 methyltransferases selective for the H3.1 histone variant. While the lysine at position 27 is conserved between H3.1 and H3.3, the presence of a threonine at position 31 in H3.3, rather than an alanine, confers the specificity of ATXR5/6 by inhibiting K27 methylation of H3.3. Incredibly, changing H3.3T31 to A31 renders H3.3 susceptible to methylation by ATXR5/6 and changing H3.1A31 to T31 lowered the levels of H3K27me1 suggesting that T31 offers H3.3 protection against heterochromatization. In another study, the H3.3-specific reader ZMYND11 modulated Pol II elongation and repressed oncogene expression and tumor growth (Wen et al., 2014). ZMYND11 specifically recognizes H3K36me3 on H3.3 (H3.3K36me3) and is recruited to gene bodies, along with Pol II, on H3.3K36me-chromatin. Interestingly, low expression levels of ZMYND11 correlated with poor prognosis breast cancer patients while its overexpression suppressed cancer cell growth in vitro and tumor formation in mice. Together, these studies emphasize the importance of histone variant specificity in establishing epigenetic landscapes conducive to normal cell biology.
1.2.4 Epigenetic landscapes and chromatin structure

Although it is widely accepted that the establishment of the epigenetic landscape is pivotal in regulating DNA-based biological processes, the correlation between epigenetic marks with large-scale chromatin structure remains elusive. What is clear, however, is that the chromatin state, defined by the patterns of histone posttranslational modification enrichment and histone variant deposition contributes to the establishment and maintenance of cell identities. Nuclear architecture, including global chromatin configurations, is tissue- and cell-type specific. Embryonic stem (ES) cells represent one model system that is widely used to correlate the changes in nuclear and chromatin architecture that accompany differentiation. A hallmark of ES cells is a unique transcriptome characterized by global, low levels of transcription. A reduction in the fraction of the genome that is transcribed occurs as cells differentiate and lose pluripotency (Efroni et al., 2008). Concomitant with this is the compaction of chromatin into heterochromatin domains (Aoto et al., 2006; Kobayakawa et al., 2007; Meshorer et al., 2006).

While the chromatin structure of ES cells was first visualized using conventional transmission electron microscopy (CTEM) and the observation that heterochromatin was more prevalent in differentiated cells compared to undifferentiated ES cells (Park et al., 2004), limited contrast and spatial resolution did not permit the elucidation of the structure of the underlying chromatin in these cells. Studies employing electron spectroscopic imaging (ESI) of undifferentiated ES cells, however, demonstrate that chromatin is, for the most part, uniformly dispersed with no apparent compact chromatin domains as observed in differentiated cells (Efroni et al., 2008; Hiratani et al., 2010). What is intriguing about ES cells is despite the uniform nature of chromatin, discrete regions of H3K9me3 are observed at the light level (Melcer et al., 2012). This is clearly demonstrated in a study aimed at determining the organization of chromatin, specifically constitutive heterochromatin, during the reprogramming of mouse embryonic fibroblast (MEF) cells into induced pluripotent stem (iPS) cells (Fussner et al., 2011b). Somatic cell reprogramming requires the expression of key transcription factors required for pluripotency (Takahashi and Yamanaka, 2006) and is accompanied by global epigenetic remodeling events and the reactivation of the X chromosome (Maherali et al., 2007). Fussner et al. (2011b) observed defined compact domains of constitutive heterochromatin enriched in H3K9me3 in the parental MEFs, prior to reprogramming. In contrast, while the H3K9me3 enrichment is localized...
in fully reprogrammed iPS cells, much like the localization observed in the parental fibroblasts, the chromocentres in iPS cells contained dispersed, rather than compact chromatin with H3K9me3-enriched chromatin domains often indistinguishable from the surrounding, H3K9me3-unmarked chromatin. The implication of these data is that the correlation between classic heterochromatic modifications and heterochromatin structure is not always observed.

The chromatin structure of a mouse lymphocyte further demonstrates the relationship between epigenetic modifications and chromatin structure (Rapkin et al., 2011). Figure 1.4 contains correlative light microscopy/ESI micrographs of an on-section gold-labeled mouse lymphocyte nucleus labeled with H3K9me3-specific antibodies. High resolution imaging revealed an H3K9me3-positive interior surrounded by a rim of heterochromatin devoid of this mark. While future studies are required to determine the structural and biochemical basis for this observation, the presence of morphologically distinct subdomains within the compact chromatin suggest that the presence of H3K9me3 is not the sole determining factor in establishing global heterochromatin organizations.
Figure 1.4: Epigenetic and morphologically distinct domains within a chromocentre.

(A) ESI micrograph of a chromocentre from a mouse lymphocyte with H3K9me3 gold-labeling. The white dots are gold particles indicating the presence of H3K9me3-modified histones. Chromatin is pseudo-colored yellow and protein-based structures cyan. Scale bar, 0.50 µm. (B) Higher magnification of the field shown in (A). The chromatin structure of the H3K9me3-enriched core differs from that of the surrounding rim of compact chromatin devoid of this mark. Scale bar, 0.2 µm. The arrowheads provide a fiduciary reference. Figure taken with permission from (Rapkin et al., 2011).
1.3 Death-domain associated protein (DAXX)

Despite implications in diverse nuclear functions, DAXX is a relatively elusive protein. I began working on DAXX at the start of my graduate training as it is a key component of PML NBs and the lab, at the time, was actively investigating the dynamic nature of PML NBs (Dellaire et al., 2006a; Dellaire et al., 2006b). Recently, however, a novel role for DAXX as an H3.3-specific histone chaperone was identified (Drane et al., 2010; Goldberg et al., 2010; Lewis et al., 2010). These findings shifted the focus of the DAXX field to chromatin biology and in time, might shed much needed insight into how the seemingly disparate functions of DAXX are related.

1.3.1 The disparate functions of DAXX

The death-domain-associated protein (DAXX) is a conserved, ubiquitously expressed 740 amino acid protein composed of an N-terminal α helix bundle and an intrinsically disordered C-terminal half (Figure 1.5) (Escobar-Cabrera et al., 2010). DAXX is a promiscuous interactor reported to associate with numerous proteins involved in disparate processes (summarized in Table 2). It has been hypothesized that the unstructured nature of the protein accounts for this behavior (Escobar-Cabrera et al., 2010; Salomoni and Khelifi, 2006). Indeed, DAXX was originally identified in a yeast two-hybrid screen designed to identify proteins that could associate with the cytoplasmic domain of the Fas receptor. This association was thought to enhance Fas-mediated apoptosis via the activation of the Jun N-terminal kinase pathway (Yang et al., 1997). In this context, DAXX functioned as a pro-apoptotic protein as its overexpression enhanced apoptosis. In a separate yeast two-hybrid screen, DAXX was shown to interact with the cytoplasmic tail of the type II TGFβ receptor and its overexpression sensitized cells to TGFβ-induced apoptosis (Perlman et al., 2001). These reports, together with the observation that RNA interference (RNAi) for DAXX rendered cells resistant to interferon γ- and arsenic trioxide-induced apoptosis (Kawai et al., 2003) confer a pro-apoptotic function for DAXX. Several reports, however, provide evidence that DAXX possesses anti-apoptotic functions. DAXX null embryos, for example, are embryonic lethal (E9.5) and exhibit extensive apoptosis as early as E7.5 (Michaelson et al., 1999). As well, the silencing of DAXX by siRNA strongly sensitized cells to Fas-, TNFα-, actinomycin D-, and ultraviolet-induced apoptosis, despite the observation that silencing alone does not cause any cytotoxic effects on cells (Chen and Chen, 2003). Together, these reports support an anti-apoptotic role for DAXX.
Figure 1.5: Domain architecture of DAXX.

(A) Linear diagram of DAXX illustrating the domain architecture of the protein. Nuclear and chromatin-associated DAXX interacting proteins are listed under the domain they interact with. SIM, SUMO-interaction motif; DHB, DAXX helix bundle; HBD, histone binding domain; Serine/Proline/Glutamic acid and Serine/Proline/Threonine rich. (B) Protein sequence alignment of the DAXX histone binding domain. Identical residues across all species are highlighted in turquoise, whereas conserved substitutions are highlighted in yellow. Human, AF039136; Mouse, AA128374; Xenopus laevis, Q2KHQ3; Drosophila melanogaster, Q8W559.
Table 1.2: DAXX interacting proteins, interaction identification method, and the functional consequence of the interaction.

<table>
<thead>
<tr>
<th>Interacting protein</th>
<th>Method of identification</th>
<th>Functional consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fas</td>
<td>Yeast two-hybrid, co-IP with exogenous proteins</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>PML</td>
<td>Yeast two-hybrid, colocalization of endogenous proteins by IF</td>
<td>Apoptosis, transcription repression</td>
</tr>
<tr>
<td>Pax3</td>
<td>Yeast two-hybrid, GST pull-down, co-IP with exogenous proteins</td>
<td>Repression of Pax3 transcription activity</td>
</tr>
<tr>
<td>ETS1</td>
<td>Yeast two-hybrid, GST pull-down, co-IP with exogenous proteins</td>
<td>Repression of ETS1 target genes</td>
</tr>
<tr>
<td>Pax5</td>
<td>co-IP with exogenous proteins, GST pull-down, co-IP with exogenous and endogenous proteins</td>
<td>Repression of Pax5 transcription activity</td>
</tr>
<tr>
<td>HIPK1</td>
<td>GST pull-down, co-IP with exogenous and endogenous proteins</td>
<td>Apoptosis, regulation of subcellular localization of DAXX</td>
</tr>
<tr>
<td>CENP-C</td>
<td>Yeast two-hybrid, colocalization by IF</td>
<td>Unknown (possibly involved in H3.3 targeting)</td>
</tr>
<tr>
<td>ATRX</td>
<td>Co-IP and colocalization by IF with endogenous proteins</td>
<td>Histone assembly complex</td>
</tr>
<tr>
<td>DEK</td>
<td>Co-IP with exogenous and endogenous proteins</td>
<td>Unknown (possibly involved in H3.3 targeting)</td>
</tr>
</tbody>
</table>

Examples of DAXX interacting proteins. Adapted from (Salomoni and Khelifi, 2006).
Shortly after the discovery of DAXX as an interactor of Fas, conflicting reports on the role of DAXX-dependent apoptotic pathways emerged (Salomoni and Khelifi, 2006). Specifically, Torii et al. failed to detect any direct interactions between DAXX and Fas by either yeast two-hybrid or coimmunoprecipitation assays, instead, they found DAXX primarily located in the nucleus where it was later confirmed to localize to PML NBs (Ishov et al., 1999; Torii et al., 1999). Interestingly, in cells lacking PML or when the PML-interacting region of DAXX is mutated, DAXX is relocalized to the nucleoplasm and within regions of pericentric heterochromatin, and its ability to enhance apoptosis is lost (Torii et al., 1999; Zhong et al., 2000b).

To date, the consensus opinion is that DAXX is a nuclear protein (Figure 1.6) that associates with several subnuclear compartments, including chromatin-containing domains. Although DAXX does not contain any canonical DNA binding domains, the presence of two N-terminal amphipathic helices originally thought homologous to those within yeast corepressors prompted studies investigating the transcription corepressor functions of DAXX. Indeed, DAXX bears transcription repressive functions by countering the activity of several transcription factors such as Pax3 (Hollenbach et al., 1999), ETS1 (Li et al., 2000b), NF-κB (Michaelson and Leder, 2003), and p73 (Kim et al., 2003). Similar to the dependence of DAXX-mediated apoptosis on its PML NB localization, the ability for DAXX to repress transcription is inhibited by its sequestration to PML NBs (Li et al., 2000a). Using a luciferase reporter assay, Li et al. showed the dose-dependent inhibition of DAXX-mediated transcription repression by PML. This finding is consistent with the PML-dependent sequestration of DAXX to PML NBs (away from chromatin), and with the enrichment of DAXX in heterochromatin in the absence of PML (Li et al., 2000a). A drawback of these studies is that they are based on either in vitro experiments or reporter assays expressing ectopic levels of DAXX, its target transcription factors, and the corresponding response elements. Boellmann et al., however, showed that overexpressed DAXX weakly enhanced basal HSF1 activity, and following stress, the induction of endogenous HSP70 was impaired in cells lacking a functional DAXX, suggesting a role for DAXX in HSF1 activation (Boellmann et al., 2004). Although reaching a conflicting conclusion with respect to DAXX and HSP70 upon heat shot, Nefkens et al., observed changes in endogenous HSP25 expression levels prior to and during heat shock recovery in the absence of DAXX, implicating DAXX in transcription regulation (Nefkens et al., 2003; Salomoni and Khelifi, 2006). What is
required, therefore, are studies aimed at identifying more endogenous gene targets of DAXX to confidently assign transcription repressor and activations functions to DAXX.

Figure 1.6: Nuclear localization of DAXX.

(Left) Mouse embryonic fibroblasts (MEFs) nuclei immunolabeled for DAXX (red) and stained with DAPI (blue). The majority of DAXX is enriched in PML NBs (discrete foci) while a fraction can be seen at pericentric heterochromatin domains (DAPI-dense regions; arrowheads). Scale bar, 5 um. (Right) ESI micrograph of a PML NB and the surrounding chromatin of a MEF nucleus labeled with DAXX primary and gold-conjugated secondary antibodies. The white dots (examples highlighted by arrowheads) are gold particles indicating DAXX. The PML NB is marked by the dashed outline. DAXX can be seen enriched in the PML NB as well as distributed throughout the surrounding chromatin. Chromatin is pseudo-colored yellow and protein-based structures cyan. Scale bar, 0.5 µm. (For an overview of the principles of ESI see Section 1.4.4.1.)
1.3.2 H3.3-specific histone chaperone function of DAXX

Despite being proposed for years to play a role in chromatin remodeling and nuclear organization by virtue of its association with HDAC II (Hollenbach et al., 2002), acetyltransferases such as CREB-binding protein (CBP) (Kuo et al., 2005), DNA methyltransferase (Dnmt1) (Puto and Reed, 2008), and CENP-C (Pluta et al., 1998), it was not until recently that DAXX was directly implicated in chromatin regulation. A number of independent studies aimed at elucidating variant-specific chromatin assembly pathways purified H3.1- and H3.3-associated complexes and identified DAXX as an H3.3 binding protein by specifically interacting with the unique AAIG motif of H3.3 (Figure 1.3) (Drane et al., 2010; Goldberg et al., 2010; Lewis et al., 2010). X-ray crystallographic studies of DAXX revealed the histone binding domain (HBD) adopts an \( \alpha \)-helical fold structure that wraps around the H3.3/H4 dimer forming a 1:1:1 ternary complex (Elsasser et al., 2012; Liu et al., 2012). Although HIRA has also been shown to associate specifically with H3.3 independent of DAXX (Tagami et al., 2004), HIRA and DAXX represent non-overlapping H3.3 subcomplexes based differing binding affinities to a negatively charged heparin matrix (Elsaesser and Allis, 2010). Interestingly, DAXX coprecipitates without apparent stoichiometric partners. This finding is consistent with \textit{in vitro} binding studies demonstrating that the direct interaction of DAXX with H3.3/H4 is not dependent on accessory proteins, such as ATRX (Lewis et al., 2010). However, ATRX is required \textit{in vivo} for DAXX to deposit H3.3 in telomeres (Goldberg et al., 2010; Lewis et al., 2010) and pericentric heterochromatin (Drane et al., 2010).

The current DAXX-dependent chromatin assembly model proposes that the diffusible pool of DAXX-H3.3/H4 complexes deliver histones to ATRX-containing complexes associated with telomeric and pericentric heterochromatin (Elsaesser and Allis, 2010). This scenario is likely given the enrichment of ATRX at both of these chromatin domains (Ishov et al., 2004; Wong et al., 2010). Furthermore, it has been suggested that the chromatin-bound complex might contain other histone chaperones and chromatin remodeling factors such as DEK and HDAC II, an intriguing possibility given the biochemical association of DAXX with these factors and a recent study demonstrating a requirement for DEK in the loading of ATRX and H3.3 on telomeres (Hollenbach et al., 2002; Ivanauskiene et al., 2014). Not surprising given the affinity of DAXX for PML NBs association, it has been shown in human cells that a fraction of the DAXX-dependent soluble pool of H3.3/H4 dimers is localized to PML NBs prior to deposition in
chromatin (Corpet et al., 2014; Delbarre et al., 2013). Based on these results, a second model has been proposed whereby PML NBs act as 'triage' centres, where DAXX-H3.3/H4 complexes may pair up with distinct chaperones that target their deposition (Delbarre et al., 2013). This scenario is also intriguing since both DEK and ATRX have been shown to associate with PML NBs and heterochromatin domains (Hollenbach et al., 2002; Ishov et al., 2004; Ivanauskiene et al., 2014).

1.4 Visualizing chromatin landscapes

There are several approaches that can be used to address questions pertaining to chromatin biology. Many, such as ChIP-Seq and the 3C technologies are biochemical-based tools that provide genome wide analysis of chromatin epigenetic states and the 3D organization of DNA within a nucleus, respectively. In contrast to microscopy that allows for single cell analysis, these techniques are more systematic in nature and can provide high resolution information about protein-DNA and DNA-DNA interactions. Furthermore, they can put data collected from cell-to-cell basis in the context of genome-wide behavior in cell populations (de Wit and de Laat, 2012). A major drawback, however, is they do not provide any information about the shape, and structure of the genome and individual genomic regions. They do not allow for the in vivo visualization of the underlying chromatin structure of the functional domains being investigated. Ideally, studies that combine microscopy and biochemistry-based genomic approaches will prove to be the most powerful in fully deciphering how the shape and structure of genomes contribute to its function in living cells.

In the following sections, I will provide an overview of a powerful genome-wide technique commonly employed in the field of chromatin biology. I will then discuss a brief history of the major findings that we have learned about the structure of chromatin from light microscopy. Finally, I will introduce electron microscopy and describe the advantages of electron spectroscopic imaging (ESI) over conventional transmission electron microscopy (CTEM). ESI is my technique of choice in addressing specific questions pertaining to the structural organization of heterochromatin domains.
1.4.1 Genomic approach to study chromatin: ChIP-Seq

In order to decipher the gene regulation networks that guide development and differentiation, a comprehensive description of protein-DNA interactions is essential. The main tool for assaying protein-DNA interactions *in vivo* is chromatin immunoprecipitation (ChIP). The technique entails shearing chromatin-protein complexes by sonication into approximately 500 bp DNA fragments. DNA fragments associated with a protein of interest are immuno-purified using specific antibodies and the associated DNA is purified. In one of the pioneering studies using this technique, Solomon et al. used an antibody against the histone H4 and performed ChIP on heat-shocked and unshocked Drosophila cells and showed that H4 remained bound to hsp70, despite the changing transcription rate (Solomon et al., 1988). The technique was later improved by the introduction of microarrays which allowed for genome-wide scale interaction studies. With this improvement, genome-wide binding profiles of transcription factors across entire genomes could be determined (Ren et al., 2000). The true power of the ChIP protocols became apparent with the advent of next-generation sequencing technologies that allowed for the sequencing of orders of magnitude greater numbers of DNA fragments in a single run. In ChIP-Seq, the obtained DNA fragments are sequenced, rather than being hybridized to a microarray. In the first large-scale study of its kind, Barski et al. used ChIP-Seq to generate high resolution maps of 20 histone post-translational modifications, as well as the histone variant H2A.Z, and RNA polymerase II in human T-cells. Not only did they map typical profiles of gene activation and repressive marks, such as H3K4me3 and H3K9me3, respectively, but they linked chromosome breakpoints observed in many T-cell cancers to specific histone modifications (Barski et al., 2007). These techniques have allowed for a global understanding of chromatin assignments as well as provided tools to identify and characterize chromatin regions and assign 'trademarks' that go beyond cytological definitions.

1.4.2 Light microscopy views of chromatin

The model of a three dimensional spatial arrangement of whole chromosomes into segregated regions has a long history dating back to Carl Rabl’s and Theodor Boveri’s first use of the term chromosome territory (CT) (Rapkin et al., 2011). The concept largely fell out of favor, however, with the advent of the electron microscope in the 1950s and the high resolution images revealing the intermingling of interphase chromatin fibres with no evidence of chromosomal separation
It was not until the development of fluorescence in situ hybridization (FISH) and confocal microscopy techniques conducive to the analysis of a single intact nucleus could whole chromosomes be visualized with the use of fluorescent "paints". Although the resolution limit of conventional confocal laser microscopy (200 nm radially, ~1µm axially) does not allow for individual fibres within the chromosome to be resolved, the segregation of individual chromosomes into discrete volumes, or CTs, appears to be a general feature of nuclear organization (Rapkin et al., 2011). Since FISH has clearly established the arrangement of chromosomes into localized domains or territories in the interphase nucleus, it has been further applied to explore the mechanisms determining the folding of DNA within individual CTs and the orientation of individual gene loci with respect to the CT volume. For example, Kupper et al. (2007) used BACs derived from several human chromosomes to determine how either transcriptional activity, local gene density within a 10 Mb window, presence in a “G” or “R” band, or replication timing, correlated with the spatial positioning of a gene locus with respect to the CT surface and the nuclear periphery. Although transcriptionally active regions have been shown to protrude from the CT surface (Chambeyron and Bickmore, 2004), Kupper and colleagues found no correlation between transcriptional activity or gene density of a chromosomal segment and its spatial relationship to the CT surface (Kupper et al., 2007). There was, however, a correlation between gene density and proximity with the nuclear interior, suggesting that gene positioning and chromosome folding may be dictated by local gene density. FISH-based methods combined with light microscopy techniques have been exploited to show that although CTs appear to remain stable in size and shape throughout interphase (Muller et al., 2010), chromatin folding is a dynamic process, with cis and/or trans genomic associations changing cellular states such as cell cycle progression (Vazquez et al., 2001) and differentiation (Chambeyron et al., 2005). In other instances, hybridization-based studies have been effectively used to delineate the spatial arrangement of distinct classes of DNA classes with respect to each other, such as the functionally independent and spatio-temporal isolation of minor and major satellite repeats of centromeres and pericentric heterochromatin, respectively (Guenatri et al., 2004).

1.4.3 Super resolution microscopy

The advent of super-resolution far field visible light microscopy techniques such as stimulated emission depletion (STED) microscopy (Hell, 2003), structured illumination (SIM) (Carlton,
and stochastic optical reconstruction microscopy (STORM) (Rust et al., 2006), have allowed us to surpass the resolution limit of conventional visible light microscopy (~200 nm) (Rapkin et al., 2011). To date, super-resolution microscopy techniques have been used in the study of chromatin domains such as telomeres (Doksani et al., 2013) and the inactive X chromosome (Smeets et al., 2014). Doksani et al. (2013) combined STORM with FISH to image telomeric DNA in situ and demonstrated that the shelterin protein TRF2 is required for the formation and maintenance of the large telomeric t-loop structures. Smeets et al. (2014), on the other hand, used 3D-SIM to investigate properties of the inactive X chromosome at high resolution and showed that rather than Xist transcripts uniformly coating the Barr body, Xist appeared as distinct focal structures within the volume of the inactive X chromosome and hypothesized that these foci mark distinct regulatory sequences that become repressed during inactivation. As these techniques become more widely available to cell biologists, we will see an explosion in their application to chromatin biology. Until that time, electron microscopy remains the most powerful tool to address questions pertaining to chromatin ultrastructure.

1.4.4 Electron Microscopy

Conventional transmission electron microscopy (CTEM) has been instrumental to our understanding of chromatin and how it is organized in the nucleus. For one, CTEM was used to initially describe heterochromatin, revealed by the presence of darkly stained, presumably densely-packed chromatin. As well, the discovery of the nucleosome along with initial experiments describing the packing of 10 nm chromatin fibres into higher order structures were dependent on CTEM (Olins and Olins, 1974; Woodcock et al., 1984). Since biological material scatters electrons poorly, CTEM requires heavy atom contrast agents, such as uranyl acetate and lead citrate. Unfortunately, significant disadvantages accompany the use of these agents in in situ experiments. First, heavy atom salts coat the biological structures to be imaged resulting in a loss of spatial resolution. Second, the various biochemical components of a biological sample possess different affinities for the contrast agents, creating the illusion of ‘electron-dense’ and mass-depleted regions that do not accurately represent the actual biological mass densities within the sample (Ahmed et al., 2009). To avoid the inherent drawbacks of contrast agents required to generate CTEM images of chromatin structures, Electron Spectroscopic Imaging (ESI) can be
employed. Since ESI produces high-resolution, high-contrast dark field images, the use of contrast-enhancing agents can be circumvented.

1.4.4.1 Visualizing chromatin using ESI

Electron spectroscopic imaging (ESI) is a specialized form of transmission electron microscopy, which is based on electron energy loss spectroscopy. When an incident electron beam encounters a thin specimen, the majority of electrons pass through the sample without scattering or scatter elastically, the latter contributing to image contrast in CTEM. A fraction of the incident electrons, however, are scattered inelastically (they lose energy through sample ionization and excitation events) and the amount of energy lost by the incident electron is element-specific. An “imaging” spectrometer can thus use these electrons to create element-specific distributions or maps with high spatial resolution and sensitivity (Bazett-Jones and Hendzel, 1999; Bazett-Jones and Ottensmeyer, 1981). In studying nucleic acid structures in biological material, phosphorus (P) and nitrogen (N) atomic imaging is particularly advantageous. Since the inherent phosphorus signal within nucleic acid is significantly higher than that contributed by protein-based structures, phosphorus images can be used to map the nucleic acid component in situ within a biological sample. Furthermore, chromatin-related structural features such as non-histone, chromatin-associated protein complexes are readily distinguished (Bazett-Jones et al., 2008).

ESI sample preparation commonly involves standard EM techniques including aldehyde fixation, dehydration, and embedding in a suitable resin. The beam intensity required to form element-specific maps is too high to perform cryo-imaging of hydrated specimens, however, cryopreservation followed by freeze-drying or freeze-substitution can be combined with ESI (Aronova et al., 2007; Grunwald et al., 2008). Although significant mass loss occurs due to the high exposure required for ESI, the information collected from the element-specific micrographs (P and N) is quantitative since rate of loss of biologically relevant elements is relatively consistent (Williams and Carter, 2008). Therefore, measurements can be made that characterize structures based on their P/N ratios, making ESI a powerful technique for delineating structural information about the nucleus. Furthermore, specific nuclear structures such as PML NBs (Boisvert et al., 2000), nucleolus (Politz et al., 2005), and the nuclear pore complex (Ahmed et al., 2010) can be easily identified and distinguished from the surrounding chromatin. An
important feature of ESI is that compact chromatin domains, such as those found at the nuclear periphery and surrounding the nucleolus, are easily distinguished from the highly dispersed fibres of open euchromatin domains in somatic cells, in contrast to CTEM where only very compact chromatin domains are contrasted (Bazett-Jones et al., 2008). Therefore, ESI is uniquely suited to studying global chromatin organization.

A summary of imaging-based approaches to study chromatin including their resolution capacity is summarized in Figure 1.7.
Figure 1.7: The scales of chromatin structural information through the lenses of the relevant microscopy techniques.

(A) A deconvolved image of a DAPI-stained C2C12 myoblast nucleus obtained with conventional light microscopy. (B) Chromosome territories within a human fibroblast nucleus detected using 3D-FISH with human chromosome paint probes. (C) Nucleus from a 4 day differentiated OS25 ES cell labeled with MMU11 chromosome paint (green) and a Hoxb1 locus gene-specific probe (red). (D) Reconstructed 3D-SIM image of a DAPI-stained C2C12 nucleus. (E) Dual color SPDM image of a human U2OS cell expressing H2A-mRFP (red) Snf2H-GFP (green). (F) Low magnification uranyl acetate- and lead citrate-stained mouse erythroleukemia nucleus acquired with CTEM. (G) 19,000× magnification ESI image of a C2C12 myoblast chromocentre. FM (fluorescence microscopy; A–C), SIM (structured illumination; D), SPDM (spectral precision distance microscopy; E), CTEM (conventional transmission electron microscopy; F), ESI (electron spectroscopic imaging; G). Figure taken with permission from (Rapkin et al., 2011).
1.5 Summary and hypothesis

The death-domain-associated protein (DAXX) is a recently identified histone chaperone, which together with ATRX, deposits the replacement histone variant H3.3 at telomeres and in regions of pericentric DNA (Drane et al., 2010; Goldberg et al., 2010). In addition to its role as a histone chaperone, DAXX can function as a transcription repressor and a mediator of apoptosis (Table 1.2). However, the mechanisms by which DAXX functions and possibly regulates these seemingly disparate pathways is largely unknown. To date, the roles of DAXX, in particular its histone chaperone function, have been investigated primarily using biochemical approaches. Therefore, a close examination of the impact of DAXX-dependent H3.3 deposition on the structural organization of chromatin may be key in assigning a comprehensive and unified function of DAXX. Global changes in the organization of constitutive heterochromatin domains into tissue-specific chromatin architectures have been observed in response to developmental and differentiation cues. This organization is dictated in part by the deposition and posttranslational modification of specific histone variants. I hypothesize that, through the deposition of H3.3, DAXX is a key factor in maintaining the structural integrity of pericentric heterochromatin domains. Furthermore, by virtue of the intricate association of heterochromatin to other nuclear structures, I hypothesize that DAXX is a global regulator of nuclear architecture. To address this hypothesis, I will use correlative LM/ESI to visualize at high-molecular resolution, chromatin domains in situ in the absence of DAXX. In addition, I will examine the role of DAXX in a tissue differentiation model to gain insight into why mice lacking DAXX fail to complete development. The results from these experiments will provide, for the first time, a visualization of the consequences that the loss of a histone chaperone has on the structural organization of chromatin and further our understanding the role of global nuclear organization on differentiation.
Chapter 2

2 The role of the histone chaperone DAXX in the structural organization of heterochromatin

In 2010, Dr. David Allis of the Rockefeller University was invited by the Biochemistry Graduate Student Union to give a seminar in the George Connell Lecture series. Dr. Allis is best known for his discovery that histone hyperacetylation correlates with active genes, observations linking epigenetics with gene regulation which paved the way for the histone code (Brownell et al., 1996; Jenuwein and Allis, 2001; Kuo et al., 1996; Kuo et al., 1998; Strahl and Allis, 2000). In his talk, Dr. Allis described the then recent findings in his lab implicating DAXX and ATRX in a novel H3.3-specific chromatin assembly complex (Goldberg et al., 2010). While DAXX/ATRX interactions have been previously reported, it was in the context of PML NBs and transcription repression, rather than histone chaperone activity (Ishov et al., 2004). Even more intriguing, was Dr. Allis’ finding that the histone variant H3.3 was being deposited into telomeric repeats, a seemingly unusual genomic location to find the variant often associated with active genes in ES cells (Ahmad and Henikoff, 2002). This, together with a paper reporting similar findings in fibroblasts and demonstrating DAXX-dependent deposition of H3.3 into pericentric heterochromatin (Drane et al., 2010) shifted the focus of DAXX-based research to chromatin biology. In this chapter, I will describe my findings demonstrating the involvement of DAXX in the organization of pericentric heterochromatin domains and show the structural consequences upon loss of this histone chaperone.

2.1 Introduction

An essential component of gene regulation is the organization of eukaryotic genomes into functionally and spatially discrete chromatin domains. Based on cytogenetic observations, euchromatin undergoes cell cycle-dependent stages of condensation and de-condensation whereas the more densely stained heterochromatin remains condensed throughout the cell cycle (Heitz, 1928). Euchromatin and heterochromatin compaction states are functionally important to partition the genome into gene activation and silencing environments, respectively (Kornberg and Lorch, 1992). A well characterized example of developmentally regulated or facultative
heterochromatin and gene silencing is the dosage compensation in females by the silencing of one X chromosome (Avner and Heard, 2001). In addition to facultative heterochromatin, eukaryotes possess constitutive heterochromatin consisting of centric and pericentric loci. In mouse cells, pericentric constitutive heterochromatin domains form 8-30 chromocentres, which are clusters of the AT-rich, 234 bp major satellite repeat contributed from multiple chromosomes (Guenatri et al., 2004; Vissel and Choo, 1989). The resulting spatially discrete structures provide a scaffold for centromere function and are proposed to act as platforms to concentrate and control heterochromatic environments (Allshire and Karpen, 2008; Almouzni and Probst, 2011).

One way to establish global chromatin environments is through the modification of the four core histones (H3, H4, H2A, H2B) of the nucleosome, particularly their N-terminal tails. Active euchromatin and silent heterochromatin are enriched in distinct sets of histone tail modifications. The methylation sites on H3K4, H3K36, and H3K79 are enriched in active regions, whereas the methylation on H3K9, H3K27, and H4K20 correlate with repressed regions of the genome (Kouzarides, 2007). Another way to distinguish chromatin is through the deposition of histone variants. For example, the H2A variant macroH2A is concentrated in the inactive X chromosome and the H3 variant CenH3 (Cenp-A) is a component of centromeric chromatin (Costanzi and Pehrson, 1998; Earnshaw and Rothfield, 1985). Despite being preferentially associated with transcriptionally active genes, the replication-independent H3 variant H3.3 is required for the formation of heterochromatin in the developing mouse embryo (Ahmad and Henikoff, 2002; Santenard et al., 2010). Furthermore, H3.3 deposition was observed in telomeric and pericentric repeats in mouse embryonic stem cells and fibroblasts, respectively (Drane et al., 2010; Goldberg et al., 2010). Whereas H3.3 is specifically deposited into active regions of the genome by the histone chaperone HIRA, it is the complex containing the chromatin remodeler ATRX and the chaperone DAXX that is responsible for H3.3 deposition into heterochromatin (Drane et al., 2010; Goldberg et al., 2010; Ray-Gallet et al., 2002; Tagami et al., 2004). DAXX is largely an unstructured protein, although the N-terminal histone binding domain adopts an α-helical fold conformation (Elsasser et al., 2012; Escobar-Cabrera et al., 2010). Through this domain, DAXX binds specifically to the H3.3 histone variant and can effectively remodel H3.3-containing nucleosomes in vitro independent of ATRX (Lewis et al., 2010).

DAXX was originally identified in a screen designed to identify proteins that could bind to the cytoplasmic domain of the Fas receptor (Yang et al., 1997). DAXX is predominantly a nuclear
protein, however, where it associates with promyelocytic leukaemia nuclear bodies (PML NBs) and ATRX-containing heterochromatic regions in S-phase of the cell cycle (Ishov et al., 1999; Ishov et al., 2004). Until the discovery of novel DAXX-containing chromatin assembly pathways, the two ascribed functions of DAXX were a mediator of apoptosis and a transcription repressor (Hollenbach et al., 2002; Khelifi et al., 2005; Michaelson and Leder, 2003; Perlman et al., 2001; Yang et al., 1997). Despite concerted efforts to elucidate the functions of DAXX, how these seemingly disparate functions are regulated and contribute to its role as a histone chaperone is unclear.

Although mechanistic details have yet to be elucidated, the involvement of the DAXX/ATRX complex in telomere stability and cancer biology, including pancreatic neuroendocrine tumors and pediatric glioblastoma, together with its role in a chromatin assembly pathway possibly specific for repetitive DNA elements, implicates DAXX as a key factor in heterochromatin maintenance (Heaphy et al., 2011; Jiao et al., 2011; Lewis et al., 2010; Schwartzentruber et al., 2012). This, together with the requirement for H3.3 in the formation of heterochromatin in the mouse embryo prompted me to explore the role DAXX plays in organizing the structure of heterochromatin domains and to determine if DAXX-dependent heterochromatin organization contributes to global nuclear architecture. To date, these roles have been investigated primarily using biochemical approaches which provide genome-wide views on cell populations. A limitation, however, is they do not provide a visualization of underlying chromatin structures. My approach, therefore, is to combine light microscopy (LM) with electron spectroscopic imaging (ESI), a specialized form of energy-loss transmission electron microscopy, to visualize chromatin domains in situ at high-molecular resolution (Dellaire et al., 2004). I found that in the absence of DAXX, there is a loss of morphological distinction between the repressive histone modification H3K9me3 and chromatin compaction. Furthermore, I see a loss of correlation between major satellite repeats and H3K9me3-enriched heterochromatin domains. Additionally, I show that the DAXX-dependent heterochromatin organization is required for the structural integrity of the nucleolus. Together, these studies implicate DAXX as a key regulator of global heterochromatin organization and provide for the first time, a link between H3.3 deposition and the structural integrity of the nucleolus.
2.2 Materials and methods

2.2.1 Cell culture and plasmid transfection

T-immortalized wild type (DAXX \(+/+)\) and DAXX null (DAXX \(-/-\)) mouse embryonic fibroblasts (Ishov et al., 2004) were cultured in DMEM (Wisent, Gibco) supplemented with 10% fetal bovine serum (FBS) (Wisent), 10 \(\mu\)g/ml penicillin and streptomycin (Wisent), and 2 mM L-glutamine (Wisent). Cells were grown on glass cover slips for immunofluorescence microscopy, electron microscopy, and transfections. For the FISH experiments, cells were grown on glass slides.

For the transfection experiment, cells were seeded to 50% confluency on glass cover slips and transiently transfected (Lipofectamine 2000 Transfection Reagent, Invitrogen) with the purified (Qiagen Plasmid Midi Kit, Qiagen) FLAG-HA-H3.3 expression vector (Morozov et al., 2012).

2.2.2 Immunofluorescence microscopy

Cells were fixed in 2% paraformaldehyde (Electron Microscopy Sciences) in PBS (Wisent) for 10 min at room temperature (RT) and permeabilized in 0.5% Triton X-100 (BioShop) in PBS for 5 min at RT. Primary antibodies used were rabbit anti-DAXX (Santa Cruz Biotechnology), goat anti-ATRX (Santa Cruz Biotechnology), mouse anti-PML (Upstate), rabbit anti-H3.3 (Abcam), rabbit anti-H3K9me3 (gift from P. Singh), mouse anti-H3K9me3 and mouse anti-H4K20me3 (gifts from H. Kimura), mouse anti-B23 (Santa Cruz Biotechnology), rabbit anti-nucleolin (Santa Cruz Biotechnology), and mouse anti-FLAG (Sigma-Aldrich). Secondary antibodies used were rabbit, sheep, or mouse Cy2, Cy3, and Cy5 (Jackson Laboratories). Cells were soaked in 50 \(\mu\)g/ml DAPI and mounted using anti-fade reagent (buffered glycerol with 4% n-propyl gallate).

Images were collected on an Olympus IX81 inverted microscope equipped with a Cascade II CCD camera (Photometrics) using either a 60\(\times\) or 100\(\times\) oil-immersion objective lenses. MetaMorph Microscopy Automation & Image Analysis Software (Molecular Devices) was used to collect images. Images were processed with Volocity 3D Image Analysis Software (PerkinElmer) and Photoshop (Adobe). Graphs and statistics were constructed using GraphPad Prism (GraphPad Software Inc) and reported as standard error of the mean. Line scans data was collected using ImageJ (National Institute of Health) and histograms constructed using GraphPad.
Prism (GraphPad Software Inc). Pearson correlation ratios between H3.3 and H3K9me3-enriched domains were computed by ImageJ software using around 270 chromocentres (Figure 2.2). Pearson coefficient values over 0.4 were considered strong correlations, between 0.3 to 0.39 were considered moderate, and values between 0.2 to 0.29 weak. Values less than 0.19 were considered negligible correlations.

### 2.2.3 Correlative LM/ESI microscopy

For a detailed sample preparation protocol and ESI procedure, see (Ahmed et al., 2009). Cells were immunolabeled with H3K9me3 and post fixed in 1% glutaraldehyde (Electron Microscopy Sciences). Following dehydration, cells were embedded in Quetol (Electron Microscopy Sciences). Samples were sectioned to 70 nm by an ultramicrotome (Leica). Grids containing the sample sections were imaged on a DMRA2 microscope (Leica) equipped with an ORCA-ER camera (Hamamatsu). Following carbon coating, electron micrographs were collected on a transmission electron microscope (Tecnai 20, FEI). To generate the phosphorus and nitrogen images, the microscope was operated at 200 kV using a post column filter (Gatan) at 120 and 155, and 385 and 415 eV, respectively. Images were recorded on a CCD camera and collected using DigitalMicrograph software (Gatan). To generate the ESI images presented in my thesis, phosphorus images were subtracted from nitrogen images to normalize the chromatin contribution in the nitrogen images to zero. Phosphorus images are pseudo-colored yellow and overlaid onto the subtracted nitrogen images that are pseudo-colored blue. In the overlay images, yellow represents nucleic acid-based structures, and the blue represents protein-based structures. To correlate the fluorescence signal with the underlying chromatin structure, the fluorescence images were overlaid onto the phosphorus-enhanced low magnification electron micrographs and H3K9me3-positive regions identified and images. For presentation, the approximate boundaries of the H3K9me3 region are marked by a dotted line. Images were processed with Photoshop (Adobe).

### 2.2.4 3D FISH

Cells grown on glass slides were immunolabeled with H3K9me3 or B23 and post fixed in 2% paraformaldehyde (Electron Microscopy Sciences) for 10 min at RT. Cells were washed three times in PBS and maintained overnight in 20% glycerol in PBS at 4°C. Cells were snap frozen, partially thawed, and then returned to the 20% glycerol solution. This was repeated five times.
The slides were washed three times in PBS, treated with 0.1 N HCl for 5 min, then washed in 2× SSC. The slides were left overnight in a solution of 50% formamide (Invitrogen) in 2× SSC at 4°C. Slides were denatured in pre-warmed 70% formamide in 2× SSC for 5 min at 75°C and then immediately placed in cold 70% formamide in 2× SSC. Major satellite and rDNA FISH probes (The Centre for Applied Genomics) were directly labeled with spectrum orange fluor-conjugated nucleotides. Probe and mouse Cot-1 DNA (2:1 ratio) were mixed in hybridization buffer (50% formamide, 10% dextran sulfate, 50 nM sodium phosphate buffer, pH 7.0, in 2× SSC) and denatured for 5 min at 80°C and then pre-annealed for 30 min at 37°C. Hybridization proceeded overnight in a humidified chamber at 37°C. Slides were washed three times in 50% formamide in 2× SSC for 5 min at 42°C, once in 0.5× SSC for 5 min at 62°C, and once in 2× SSC for 5 min at RT. Slides were mounted using Vectashield (Vector Laboratories) containing DAPI). Images were collected on an Olympus IX81 inverted microscope equipped with a Cascade II CCD camera (Photometrics) using either a 60× or 100× oil-immersion objective lenses. MetaMorph Microscopy Automation & Image Analysis Software (Molecular Devices) was used to collect images. Images were processed and quantified with Volocity 3D Image Analysis Software (PerkinElmer) and Photoshop (Adobe). Graphs and statistics were constructed using GraphPad Prism (GraphPad Software Inc). For the major satellite FISH experiment, data was presented as standard error of the mean. Line scans data was collected using ImageJ (National Institute of Health) and histograms constructed using GraphPad Prism (GraphPad Software Inc). For the rDNA experiment, data was reported as a maximum/minimum box plot.

2.2.5 Western blot

Cells were grown in 10-cm culture dishes were harvested and resuspended with 9M Urea in 10 mM Tris-Cl, pH 6.8. Samples were quantified using the Bio-Rad Protein Concentration Assay (Bio-Rad Laboratories) and diluted to 10 µg/20µl in 9M Urea containing a 6× SDS-PAGE loading buffer. Protein samples were resolved on either 10% (GAPDH) or 15% (H3K9me3 and H3.3) SDS-PAGE gels and transferred onto nitrocellulose membranes (GE Healthcare). Membranes were blocked overnight in 5% skim milk powder in TBST (0.05% Tween-20, Bio-Rad) Primary antibodies used were mouse anti-H3K9me3 (gift from H. Kimura), rabbit anti-H3.3 (Abcam), rabbit anti-DAXX (Sigma), and rabbit anti-GAPDH (Sigma). Secondary antibodies used were mouse and rabbit anti-HRP (Sigma). Detection was performed using the Western Lightning Plus ECL system (PerkinElmer).
2.3 Results

2.3.1 Nuclear localization of DAXX

As a prelude to addressing the role of DAXX as a regulator of heterochromatin structure via its H3.3 chaperone activity, I wanted to characterize its localization in the control fibroblasts that will be exploited in this chapter of my thesis. It has been shown that a fraction of DAXX localizes to PML NBs and to pericentric heterochromatin domains in the S-phase of the cell cycle (Ishov et al., 1999; Ishov et al., 2004; Zhong et al., 2000b). I performed an immunofluorescence experiment using wild type fibroblasts and antibodies against DAXX, PML to mark PML NBs, and ATRX, DAXXs accessory protein in the H3.3 histone chaperone complex. Consistent with previous findings, I observed DAXX enriched in PML NBs (Figure 2.1A, arrowheads). Close examination of the 'background' DAXX shows that a fraction is enriched in DAPI-dense regions (Figure 2.1A, B). To confirm that these structures are indeed pericentric heterochromatin domains, I prepared the same cells with antibodies against DAXX, ATRX, and H3K9me3, the post translational histone modification enriched in chromocentres. While the majority of chromocentres do not contain large accumulations of DAXX, a fraction of DAXX does indeed localize to chromocentres (Figure 2.1B, arrowheads). Western blot and immunofluorescence analysis of wild type (control) and DAXX null (experimental) cells confirms that the experimental cells are indeed null for the DAXX protein (Figure 2.1C, D).
Figure 2.1: Localization of endogenous DAXX.

(A) Wild type cells immunolabeled for DAXX (red), PML (green), and ATRX (blue). Arrowheads and enlarged regions indicate representative DAXX-containing PML NBs. Scale bar, 5 µm. (B) Wild type cells immunolabeled for DAXX (red), H3K9me3 (green), and ATRX (blue). Arrowheads and enlarged regions indicate DAXX-enriched chromocentres. DAXX levels in the enlarged regions were enhanced in photoshop. Scale bar, 5 µm. (C) Western blot analysis of whole cell lysates from wild type (WT) and DAXX null (DN) fibroblasts. (D) Wild type and DAXX null cells immunolabeled with DAXX antibodies. DAXX protein is not detectable in the null cells. Scale bar, 5 µm.
2.3.2 H3.3 is enriched in a fraction of H3K9me3-enriched domains

The discovery of DAXX as an H3.3-specific chaperone responsible for its deposition in telomeres and pericentric heterochromatin (Drane et al., 2010; Goldberg et al., 2010) was evidence that H3.3 is not restricted to active regions of the genome. To determine the DAXX-dependent nuclear localization of endogenous H3.3, I performed an IF experiment using wild type and DAXX null MEFs (Ishov et al., 2004). Using H3K9me3 to mark chromocentres, I observed that, within the resolution limit of fluorescence microscopy, H3.3 had similar distribution patterns in both cell lines (Figure 2.2A). Line scan intensity plots were used to show H3.3 enrichment in chromocentres. Histograms of the regions marked by arrows show that a fraction of the H3K9me3 peaks contained H3.3 enrichments in a similar distribution pattern (peaks marked by asterisks). Pearson coefficients were determined between H3.3 and H3K9me3 (Figure 2.2B). Values over 0.4 were considered strong correlations, between 0.3 to 0.39 were considered moderate, and values between 0.2 to 0.29 weak. Values less than 0.19 were considered negligible correlations. The percentage of strong correlations in wild type and DAXX null cells were similar at 4.8 and 5.6 %, respectively. Moderate positive correlations went from 4.4 % in wild type cells to 7.0 % in the DAXX null cells. Weak positive correlations were similar between the two cell types at 9.6 % in wild type and 11.2 % in DAXX null. Negligible correlations decreased from 81.1 % in the wild type to 76.2 % in the DAXX null cells. Mean values of the complete data set were significantly different (P < 0.002). This data is consistent with previous reports demonstrating a DAXX-dependent deposition of H3.3 at pericentric heterochromatin (Drane et al., 2010).

As a control for epitope accessibility within compact chromatin domains, I transiently transfected wild type and DAXX null cells with a FLAG-H3.3 plasmid and repeated the immunofluorescence using anti-FLAG primary antibodies (Figure 2.2C). Consistent with the endogenous labeling of H3.3, over-expressed H3.3 had similar nuclear diffuse staining patterns with respect to endogenous distributions. Line scan intensity plots revealed only a fraction of chromocentres contained an enrichment of FLAG-H3.3, suggesting the forced expression of H3.3 is not sufficient to drive H3.3 into constitutive heterochromatin domains. Western blot analysis of whole-cell lysates (Figure 2.2D, E) showed that the expression levels of H3.3 and H3K9me3 are similar in both cells lines.
Figure 2.2: Distribution of H3.3 in wild type and DAXX null fibroblasts.

(A) Immunolabeled wild type and DAXX null fibroblasts with H3.3 (red) and H3K9me3 (green). White arrows through MERGE images indicate intensity linescan plot and direction. Asterisks mark chromocentres containing H3.3 enrichment. Scale bar, 5 µm. (B) Pearson correlations between H3.3 and H3K9me3-enriched chromocentres. Data grouped according to Pearson
correlation relationship (see Section 2.2.2) and reported as percentages of the total number of chromocentres analyzed. Percentages of strong correlations in wild type and DAXX null cells are 4.8 and 5.6 %, respectively. Mean values of the complete data set are significantly different (P < 0.002). Over 250 chromocentres analyzed for each cell type. (C) FLAG-H3.3 transfected wild type and DAXX null fibroblasts and immunolabeled for FLAG (red) and H3K9me3 (green). White arrows through MERGE images indicate intensity linescan plot and direction. Asterisks mark chromocentres containing H3.3 enrichment. Scale bar, 5 µm. (D-E) Western blot analysis of whole cell lysates of H3.3 (C) and H3K9me3 (D).

2.3.3 DAXX maintains the structural integrity of pericentric heterochromatin domains

DAXX is part of an H3.3 chromatin assembly pathway possibly specific for repetitive regions of the genome (Lewis et al., 2010). This, together with the association of DAXX with major satellite repeats and the requirement for H3.3 deposition in the formation of heterochromatin in early mouse development (Drane et al., 2010; Santenard et al., 2010), led me to ask if DAXX is involved in the structural organization of pericentric heterochromatin domains. I immunolabeled wild type and DAXX null mouse embryonic fibroblasts with antibodies specific to the repressive histone modifications H3K9me3 and H4K20me3 (Figure 2.3A). Consistent with previous reports (Bulut-Karslioglu et al., 2012), I found that chromocentres from both cell lines are enriched in H3K9me3 and H4K20me3, two signature marks of pericentric heterochromatin. I found the number of chromocentres is indistinguishable between the two cell types with an average of 30.5 ± 5.1 and 33.0 ± 5.1 per nucleus in wild type and DAXX null cells, respectively (Figure 2.3B).
Figure 2.3: Distribution of H3K9me3 and H4K20me3 in wild type and DAXX null fibroblasts.

(A) IF of wild type and DAXX null fibroblasts labeled with antibodies against H3K9me3 (red) and H4K20me3 (green). Scale bar, 5 µm. (B) Quantification of the average number of chromocenters. Error bars represent SEM from three independent experiments. Over 1200 chromocenters from a minimum of 40 cells analyzed.
To determine the underlying chromatin structure of chromocentres in the absence of DAXX, I prepared wild type and DAXX null MEFs for LM/ESI using the H3K9me3 histone modification as a marker for chromocentres. EM grids containing fluorescent H3K9me3 labeling were imaged (Figure 2.4A, upper left panel) and overlayed onto the corresponding low magnification EM micrographs recorded from the same physical section (Figure 2.4A, bottom left panel). H3K9me3-positive regions were identified and imaged (Figure 2.4A, center panel). ESI micrographs of H3K9me3-enriched domains were obtained and the approximate boundaries of the H3K9me3 signal outlined by dashed lines (Figure 2.4A, right panel). Consistent with previous studies (Efroni et al., 2008; Fussner et al., 2011b), chromocentres from wild type cells, are radially symmetric structures comprised of compact chromatin (Figure 2.4A). They occupy discrete regions within the nucleus and are readily discernible from the surrounding euchromatin. Furthermore, the boundaries of the chromocentres, as approximated by the H3K9me3 fluorescence (marked by a dashed line) correlate with the regions of compact chromatin. In contrast to these observations in the wild type cells, the loss of DAXX resulted in the loss of the spatial correlation between H3K9me3 enrichment and compact chromatin (Figure 2.4B, right panel). In this example, the H3K9me3 signal shows two regions of enrichment (arrowhead, dashed lines), whereas these plus a third connected domain of compact chromatin can be discerned that does not contain the mark. Despite not containing this heterochromatin mark, the domain can be readily distinguished morphologically from the surrounding chromatin.
Figure 2.4: Disruption of chromocentres in the absence of DAXX.
Correlative LM/ESI micrographs of wild type (A) and DAXX null (B) fibroblasts. H3K9me3 fluorescence (left panel, top) overlayed onto the P-enhanced mass image (left panel, bottom) generating the correlative image (centre panel). ESI micrograph (right panel) of the corresponding H3K9me3-containing structure (arrowhead). Approximate boundaries of the H3K9me3 region are indicated by a dashed line. Chromatin is pseudo colored yellow and protein-based structures cyan. Scale bar, 0.5 µm.
In addition to discrete chromocentres (Figure 2.4A), centric and pericentric regions of the genome can be found adjacent to the nucleolus (Carvalho et al., 2001; Guenatri et al., 2004). Despite these associations, these regions remain as discrete structures respecting structural and biochemical boundaries. In Figure 2.5A, an ESI micrograph obtained from a wild type nucleus shows the association of H3K9me3-enriched chromatin (dashed line) and the nucleolus (Nu). What should be appreciated in this image, is the "respect" for spatial boundaries, where both structures occupy discrete regions, albeit closely associated. Figure 2.5B (Cell 1-4) shows representative images from DAXX null cells demonstrating the loss of spatial boundaries between perinucleolar heterochromatin and the nucleolus as well as the range of chromatin-related phenotypes caused by the loss of DAXX. In Cell1, not only do the three regions of H3K9me3 signal (dashed lines) correlate with ranges of chromatin compaction, the distinction between the nucleolus and the surrounding heterochromatin is lost. In Cell 2, despite the radial nature of the structure, the chromocentre contains levels of non-chromatin protein-based components not typically observed in chromocentres. Furthermore, the H3K9me3 signal does not span the entire area of compaction. A similar pattern is seen in Cell 3. In this example, the chromocentre is found between two nucleoli (Nu). Although compact chromatin that correlates with the H3K9me3 labeling are observed, they are in contact with a compact chromatin domain that does not correlate with the H3K9me3 modification and the two domains (containing H3K9me or not) can be distinguished morphologically in the phosphorus distribution image. Cell 4 shows another phenotype characterized by large regions of compaction containing protein-based structures with H3K9me3-enriched boundaries morphologically indistinguishable from the surrounding chromatin.

I conclude that in the absence of DAXX, typical H3K9me3-containing chromocentres, which are normally discrete domains, become physically linked to compact chromatin domains lacking this heterochromatin mark. As well, a loss of distinction can frequently be observed between perinucleolar heterochromatin domains and the nucleolus.
Figure 2.5: Loss of spatial distinction between pericentric heterochromatin and nuclear structures in the absence of DAXX.

(A) Correlative LM/ESI micrograph from a wild type nucleus. H3K9me3 fluorescence was overlayed onto the P-enhanced mass image generating the correlative image (left). ESI micrograph (right panel) of the corresponding H3K9me3-containing structure. Approximate boundaries of the H3K9me3 region are indicated by a dashed line. This example demonstrates the respect for spacial boundaries as the structures remain as separate entities. (B) Correlative LM/ESI images from four DAXX null cells demonstrating the range of phenotypes associated with the loss of DAXX. Chromatin is pseudo colored yellow and protein-based structures cyan. Nu, nucleolus. Scale bar, 0.5 µm.
2.3.4 Aberrant spatial relationships of H3K9me3-enriched chromatin and major satellite DNA

To further characterize chromocentres from cells devoid of DAXX, I performed an immuno-FISH experiment using antibodies against H3K9m3 and DNA FISH probes against major satellite DNA. Consistent with previous observations (Guenatri et al., 2004), chromocentres from wild type cells contain a core of major satellite repeat DNA embedded in H3K9me3-enriched chromatin (Figure 2.6A). Line scan analysis of representative chromocentres demonstrates strong correlations in intensity peak profiles between the H3K9me3 and major satellite signals. Independent of the direction of the line scan, the major satellite signal is contained within the H3K9me3 area. In the DAXX null cells, however, while approximately half of the chromocentres display a typical spatial relationship between H3K9me3 and major satellite DNA, approximately 50% display aberrant spatial relationships (Figure 2.6B). In these chromocentres, major satellite DNA peak profiles are no longer contained within the H3K9me3-defined core of the chromocentre but extend asymmetrically beyond the H3K9me3 distribution. The percentage of the typical chromocentres in DAXX nulls (centrally-located major satellite DNA) is 63.5 ± 10.3 (n=1301) compared to wild type cells where 92.4 ± 7.6 (n=1222) chromocentres display typical H3K9me3 and major satellite spatial relationships (Figure 2.6C). I conclude that pericentric satellite repeat DNA becomes uncoupled from its association with the H3K9me3 histone mark when DAXX is absent.

Taken together, these data led me to conclude that DAXX plays a role in maintaining the relationships between compact chromatin, compact chromatin biochemically marked as constitutive heterochromatin by H3K9me3, and major satellite repeat DNA.
Figure 2.6: Aberrant spatial relationships between H3K9me3 and major satellite DNA.
FISH of major satellite DNA (red) and IF microscopy of H3K9me3 (green) on wild type (A) and DAXX null (B) fibroblasts. Magnified chromocentres are marked by white boxes in the merged images. Two independent histograms of the line scan intensity plots are shown for each enlarged chromocentre. Dashed solid arrows indicate the line scan plot and direction of the first (left) and
second (right) histograms, respectively. Scale bar, 5 µm. (C) Quantification of the percentage of typical vs. atypical chromocentres from each cell line. Scale bars represent SEM. Over 1000 chromocentres from a minimum of 30 cells analyzed.

2.3.5 Increased number of cells containing mini nucleoli.

A structural relationship exists between centric and pericentric heterochromatin and the nucleolus (Carvalho et al., 2001; Guenatri et al., 2004; Guetg et al., 2010; Peng and Karpen, 2007). In Su(var)3-9 mutants that lack H3K9me chromatin, the cells displayed disorganized nucleoli (Peng and Karpen, 2007). I therefore wanted to determine if the observed disruptions in the organization of heterochromatin in the absence of DAXX, including the frequently observed loss of discrete boundaries between perinucleolar heterochromatin domains and nucleoli, result in changes in the structural integrity of the nucleolus. I immunolabeled wild type and DAXX null cells with antibodies against B23, a protein found in the granular component (GC) and the dense fibrillar component (DFC) of the nucleolus, yet is enriched in the GC (Biggiogera et al., 1990; Boisvert et al., 2007). In both normal and DAXX null fibroblasts, I observed 6.0 ± 0.15 and 5.6 ± 0.13 large nucleoli per cell, respectively. However, I observed, in some cells, between 1 and 9 very small accumulations of B23 which I refer to as "mini nucleoli" (Figure 2.7A, arrowheads). Since the GC forms the outermost region of the nucleolus, B23 appears as a ring-like pattern. Line scan analysis of intact nucleoli show the doublet pattern of B23 intensity whereas mini nucleoli, due to their small size, appear as singlets in the line scan intensity plots. Using this feature of B23, the quantification revealed that 83% of DAXX null cells contain one or more mini nucleolus in contrast to the 15% of wild type cells at one mini nucleolus (Figure 2.7B).
Figure 2.7: DAXX maintains the structural integrity of nucleoli and the organization of rDNA.

(A) B23-labeled IF images. Arrowheads indicate the mini nucleoli. Arrows indicate the line scan intensity plot and direction. Scale bar, 5 µm. (B) Quantification of the percentage of cells containing a minimum of one mini nucleolus. Scale bars represent SEM. Over 100 cells analyzed. (C) FISH of rDNA (red) and IF microscopy of B23 (green). Arrowheads indicate rDNA foci found outside of the B23-defined nucleolar boundaries. Scale bar, 5 µm. (B) Box plot of the fraction of rDNA foci found outside of the nucleolar boundaries. 50 cells analyzed.
2.3.6 Dispersed rDNA genes in the absence of DAXX.

Ribosomal DNA (rDNA) genes associate with the cytogenetically discrete nucleolus organizer regions (NORs) (McClintock, 1934). As nucleoli form from NORs, it is assumed that rDNA alone is sufficient to establish a functional nucleolus (Grob et al., 2014; Karpen et al., 1988). Since perinucleolar heterochromatin is intimately linked to rDNA gene regulation and stability (Guett et al., 2010; Peng and Karpen, 2007), I wanted to determine if the organization of rDNA genes was disrupted in the absence of DAXX. To test this, I performed an immuno-FISH experiment using antibodies against B23 as a marker for the nucleolus and FISH probes against rDNA genes (Figure 2.7C). In wild type cells, rDNA repeats are clustered and localized within the confines of the nucleolus as visualized by B23. In the absence of DAXX, however, I observed an increase in the fraction of rDNA foci localized outside of the nucleolar boundaries (Figure 2.7C, D).

Taken together, these data demonstrate that DAXX-dependent heterochromatin organization is intimately linked to the structural integrity of the nucleolus. Furthermore, the observed increase in the number of mini nucleoli could be caused at least in part, to a dispersal of rDNA genes.

2.4 Discussion

The incorporation of histone variants into specific genomic regions by their corresponding chaperones and chromatin assembly pathways, together with the particular patterns of histone post translational modifications establish heterochromatin domains required for genome integrity and the formation of cell type-specific chromatin landscapes. By using a model system that targets an H3.3-specific histone chaperone and a technique that allows us to visualize chromatin in situ, my study provides insight into the role DAXX plays on the structural organization of pericentric heterochromatin domains in mouse cells.

2.4.1 H3.3 enrichment at chromocentres

While originally found accumulated at active sites of transcription, including rDNA (Ahmad and Henikoff, 2002), the discovery of novel chromatin assembly pathways containing the nuclear protein DAXX (Drane et al., 2010; Goldberg et al., 2010) have placed H3.3 into repetitive
regions of the genome including telomeres (Goldberg et al., 2010) and the satellite repeats of pericentric heterochromatin domains (Corpet et al., 2014; Drane et al., 2010; Morozov et al., 2012). These findings prompted me to investigate the distribution pattern of H3.3 with respect to H3K9me3-enriched chromocentres in DAXX null and the corresponding wild type MEFs. Using immunofluorescence microscopy, I show that endogenous H3.3 is distributed throughout the nucleus (Figure 2.2A). While the majority of the signal has a diffuse-type pattern, foci or regions of H3.3 enrichments are present. Using intensity line scan analyses, I show that a fraction though not all of these H3.3-enriched regions correspond to H3K9me3 peaks. A similar result is seen in the ectopically expressing H3.3 cells (Figure 2.2C). As well, results from the Pearson correlation analysis of H3.3 and H3K9me3-enriched chromocentres indicate that there are statistically significant differences between wild type and DAXX null cells (Figure 2.2B). Drane et al. (2010) show that reintroduced DAXX into MEFs localizes ectopically expressed H3.3 to PML NBs. Similarly, Corpet et al. (2014) demonstrate the DAXX-dependent targeting of newly synthesized H3.3 to PML NBs in both proliferating and senescent human cells. It was later shown, again in human cells, that DAXX is targeting the soluble pool of (H3.3-H4) dimers to PML NBs pending chromatin deposition, rather than dragging H3.3-incorporated chromatin to PML NBs (Delbarre et al., 2013). While these studies make use of imaging techniques and show H3.3 at PML NBs, H3.3 at pericentric heterochromatin has only been demonstrated using ChIP-based methods (Corpet et al., 2014; Drane et al., 2010; Morozov et al., 2012). A drawback of biochemistry-based techniques in the context of pericentric heterochromatin domains (chromocentres), however, is chromocentres are studied as a population and with that comes the assumption that chromocentres are homogeneous. I show, however, that H3.3 enrichment occurs in a fraction of chromocentres, implying that not all chromocentres are equal. This makes sense since the number of chromocentres is variable not only between cell types, but within cells of a given cell type (Cerda et al., 1999; Mayer et al., 2005). Therefore, the question remains as to which specific genomic regions is DAXX targeting H3.3 to.

2.4.2 The loss of DAXX causes an uncoupling of an epigenetic mark from the underlying chromatin structure

Since DAXX is the chaperone responsible for the pericentric heterochromatin deposition of H3.3 in mouse and human cells (Corpet et al., 2014; Drane et al., 2010), I wanted to determine if its loss had any structural consequences on heterochromatin domains in mouse cells. Using LM/ESI
and the H3K9me3 histone modification as the correlative marker for pericentric heterochromatin, I imaged DAXX null and the corresponding wild type fibroblasts, targeting the compact chromatin of chromocentres and perinucleolar heterochromatin. Consistent with previously published data (Guenatri et al., 2004; Hsu et al., 1971; Lachner et al., 2001; Peters et al., 2003), in wild type cells, we observed a correlation between the compact chromatin of chromocentres and the dense regions surrounding the nucleolus. In the absence of DAXX, however, we observed changes in the structural organization of H3K9me3-enriched pericentric heterochromatin domains including the loss of radial symmetry and aberrant spatial relationships between the repressive histone modification and major satellite repeats. More intriguing, however, was the loss of correlation between the H3K9me3 epigenetic mark and compact chromatin in the absence of DAXX. The uncoupling of a biochemical mark of heterochromatin and the underlying chromatin structure is seen in ES cells which are characterized by a signature open chromatin structure that is indicative of their pluripotency state (Efroni et al., 2008; Fussner et al., 2011b). Despite the presence of the H3K9me3 signal in either a typical ES or an induced pluripotent (iPS) cell, ESI reveals that the chromocentres contain dispersed chromatin, rather than being compact structures. This contrasts with somatic and partially reprogrammed cells where the densely packed chromatin of chromocentres is enriched in the H3K9me3 mark.

While the placement and redistribution of repressive marks can coincide with the establishment of heterochromatin domains during differentiation (Fussner et al., 2011b; Guenatri et al., 2004; Hawkins et al., 2010; Wen et al., 2009), the observation that epigenetic landscapes and higher order chromatin organizations are mutually exclusive is consistent with published data. Senescence-associated heterochromatin foci (SAHFs), which form after the induction of senescence, are DAPI-dense foci enriched in condensed heterochromatin comprised of a tightly packed H3K9me3-rich core, surrounded by a less densely packed H3K27me3 ring (Chandra et al., 2012; Narita et al., 2003). Despite the extensive reorganization of chromatin domains that accompany senescence, the landscapes of H3K9me3 and H3K27me3 remain unchanged. Furthermore, SAHF formation was observed in the presence of reduced levels of H3K9me3 and H3K27me3 suggesting that the formation of higher order chromatin structures and the establishment of epigenetic profiles are discrete events (Chandra et al., 2012). Another example is the distribution pattern of repressive histone modifications within the heterochromatin domains of a mouse lymphocyte. Unlike the open chromatin of an ES cell, lymphocytes contain
large domains of compact chromatin that occupy the majority of the nuclear volume. Within these domains are H3K9me3-enriched chromocentres. Unlike canonical chromocentres from MEFs, those from lymphocytes contain the H3K9me3-enriched core surrounded by heterochromatin devoid of this repressive mark (Rapkin et al., 2011). Future studies are required to determine the basis for the relationship between repressive histone modification profiles and their impact on higher-order chromatin organizations, such as those seen in pericentric heterochromatin domains.

2.4.3 DAXX maintains the structural integrity of nucleoli

I show here that the structural integrity of the nucleolus is compromised in the absence of DAXX. The phenotype is characterized by an increase in the number of cells containing mini nucleoli as well as the dispersal of rDNA genes. I speculate that these observations are caused by the loss of the boundary between the nucleolus and perinucleolar heterochromatin. The nucleolus, the most prominent structure in the nucleus and the site of ribosome biogenesis, is surrounded by blocks of condensed heterochromatin (Haaf and Schmid, 1991). rRNA synthesis and the formation of centric-pericentric heterochromatin domains requires the silencing of perinucleolar association of rDNA repeats (Akhmanova et al., 2000; Guetg et al., 2010). In this context, the nucleolus is a structural platform for the maintenance and possibly the establishment of heterochromatin domains (Guetg and Santoro, 2012). It was shown that Su(var) mutants in Drosophila that have reduced levels of H3K9 methylation exhibit multiple ectopic nucleoli. While they could have formed from dispersed nucleolar material, it was equally likely that they were caused by a mislocalization of rDNA (Peng and Karpen, 2007). The principle rDNA silencing pathway is NoRC (nucleolar remodeling complex), consisting of TIP5 and ATPase SNF2h (Santoro et al., 2002; Strohner et al., 2001). Depletion of TIP5 not only reduces rDNA silencing but it impairs the formation of perinucleolar heterochromatin (Guetg et al., 2010). It was recently discovered that PARP1 (poly(ADP-ribose)-polymerase-1) associates with TIP5 and it is involved in rDNA silencing (Guetg et al., 2012). Interestingly, PARP1 was purified from H3.1- and H3.3-containing complexes (Drane et al., 2010). Whether the disruptions of heterochromatin result from a mislocalization of rDNA genes or changes in nucleolar integrity due to disruptions in heterochromatin formation remain to be elucidated. It would be intriguing, however, to perform ESI experiments on the TIP5 and PARP1 null MEFS and compare the
phenotype to that observed in DAXX null cells to gain insight into the structural consequences on pericentric heterochromatin upon changes in rDNA repression.

2.4.4 DAXX-dependent selective methylation of H3.3K9me3

I propose a model where the DAXX-dependent deposition of H3.3 into pericentric repeats is, at least in part, responsible for marking appropriate H3K9me3 boundaries in order to establish heterochromatin domains (Figure 2.8). This model is consistent with the 'H3 barcode hypothesis' which proposes that H3 variants index the genome by creating different chromosomal regions (Hake and Allis, 2006). Once the variants are in place, histone PTMs would serve to regulate gene expression profiles and establish genomic nuclear architectures. Indeed evidence is emerging supporting the importance of the selective modification of histone variants. Santenard et al. (2010) reported that the ectopic expression of a mutant H3.3 (H3.3K27R) impairs heterochromatin formation and development at the blastocyst stage. It was also shown that the balance between H3.3 and H1 is required for chromosome segregation and development to the blastocyst stage, and key to these processes, is the methylation of H3.3K36 (Lin et al., 2013). Consistent with this model, it is possible that DAXX, through the selective deposition of H3.3, indexes the genomic regions comprising pericentric constitutive heterochromatin, driving the pattern of H3.3K9me3. In this context, H3.3K9me3 establishes both chromocentre and perinucleolar heterochromatin domains. In the absence of DAXX, the pattern of H3.3K9me3 results in the misappropriation of heterochromatin boundaries causing the disruption of pericentric heterochromatin domains. Future studies are required to confirm the boundaries of H3.3K9me3 with and without DAXX. As well, it will be important to investigate the structural consequences upon ectopic expression of H3.3K9 mutants.
Figure 2.8: DAXX establishes essential pericentric heterochromatin genomic boundaries. DAXX-dependent selective methylation of H3.3K9me3 establishes genomic boundaries required for the structural organization of heterochromatin and heterochromatin-dependent domains such as the nucleolus. In the absence of DAXX, pericentric heterochromatin domains and the structural integrity of the nucleolus are jeopardized due to inappropriate H3.3K9me3 boundaries.
Chapter 3

3 Elucidating a role for DAXX in muscle differentiation

3.1 Introduction

A key question in the field of developmental biology is how different cell types arise from a single fertilized egg. The two main views of cell commitment in which the differentiation of somatic tissues is achieved involve either transcriptional activation or repression processes (Fisher and Merkenschlager, 2002). The former describes the increasing transcriptional competence of lineage-specific factors such as MyoD, that is not only required to drive the expression of the myogenic basic helix-loop-helix (bHLH) proteins but has been shown to activate muscle-specific genes and redirect the development of fibroblasts into muscle cells (Weintraub et al., 1989). The latter and alternative view describes differentiation as a series of gene silencing events that effectively restrict the expression of a fraction of the genome. For example, Pax5 plays an essential role in B-cell commitment by repressing the transcription of genes of alternate lineages (Nutt et al., 1999). Transcription regulation alone, however, is not sufficient, rather, it is the combination of nuclear organization and gene activity that is required for a more comprehensive understanding of the complexity of establishing cell-type specificities (Kosak and Groudine, 2004). Included in this, is higher order chromatin organizations, such as genes spatial relationships to intranuclear structures. For example, to address the role of higher order organizations with respect to development and cell type-specific gene expression, Moen at al. showed that a subset of muscle-specific genes repositioned relative to the periphery of structurally discrete, splicing factor rich (SC-35) domains during skeletal muscle differentiation (Moen et al., 2004). Additionally, global changes in the organization of constitutive heterochromatin domains have been observed in response to differentiation. In mouse cells, concomitant with differentiation of Purkinje neurons is the rearrangement of centromeric domains (Martou and De Boni, 2000) and similarly, the clustering of centric and pericentric heterochromatin was observed in terminally differentiated myotubes (Brero et al., 2005; Terranova et al., 2005).

While an accessible, completely sequenced genome is imperative in understanding development and differentiation, its arrangement in the nucleus and thus its higher-order chromatin structure, may impart crucial information about its function. Indeed, the structure and function of
chromatin and nuclear compartments are not mutually exclusive where a disruption in function can lead to structural perturbations, and vice versa (Kosak and Groudine, 2004). The inhibition of rDNA transcription, for example, results in the disruption and ultimate disappearance of the nucleolus (Lewis and Tollervey, 2000; Scheer and Hock, 1999). On the other hand, others have shown that disruption of the nuclear lamina inhibits DNA synthesis and RNA polymerase II-dependent transcription (Spann et al., 2002; Spann et al., 1997). Therefore, it is thought that the integration of structure and function not only enables the sequestering and conservation of resources, but facilitates the regulation of essential cellular processes (Kosak and Groudine, 2004).

Nuclear organization, including the amount and distribution of compact chromatin, is cell-type specific. This topological organization not only reflects differentiation states but is required to establish tissue-specific gene expression profiles (Francastel et al., 2000). The relationship between cell-type specificity and chromatin domain organization can be visualized in ESI micrographs of nuclei from different mouse tissues (Figure 3.1). Although containing the same genetic material, there are clear differences in the way the genetic material is organized in each cell type (Rapkin et al., 2011). In nuclei of E3.5 mouse epiblasts (from which ES cells are derived), for example, chromatin is uniformly dispersed throughout the nucleus. Hepatocytes, on the other hand, contain blocks of compact chromatin along the nuclear envelope and dispersed fibres in the nucleoplasmic space. In contrast to both epiblasts and hepatocytes, the chromatin of a spleen lymphocyte is packaged into highly compact domains throughout the entire nuclear volume. While these cells contain the same amount of chromatin, the volume occupied by chromatin is vastly different with an ES cell, a lymphocyte, and a hepatocyte occupying approximately 37%, 48%, and 14%, respectively (Fussner et al., 2010). Although the functional significance of these differences is not yet fully understood, the organization of compact chromatin may be the foundation for the creation of functional domains that characterize different cell types (Rapkin et al., 2011).
Figure 3.1: Global chromatin organization in mouse tissue nuclei.

ESI micrographs of sections of mouse tissue nuclei. In all images, chromatin is pseudo-colored yellow and protein-based structures cyan. Arrows indicate compact chromatin along the edge of the nuclear envelope. Arrowheads point to the protein-rich nuclear pores. (A) Open, mesh-like chromatin of a mouse E3.5 epiblast. The arrows point to the rim of chromatin along the nuclear envelope. (B) Nucleus of a mouse hepatocyte. There is an intermediate level of chromatin compaction. (C) A spleen lymphocyte contains extensive blocks of compact chromatin extending into the interior of the nucleus. Scale bar, 0.5 µm. Figure taken with permission from (Rapkin et al., 2011).
While cell type specification is achieved by establishing appropriate global chromatin landscapes, it also relies on local chromatin structural details, such as histone methylation and acetylation patterns as well as the incorporation of histone variants at lineage-specific genes. For example, in hepatocytes, histone modifications including H3K4me3, persist through mitosis and upon transcription inhibition across the promoter and coding regions of constitutively active hepatic genes (Kouskouti and Talianidis, 2005). Consistent with its involvement in gene silencing, H3K27me3 has been shown to be a critical regulator of muscle-specific gene expression and myogenesis (Caretti et al., 2004; Seenundun et al., 2010). In an attempt to further understand the interplay between lineage-specific transcription factors and histone modifications during differentiation, Asp et al. (2011) performed a genome-wide study looking at histone modifications and factors in muscle differentiation using the C2C12 myogenesis system. Whereas H3K9me3 did not contribute to the differential regulation of gene expression during differentiation, H3K27me3 was widely distributed throughout the genome in both the precursor cells (myoblasts) and the fully differentiated cells (myotubes) and was inversely correlated with active chromatin marks. Furthermore, the class of genes exhibiting the highest levels of H3K27me3 were committed to non-muscle lineages whereas genes induced during differentiation were less highly enriched in H3K27me3. Interestingly, they found that genes upregulated in myotubes already adopted features of active chromatin, such as PolII enrichment, in the cycling myoblasts, suggesting these genes were poised for activation prior to achieving maximal expression.

Consistent with this finding, the same group investigated chromatin marks on regulators of osteogenesis and adipogenesis, since, upon exposure to appropriate inducers, C2C12 cells can transdifferentiate into osteoblasts and adipocytes (Hu et al., 1995; Lee et al., 2000). They found that lineage-specific factors such as Runx2 and PPARγ (osteogenesis and adipogenesis, respectively) were not only expressed but bore signatures of gene activation such as PolII and H3K4me3 enrichment. What was remarkable, however, was the retention of the positive signatures on these factors in myotubes, despite not being myogenesis-required genes. As well, downstream effectors of these pathways were enriched in H3K27me3, suggesting that although key regulatory factors are expressed and primed for activation, additional induction events are required to drive the expression of those lineage-specific factors. It was proposed that, similar to the presence of bivalent domains on development regulators in ES cells, the presence of active
marks on non-muscle factors could be the underlying basis for the observed plasticity in muscle cells (Asp et al., 2011).

Despite the various cell-specific lineages and differentiation programs, intriguing similarities, such as the reorganization of heterochromatin domains, suggest that conservative principles underlie the lineage restrictions required for cell differentiation (Fisher and Merkenschlager, 2002). While the fibroblasts used in my experiments are a powerful tool in delineating the consequences of the loss of DAXX on nuclear structure and chromatin organization, they are not an ideal system to address the role of DAXX in differentiation. Since skeletal muscle cells are often used as a paradigm for discerning the underlying mechanisms of cellular differentiation, I used the mouse C2C12 myogenesis system as a model to further our understanding of the role of DAXX in differentiation. Vertebrate skeletal muscle, composed of functionally discrete structures called myofibers, arise from somites during development (Saccone and Puri, 2010). During somite development, a fraction of progenitor cells become myoblasts and are committed to the myogenic lineage (Cossu et al., 1996). The fraction of muscle progenitors that do not differentiate into skeletal muscle become quiescent satellite cells, that in response to muscle damage, can be activated to repair and mediate postnatal muscle growth (Kuang and Rudnicki, 2008; Morgan and Partridge, 2003). When C2C12 cells are grown in culture, in the presence of mitogens, the inhibitor of differentiation (Id) protein is expressed and blocks differentiation by inhibiting myogenic factors (Benezra et al., 1990). Under conditions of mitogen deprivation, proliferating myoblasts can either withdraw permanently from the cell cycle and differentiate into multinucleated myotubes or undergo apoptosis (Wang and Walsh, 1996). Myoblasts and a fully differentiated (5 day) multinucleated myotube are shown in Figure 3.2.
Figure 3.2: C2C12 myogenesis.
Representative immunofluorescence image demonstrating C2C12 differentiation. Serum removal induces the formation of terminally differentiated multinucleated myotubes (right) from cycling precursor myoblasts (left). Myoblasts are morphologically distinct from myotubes as visualized by DAPI (blue, nuclei) and phalloidin (red, actin filaments). Scale bar, 10 µm.

Concomitant with terminal differentiation is the large scale reorganization of constitutive heterochromatin domains including the clustering of chromocentres (Brero et al., 2005; Terranova et al., 2005). Since myoblasts are morphologically distinct from myotubes with respect to cell structure as well as chromatin organization, myogenesis and the in vitro differentiation of C2C12 cells provides an ideal system to study factors influencing chromatin organization during differentiation. Importantly, the chromatin reorganization events that follow differentiation, such as chromocentre clustering, can be visualized using standard immunofluorescence and electron microscopy (i.e. ESI) techniques (Figure 3.3).
Figure 3.3: Large scale reorganization of constitutive heterochromatin domains. Concomitant with myogenesis is the clustering of chromocentres (DAPI-dense foci) into fewer and larger structures. Since myogenin levels are increased after the induction of differentiation, it was used to monitor the progress of the differentiation program. Scale bar, 10 µm.
To date, there is only one reported study exploring DAXX's role in skeletal muscle differentiation. Gupta et al. (2009) showed DAXX, through the interaction with E2A proteins, inhibits muscle differentiation. E2A proteins are transcription factors that pair with the muscle-specific bHLH transcription factors forming heterodimers that drive the differentiation of myoblasts into myotubes (Arnold and Winter, 1998; Weintraub, 1993). This work presents the first example of DAXX in the control of muscle differentiation specifically, but when combined with the findings that DAXX-deficient mice fail to develop specific tissues, a role as a regulator of cellular differentiation emerges (Michaelson et al., 1999).

While mechanistic details are rather preliminary, the involvement of DAXX in the control of myogenesis and T-cell homeostasis implicate DAXX as factor in tissue differentiation (Gupta et al., 2009; Leal-Sanchez et al., 2007). This, together with the published observations that pericentric heterochromatin domains reorganize upon muscle differentiation and my findings that DAXX is a key regulator of the structural organization of heterochromatin and nuclear organization (Chapter 2), prompted me to explore the role DAXX plays in C2C12 myogenesis, a model system of tissue differentiation. In myoblasts, DAXX is predominantly found at PML NBs, whereas in myotubes, it is enriched in chromocentres possibly due to the reduction of PML NBs in the multinucleated structures. By manipulating the levels of PML NBs in myotubes, I found a fraction of DAXX at both PML NBs and chromocentres, from which I conclude that there is an active process that regulates the relocazation of DAXX during differentiation.

### 3.2 Materials and methods

#### 3.2.1 Cell culture

C2C12 myoblasts were cultured in growth media (GM): DMEM (Wisent, Gibco) supplemented with 10% FBS (Wisent), 10 µg/ml penicillin and streptomycin (Wisent), and 2 mM L-glutamine (Wisent). To induce myogenesis, and generate fully differentiated myotubes, myoblasts were grown to confluence and cultured in differentiation media (DM): DMEM (Wisent, Gibco) supplemented with 2% horse serum (Wisent), 10 µg/ml penicillin and streptomycin (Wisent), and 2 mM L-glutamine (Wisent). Cells were differentiated for 5 days before analysis. In all differentiation experiments, media was replaced every 48 hours. Cells were grown on glass cover slips for immunofluorescence microscopy, electron microscopy, and transfections.
For transfections, myoblasts were seeded to 50% confluence on glass cover slips and transiently transected (Lipofectamine 2000 Transfection Reagent, Invitrogen) with the specified purified (Qiagen Plasmid Midi Kit, Qiagen) FLAG plasmids. For the interferon experiment, myoblasts were first differentiated for 5 days in DM. Interferon-α was added to fresh DM to a final concentration of either 500 or 1000 U/ml. Myotubes were treated with interferon for 24 hours prior to preparation for immunofluorescence and western blot analysis. The treatment was carried out in triplicate and quantification statistics reported as standard error of the mean.

To prepare the low calcium GM and DM, calcium-free DMEM (Wisent) was used and supplemented as described above. Low calcium GM and DM was achieved by supplementation with CaCl₂ to a final concentration of 0.05 mM. The concentration of CaCl₂ in regular DMEM is 1.8 mM. Prior to immunofluorescence and western blot analysis, cells were cultured as described above. The experiment was repeated in triplicate and quantification statistics reported as standard error of the mean.

TSA (Sigma-Aldrich) was diluted to a final concentration of 50 nM in fresh GM or DM. Control myoblasts (0 (-)) and myotubes (5 (-/-)) were cultured as described above. Experimental myoblasts (0 (+)) were grown in GM supplemented with TSA for 24 hrs prior and then fixed. To prepare 5 (+/-) samples, myoblasts were seeded and cultured in fresh GM for 24 hrs. They were then treated with TSA-supplemented media for 24 hours and differentiated in regular DM for 5 days. To prepare 5 (-/+ 24 hrs) samples, myoblasts were seeded and cultured for 48 hours and differentiated in DM. After 24 hours, DM was replaced with TSA-containing DM and cultured for 4 days. A summary of the experimental time line is shown in Figure 3.7. The experiment was repeated in triplicate and quantification statistics reported as standard error of the mean.

### 3.2.2 Transfection and plasmid constructs

Full length mouse PML cDNA (Mammalian Gene Collection) was obtained from The Centre for Applied Genomics (TCAG, The Hospital for Sick Children) and subcloned into the pcDNA3.1(+)−FLAG expression construct (Life Technologies Inc.). To clone full length mouse DAXX, total RNA was isolated from wild type MEFs using TRIzol Reagent (Life Technologies Inc.) and first strand cDNA was generated using a long-range cDNA synthesis kit (Qiagen). Full
length DAXX, deletion constructs, and the SIM mutant were subcloned into the pcDNA3.1(+)–FLAG expression construct (Life Technologies Inc.). All cloned plasmids were confirmed by sequencing. The oligonucleotides used for subcloning the various constructs are described in Table 3.

For transfections, myoblasts were seeded to 50% confluency on glass cover slips and transiently transfected (Lipofectamine 2000 Transfection Reagent, Invitrogen) with the specified purified (Qiagen Plasmid Midi Kit, Qiagen) FLAG plasmids.

Table 3.1: Primers used in plasmid construction.

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<th>Name</th>
<th>Sequence (5’-3’)</th>
<th>Description</th>
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<td>mP-F1</td>
<td>ATACGAGGATCCAAATGGAACTGAACCACTTTCC</td>
<td>Forward primer PML</td>
</tr>
<tr>
<td>mP-R1</td>
<td>TCGTATGATATCCTAGGAGATGATTCCTTTTA</td>
<td>Reverse primer PML</td>
</tr>
<tr>
<td>mD-F1</td>
<td>ATACGAGGATCCAAATGGCCACGATGACAGC</td>
<td>Forward primer FL DAXX, 1-423</td>
</tr>
<tr>
<td>mD-R1</td>
<td>TCGTATGATATCCTAATCAGATGCTGAAAGCAAGATG</td>
<td>Reverse primer FL DAXX, 490-740</td>
</tr>
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<td>mD-R2b</td>
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<td>Reverse primer 1-423</td>
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<td>mD-ΔSIM-R</td>
<td>TCGTATGATATCCTACTCCCGGGTGCAGCTGTG</td>
<td>Reverse primer ΔSIM</td>
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</table>
3.2.3 Immunofluorescence microscopy

Cells were fixed in 2% paraformaldehyde (Electron Microscopy Sciences) in PBS (Wisent) for 10 min at room temperature (RT) and permeabilized in 0.5% Triton X-100 (BioShop) in PBS for 5 min at RT. Primary antibodies used were rabbit anti-DAXX (Santa Cruz Biotechnology), mouse anti-PML (Upstate), goat anti-ATRX (Santa Cruz Biotechnology), goat anti-myogenin (Santa Cruz Biotechnology), and mouse anti-FLAG (Sigma-Aldrich). Secondary antibodies used were rabbit, sheep, or mouse Cy2, Cy3, and Cy5 (Jackson Laboratories). Cells were soaked in 50 µg/ml DAPI and mounted using anti-fade reagent (buffered glycerol with 4% n-propyl gallate). Images were collected on an Olympus IX81 inverted microscope equipped with a Cascade II CCD camera (Photometrics) using either a 60× or 100× oil-immersion objective lenses. MetaMorph Microscopy Automation & Image Analysis Software (Molecular Devices) was used to collect images. Images were processed with Volocity 3D Image Analysis Software (PerkinElmer) and Photoshop (Adobe). Graphs and statistics were constructed using GraphPad Prism (GraphPad Software Inc). Line scans data was collected using ImageJ (National Institute of Health) and histograms constructed using GraphPad Prism (GraphPad Software Inc). Quantification statistics were reported as standard error of the mean.

3.2.4 ESI microscopy

For a detailed sample preparation protocol and ESI procedure, see Ahmed et al. (2009). Myoblasts or 5 day differentiated myotubes were seeded on glass coverslips, fixed in 2% paraformaldehyde for 10 min at RT, permeabilized in 0.5% Triton X-100 (BioShop) in PBS for 5 min at RT, and post fixed in 1% glutaraldehyde (Electron Microscopy Sciences). Following dehydration, cells were embedded in Quetol (Electron Microscopy Sciences). Samples were sectioned to 70 nm by an ultramicrotome (Leica). Following carbon coating, electron micrographs were collected on a transmission electron microscope (Tecnai 20, FEI). To generate the phosphorus and nitrogen images, the microscope was operated at 200 kV using a post column filter (Gatan) at 120 and 155, and 385 and 415 eV, respectively. Images were recorded on a CCD camera and collected using DigitalMicrograph software (Gatan). To generate the ESI images presented in my thesis, phosphorus images were subtracted from nitrogen images to normalize the chromatin contribution in the nitrogen images to zero. Phosphorus images are pseudo-colored yellow and overlayed onto the subtracted nitrogen images that are pseudo-colored blue. In the overlay images, yellow represents nucleic acid-based structures, and the cyan represents protein-
based structures. Images were processed with Photoshop (Adobe). Average phosphorus density analysis was performed using ImageJ (National Institute of Health).

### 3.2.5 Western blot

Cells grown in 10-cm culture dishes were harvested and resuspended with 9M Urea in 10 mM Tris-Cl, pH 6.8. Samples were quantified using the Bio-Rad Protein Concentration Assay (Bio-Rad Laboratories) and diluted to 10 µg/20µl in 9M Urea containing a 6× SDS-PAGE loading buffer. Protein samples were resolved on either 10% (GAPDH) or 15% (H3K9me3 and H3.3) SDS-PAGE gels and transferred onto nitrocellulose membranes (GE Healthcare). Membranes were blocked overnight in 5% skim milk powder in TBST (0.05% Tween-20, Bio-Rad). Primary antibodies used were mouse anti H3K9me3 (gift from H. Kimura), rabbit anti-H3.3 (Abcam), and GAPDH (Sigma). Secondary antibodies used were mouse and rabbit anti-HRP (Sigma). Detection was performed using the Western Lightning Plus ECL system (PerkinElmer).

### 3.3 Results

#### 3.3.1 DAXX localization in myoblasts and myotubes

DAXX is predominantly a nuclear protein where it associates with PML NBs and with pericentric heterochromatin domains in a cell cycle-dependent manner as well as in the absence of PML (Ishov et al., 1999; Ishov et al., 2004). To determine the localization of DAXX during myogenesis, I performed an IF experiment using myoblasts and 5 day differentiated myotubes (Figure 3.4). Using myogenin and the presence of multinucleated myotubes to track differentiation, I observed a fraction of DAXX enriched in PML NBs (Figure 3.4A). Surprisingly, PML NB number was greatly reduced to barely detectable levels in myotubes and DAXX relocalized to DAPI-enriched structures. To confirm that DAPI-rich structures are in fact chromocentres, I immunolabeled myoblasts and myotubes with antibodies against HP1α, a protein involved in gene silencing and heterochromatin formation that binds with affinity to the H3K9me3 repressive histone modification (Bannister et al., 2001; Lachner et al., 2001) and found DAXX relocalized to HP1α-containing chromocentres (Figure 3.4B). Western blot analysis of whole cell myoblast and myotube lysates showed a reduction in PML protein expression and the consistent expression of DAXX in both differentiation states, results consistent with my immunofluorescence observations (Figure 3.4C). The remaining PML in the
myotube samples were likely from myocytes that failed to differentiate. Myocytes can be eliminated with $10^{-5}$ M Ara-C (Cytosine β-D-arabino furanoside) however since I was scoring the cells based on an obvious morphological distinction, I did not use the treatment (Terranova et al., 2005).
Figure 3.4: DAXX localizes to PML NBs in myoblasts and chromocentres in myotubes.
(A) IF of C2C12 myoblasts and myotubes. DAXX (red) is localized to some PML NBs (green) in myoblasts. No PML NBs were detected in myotubes. (B) In myotubes, DAXX (red)
colocalized with HP1α (green). HP1α was used as a marker for chromocentres. Myogenin was used to mark differentiated cells. Scale bar, 10 µm. (C) Western blot analysis of DAXX and PML protein levels during myogenesis.

3.3.2 Interferon relocalizes DAXX in myotubes

During the course of differentiation, I observed a reduction in PML NB number and decrease in PML protein expression (Figure 3.4), and with that, a relocalization of DAXX from PML NBs to chromocentres. It is unclear, however, if the relocalization of DAXX was a consequence of terminal differentiation or due to the loss of PML NBs. It has been shown that PML protein is induced by interferons (Chelbi-Alix et al., 1995). Therefore, in an attempt to understand the cause of the relocalization, I differentiated myoblasts for 5 days and then treated them with interferon for 24 hours. In control myotubes, DAXX was enriched in chromocentres but in the presence of interferon, however, the number of PML NBs increased and a fraction of DAXX relocalized to the reformed PML NBs (Figure 3.5A, arrowheads). I quantified the number of PML NBs and DAXX foci at PML NBs in the absence or presence of interferon and found that both increased in a dose-dependent manner with respect to the concentration of interferon (Figure 3.5B). Western blot analysis of whole cell lysates from the control and interferon-treated myotubes also showed an increase in PML protein expression. Similarly, and consistent with previous studies investigating the role of DAXX in B lymphophoiesis and avian sarcoma virus integration, interferon induced the expression of DAXX (Gongora et al., 2001; Greger et al., 2005) (Figure 3.5C).
Figure 3.5: Interferon induces the reformation of PML NBs in myotubes.
(A) Fully differentiated C2C12 myotubes treated with interferon for 24 hours and then prepared for IF. In control myotubes, DAXX (red) localizes to chromocentres. In the presence of interferon, the number of PML NBs increases (green) and a fraction of DAXX relocalizes to the
reformed PML NBs (arrowheads). Scale bar, 10 µm. (B) Quantification of the number of PML NBs and DAXX foci at PML NBs in the presence or absence of interferon. Error bars represent SEM from three independent experiments. A minimum of 120 cells analyzed for each treatment. (C) Western blot analysis of DAXX and PML protein levels. Interferon increases the expression of PML.

3.3.3 DAXX is retained at PML NBs under low calcium conditions

Calcium is a key regulator of muscle development. Lowering extracellular calcium concentrations has been shown to inhibit differentiation and interfere with myoblast fusion (Knudsen and Horwitz, 1977; Morris and Cole, 1979; Shainberg et al., 1969). I wanted to exploit these findings to manipulate myoblast fusion in an attempt to determine if the loss of PML NBs and the subsequent relocalization of DAXX to chromocentres during myogenesis was due to the formation of multinucleated myotubes. I differentiated C2C12 myoblasts for 5 days in control (1.8 mM) or low (0.05 mM) calcium conditions. I then performed an IF experiment to determine the localization of DAXX by labeling for DAXX, myogenin to confirm molecular activation of the differentiation program, and PML to mark PML NBs. As stated in the previous sections, after 5 days in control differentiation media, a reduction in the number of PML NBs and relocalization of DAXX to chromocentres was observed in the fully differentiated, multinucleated myotubes (Figure 3.6A). Consistent with previous findings, under low calcium conditions, multinucleation was impaired despite the expression of myogenic transcription factors (myogenin) and I observed an increase in the number of PML NBs relative to control cells. Furthermore, a fraction of DAXX remained at PML NBs. Quantification of the number of PML NBs under both conditions revealed the increase in PML NB number was statistically significant (P<0.001) (Figure 3.6B). Western blot analysis of whole cell lysates from myoblasts, control, and experimental myotubes showed similar PML protein expression levels in the myotube samples, and the decrease relative to myoblasts (Figure 3.6C). The expression level of DAXX remained unchanged under low calcium conditions.
Figure 3.6: DAXX is retained at PML NBs under low calcium conditions.

(A) IF of C2C12 cells differentiated for 5 days under control (1.8 mM) or low calcium (0.05 mM) conditions. Low calcium impairs myotube formation. Under low calcium conditions, PML NBs (green) were increased relative to control cells and a fraction of DAXX (red) was retained there. Scale bar, 10 µm. (B) Quantification of the number of PML NBs and DAXX foci at PML NBs. Error bars represent SEM from three independent experiments. A minimum of 100 cells was analyzed for each treatment. (C) Western blot analysis of DAXX and PML protein levels following differentiation under the two conditions. B, myoblasts; T, myotubes.
3.3.4 The relocalization of DAXX to constitutive heterochromatin is dependent on HDAC activity

Histone acetylation is a posttranslational modification involved in chromatin structure, DNA replication, DNA repair, heterochromatin silencing and gene transcription (Groth et al., 2007; Shahbazian and Grunstein, 2007). Whereas a hyperacetylation state marks transcriptionally active genes and promotes a more open, decondensed chromatin state, hypoacetylation, achieved by the removal of acetyl groups by histone deacetylases (HDACs) is implicated in the formation of a more compacted, transcriptionally repressed chromatin structure (Lee and Workman, 2007; Shogren-Knaak et al., 2006; Tse et al., 1998; Wang et al., 2001). Currently, four classes and a total of 18 mammalian HDACs have been identified and, based on their requirements for specific co-factors and unique protein structures, class-specific inhibitors have been identified and have been instrumental in deconstructing the functions of individual HDAC classes (Delcuve et al., 2012). Since chromatin remodeling and histone acetylation are key factors in regulating gene expression and differentiation, it isn't surprising that HDAC activity is required for skeletal muscle development (McKinsey et al., 2001; Sartorelli and Puri, 2001). Interestingly, the effects of the class I and II HDAC inhibitor Trichostatin A (TSA) are stage specific: the addition of TSA prior to differentiation does not impair myotube formation whereas its presence in the differentiation media blocks chromocentre clustering (Iezzi et al., 2002; Terranova et al., 2005).

I wanted to determine if the relocalization of DAXX to pericentric heterochromatin domains was a consequence of PML NB loss or the differentiation-induced chromocentre clustering. Since TSA has been shown to inhibit the reorganization of chromocentres, I designed an experiment where 50 nM TSA was added in a stage-specific manner (Figure 3.7A). The addition of TSA did not affect the survival of myoblasts, nor did it inhibit the formation of myotubes when added prior to differentiation (5 (+/−)). Since the addition of TSA at the start of differentiation interfered with myotube formation, I added it 24 hours after the induction of differentiation (5 (+/− 24 hrs)). A summary of the experiment is shown in Figure 3.7A. To confirm that TSA did indeed interfere with chromocentre clustering, I quantified the number of DAPI foci at each stage (Figure 3.7B). The average number of chromocentres in myoblasts with or without TSA was 27.7 ± 0.6 and 28.1 ± 0.7, respectively. The addition of TSA prior to differentiation (5 (+/−)) resulted in a significant increase (P < 0.001) in the number of chromocentres (13.4 ± 0.4) compared to the control (5 (−/−), 9.5 ± 0.3). In addition, the number of chromocentres (19.5 ± 0.6) was
significantly greater than both the control and the 5 (+/-) treatment when the cells were treated with TSA 24 after differentiation was started (5 (-/+ 24 hrs)). From this, I confirmed that TSA did interfere with chromocentre clustering during myogenesis.
Figure 3.7: Stage-specific addition of TSA.

(A) Schematic of the experimental design. GM, growth media; DM, differentiation media; d, day. (B) Quantification of the average number of chromocentres under the various differentiation and TSA conditions. Error bars represent SEM from three independent experiments. A minimum of 100 cells analyzed for each treatment. [0 (-), untreated myoblasts; 0 (+) TSA treated myoblasts; 5 (-/-), untreated myotubes; 5 (+/-), TSA added to the growth media for 24 hrs prior to differentiation under control conditions; 5 (-/+ 24 hrs), TSA added to the differentiation media after 24 hrs of differentiation].
To address PML NB loss and DAXX relocalization during myogenesis, I performed the TSA experiment outlined above and quantified the number of PML NBs and DAXX foci at PML NBs after each treatment (Figure 3.8B). In myoblasts grown in the presence of 50 nm TSA (0 (+)), DAXX was predominantly enriched in PML NBs (Figure 3.8A). Treating myoblasts with TSA for 24 hours prior to a 5 day differentiation under normal conditions (5 (+/-)) resulted in myotubes with reduced numbers of PML NBs and the concomitant relocalization of DAXX to chromocentres. The addition of TSA 24 hours after the initiation of differentiation (5 (-/+ 24 hrs)), however, resulted in the inhibition of myotube formation. In myogenin-positive non-multinucleated cells, I observed an increase in both PML NB number and the fraction of DAXX at PML NBs. Western blot analysis of whole cell lysates from samples from all conditions was performed (Figure 3.8C). The expression level of DAXX remained unchanged in myoblasts and myotubes with or without TSA. I observed an increase in the expression of PML protein in myoblasts exposed to TSA as well in myotubes differentiated in the presence of TSA, a finding consistent with a previous study reporting PML protein acetylation is induced by TSA (Hayakawa et al., 2008).
**Figure 3.8: DAXX is retained at PML NBs in the presence of TSA.**

(A) IF of C2C12 myoblasts and myotubes subject to the stage-specific addition of TSA. Multinucleation was inhibited when TSA was added 24 hrs after the induction of differentiation (5 (-/+ 24 hrs)). In the myogenin positive cells, DAXX (red) was at PML NBs. Scale bar, 10 µm.

(B) Quantification of the number of PML NBs and DAXX foci at PML NBs under the various TSA conditions. Error bars represent SEM from three independent experiments. A minimum of 100 cells analyzed for each treatment.

(C) Western blot analysis of DAXX and PML protein levels. TSA treatment increased PML protein expression. [0 (-), untreated myoblasts; 0 (+) TSA treated myoblasts; 5 (-/-), untreated myotubes; 5 (+/-), TSA added to the growth media for 24 hrs prior to differentiation under control conditions; 5 (-/+ 24 hrs), TSA added to the differentiation media after 24 hrs of differentiation].

It has been shown that DAXX physically interacts with HDACs and it was suggested that DAXX recruits one of the three HDAC corepressor complexes to target genes and via histone deacetylation, represses transcription of the targets (Hollenbach et al., 2002; Kelly and Cowley, 2013; Li et al., 2000a). Furthermore, it has been shown that the heterochromatin-associated protein HP1 dissociates from pericentromeric heterochromatin and can be recruited to alternate heterochromatin domains in response to TSA (Bartova et al., 2005; Taddei et al., 2001; Zhang et al., 2007). Since DAXX relococalizes to HP1-containing chromocentres in myotubes (Figure 3.4), I wanted to determine if, despite the increased number of PML NBs in the myogenin-positive, TSA-treated cells, a fraction of DAXX remained at chromocentres. I performed the same stage-specific TSA experiment as outlined Figure 3.7 and prepared cells for immunofluorescence labeling using antibodies against DAXX and HP1α (Figure 3.9). Consistent with previous reports, exposure to TSA in myoblasts and myogenin-positive differentiated cells caused a dissociation of HP1α from DAPI-enriched pericentric heterochromatin domains. In addition, DAXX no longer colocalized with HP1α foci.
Figure 3.9: DAXX and HP1α fail to colocalize in chromocentres in the presence of TSA.

IF of C2C12 myoblasts and myotubes subject to the stage-specific addition of TSA. In the presence of TSA, HP1α (green) dissociates from DAPI-dense heterochromatic foci (0 (+)). While a fraction of DAXX (red) is localized to chromocentres post TSA treatment (5 (0/+ 24)), HP1α is not enriched in those foci. Scale bar, 10 µm. [0 (-), untreated myoblasts; 0 (+) TSA treated myoblasts; 5 (-/-), untreated myotubes; 5 (+/-), TSA added to the growth media for 24 hrs prior to differentiation under control conditions; 5 (-/+ 24 hrs), TSA added to the differentiation media after 24 hrs of differentiation].
The enrichment of HP1 proteins to pericentric heterochromatin is mediated by their interaction with the H3K9me3 histone modification (Bannister et al., 2001; Jacobs et al., 2001; Lachner et al., 2001). To determine if the dissociated HP1α I observed in the presence of TSA was a result of changes in the H3K9me3 distribution pattern, I labeled TSA-treated myoblasts and myotubes with antibodies against HP1α and H3K9me3 (Figure 3.10). In the absence of TSA, H3K9me3 and HP1α are enriched in DAPI-dense chromocentres. In the presence of TSA, however, H3K9me3 levels are decreased in chromocentres, in contrast to HP1α which is dissociated from chromocentres. This finding is in contrast to observations in primary human Wi38 fibroblasts where the abundance of H3K9me3 at pericentromeres did not change upon exposure to TSA (Zhang et al., 2007). It should be noted, however, that their observations were made by ChIP and did not perform the corresponding imaging experiment.
Figure 3.10: Reduced levels of H3K9me3 at chromocentres in the presence of TSA.

IF of C2C12 myoblasts and myotubes subject to the stage-specific addition of TSA. In the presence of TSA, the levels of H3K9me3 (red) at chromocentres are reduced, accounting for the partial dissociation of HP1α (green) from these structures. Scale bar, 10 µm. [0 (-), untreated myoblasts; 0 (+) TSA treated myoblasts; 5 (-/-), untreated myotubes; 5 (+/-), TSA added to the growth media for 24 hrs prior to differentiation under control conditions; 5 (-/+ 24 hrs), TSA added to the differentiation media after 24 hrs of differentiation].
3.3.5  Ectopic PML does not relocalize DAXX from chromocentres

It has been shown that PML recruits DAXX to PML NBs (Ishov et al., 1999) and in cells which lack PML, DAXX can be found within pericentric heterochromatin regions (Ishov et al., 1999; Li et al., 2000a; Zhong et al., 2000b). Intriguingly, my observations of DAXX at PML NBs in myoblasts and at chromocentres in myotubes where PML NBs are absent, mirror these findings. I next wanted to determine if ectopic expression of PML could recruit DAXX to PML NBs in myotubes. I cloned an N-terminal FLAG-tagged full-length mouse PML cDNA expression vector and transiently transfected it into myoblasts. I prepared transfected myoblasts and 5 day differentiated myotubes for immunofluorescence using antibodies against FLAG (PML) and DAXX. In the transfected cells, a fraction of DAXX localized with ectopic PML NBs (Figure 3.11, arrowheads). Interestingly, DAXX remained at chromocentres in the transfected myotubes, despite the presence of ectopic PML NBs. This finding supports the hypothesis that the relocalization of DAXX to chromocentres is an active process, and not merely a default state when PML NBs are absent.
Figure 3.11: DAXX remains at chromocentres in myotubes in the presence of ectopic PML. C2C12 myoblasts transfected with FLAG-PML and differentiated according to standard conditions. In myoblasts, DAXX (red) is enriched in ectopic PML (FLAG, green) structures (arrowheads). The presence of PML-containing structures in differentiated myotubes failed to recruit DAXX. Under these conditions, DAXX remained at chromocentres. Scale bar, 10 µm.
3.3.6 The SUMO-interacting motif of DAXX is required for chromocentre localization in myotubes

SUMOs (small ubiquitin-related modifiers) constitute a conserved family of proteins that, like ubiquitin, are covalently attached to target proteins and regulate diverse cellular processes including transcription, chromosome organization, DNA repair, and nuclear transport (Johnson, 2004). The PML protein is modified by SUMO (Muller et al., 1998) and sumoylation of PML is essential for the formation of PML NBs (Zhong et al., 2000a). It is thought that sumoylated targets impart their functions by specifically interacting with effector proteins containing SUMO-interacting motifs (SIM) (Hecker et al., 2006). Indeed, SUMO-modified PML recruits DAXX to PML NBs and this interaction is required to inhibit DAXX-mediated transcription repression (Ishov et al., 1999; Li et al., 2000a). Furthermore, a SIM in DAXX was identified that conforms to the generic S-X-S motif and it was shown to be critical in targeting DAXX to PML NBs (Lin et al., 2006; Minty et al., 2000). I showed that DAXX relocalizes to chromocentres in the fully differentiated myotubes and in those same cells, I observed a dramatic loss in PML NB number. I therefore wanted to determine the consequences on the nuclear localization of DAXX in myotubes in the absence of its SIM. To test this, I cloned an N-terminal FLAG-tagged full length (1-740) DAXX construct as well as a mutant construct (ΔSIM) lacking residues 733-740 (Figure 3.12A). The constructs were transiently transfected into myoblasts, differentiated for 5 days, and then prepared for immunofluorescence labeling with antibodies against FLAG and ATRX (Figure 3.12B). Similar to endogenous DAXX, the full length construct was enriched in ATRX-containing chromocentres. Unlike the full length construct, ΔSIM had a nuclear diffuse signal and was not enriched in chromocentres. Therefore, the SIM of DAXX is required for the targeting of DAXX to pericentric heterochromatin domains in myotubes.
Figure 3.12: C-terminal SIM is required for DAXX to sequester at chromocentres in myotubes.

(A) Schematic of the design of the full length and SIM mutant (ΔSIM) DAXX constructs. A stop codon introduced at amino acid 733 removes the previously identified SIM. N-terminal FLAG, black shaded region; SIM, orange shaded region. (B) C2C12 myoblasts transfected with the full length of ΔSIM construct and then differentiated for 5 days under normal conditions. The cells were prepared for IF and labeled for FLAG (green) and ATRX (red) to mark chromocentres. Full length DAXX localizes to ATRX-enriched chromocentres. ΔSIM mutant is not enriched in chromocentres. The presence of the ΔSIM construct does not relocalize ATRX from chromocentres. Scale bar, 10 µm.
3.3.7 The N-terminal domain is sufficient to localize DAXX to chromocentres in myotubes

To map the region of DAXX that is required for localization at chromocentres, I generated N-terminal FLAG-tagged DAXX constructs corresponding to amino acids 1-423 (N-terminus) and 490-740 (C-terminus) of DAXX (Figure 3.13A). The N-terminus of DAXX contains two identified regions: the structural helix bundle (Escobar-Cabrera et al., 2010) and the H3.3-specific histone binding domain (Elsasser et al., 2012; Lewis et al., 2010). The C-terminus, however, contains the PML-interacting region and is sufficient for localization at PML NBs (Ishov et al., 1999). The mutant constructs, as well as full length DAXX were transiently transfected into myoblasts and differentiated for 5 days 24 hours post transfection. The resulting myotubes were prepared for immunofluorescence labeling with antibodies against FLAG and ATRX. Similar to endogenous DAXX, transfected full length and C-terminal DAXX localized to PML NBs in myoblasts (Figure 3.13B). N-terminal DAXX, on the other hand, appeared to be slightly enriched in a fraction of ATRX-containing heterochromatic foci. This observation was exaggerated in myotubes where N-terminal DAXX was visibly enriched in a fraction of chromocentres (Figure 3.13C). In myotubes, the distribution pattern of full length DAXX resembled that of endogenous DAXX. In contrast to myoblasts where both the full length and C-terminal construct localized to PML NBs, in myotubes, the C-terminal construct was nuclear diffuse. Taken together, these data demonstrate that the N-terminus of DAXX is sufficient for chromocentre localization in myotubes.
**A**

![Diagram of protein domain structure](image)

**B** Myoblasts

<table>
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- Full length (1-740)
- N-terminus (1-423)
- C-terminus (490-740)

**C** Myotubes

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- Full length (1-740)
- N-terminus (1-423)
- C-terminus (490-740)
Figure 3.13: N-terminus of DAXX containing the histone binding domain is required for DAXX to localize to chromocentres.

(A) Schematic of the domain architecture of the DAXX deletion constructs. DHB, DAXX helix bundle; HBD, histone binding domain. (B) Myoblasts transfected with the various constructs and prepared for IF. The full length and C-terminus constructs (red) are enriched in PML NBs and form distinct foci lacking ATRX (green). The N-terminal construct demonstrates colocalization with ATRX, albeit, not much higher than background. (C) Myoblasts transfected with the various constructs and then differentiated for 5 days under standard conditions. In myotubes, full length DAXX colocalizes with ATRX at chromocentres whereas the C-terminal construct is nuclear diffuse. The N-terminal construct has enrichment in ATRX-containing chromocentres. Scale bar, 10 µm.

3.3.8 Changing chromatin landscape during myogenesis

In Chapter 2 of my thesis, I describe a novel role for the histone chaperone DAXX in the structural organization of pericentric heterochromatin domains. This, together with documented changes of chromatin organization during development (Ahmed et al., 2010; Fussner et al., 2011b) and differentiation (Fussner et al., 2010; Rapkin et al., 2011), and with the known clustering of chromocentres during muscle differentiation (Brero et al., 2005; Terranova et al., 2005), prompted me to characterize the chromatin changes associated with myogenesis. In particular, I wanted to determine the heterochromatin organization in the two cell types at high molecular resolution. To accomplish this, I performed an ESI experiment on myoblasts and fully differentiated myotubes (Figure 3.14A). Myoblast chromocentres are radially symmetric, compact, spatially discrete structures from nucleoli and from the surrounding chromatin (Figure 3.14A, top row). Clustered chromocentres from myotubes are structurally similar, despite being larger in size (Figure 3.14A, bottom row). The surrounding chromatin of the two cell types, however, is strikingly different. Myoblasts contain 'clumpier' chromatin while the surrounding chromatin of myotubes is much more open. To quantify the ESI observations, I analyzed the phosphorous density of the images in Figure 3.14 by determining the mean phosphorus signal of the compact heterochromatin and surrounding chromatin domains (Figure 3.14B). The ratio of
the average phosphorus intensity in compact chromatin to surrounding chromatin in myoblasts is 2.6 whereas in myotubes it is 5.7.
Figure 3.14: Chromatin landscape of myogenesis.

(A) ESI micrographs of C2C12 myoblast (top) and myotube (bottom) nuclei. Arrows point to the regions of compact chromatin (CC). The surrounding chromatin (SC) of myotube nuclei is more open, relative to the clumpier chromatin observed in the myoblast nuclei. Arrowheads point to the protein-rich nuclear pores. Chromatin is pseudo-colored yellow and protein-based structures cyan. Scale bar, 0.5 µm. (B) Average phosphorus density analysis of the images in (A) showing the distribution of chromatin within the CC and SC domains.
3.4 Discussion

3.4.1 PML NB-dependent localization of DAXX during myogenesis

Despite being initially identified as a cytoplasmic binding partner of Fas-induced apoptosis (Yang et al., 1997), DAXX is a nuclear protein that localizes to both PML NBs and heterochromatin (Ishov et al., 1999; Ishov et al., 2004). DAXX has been implicated in variety of disparate functions, many of which are PML NB-dependent. For example the C-terminal domain of DAXX is required to enhance apoptosis and to localize DAXX at PML NBs (Torii et al., 1999). In addition, the ability for DAXX to mediate apoptosis is abrogated in the absence of PML (Zhong et al., 2000b). Furthermore, the sequestration of DAXX to PML NBs inhibits its repressor activity, and in the absence of PML, DAXX is found at chromocentres where its repressor function is restored (Li et al., 2000a). Recently it has been demonstrated that the soluble pool of (H3.3-H4) dimers accumulate at PML NBs pending deposition into chromatin (Delbarre et al., 2013). Although the accumulation was specific to H3.3 as H3.1- and H3.2-containing dimers failed to localize to PML NBs, it is still unclear if this occurs at physiologically-appropriate levels and if PML is directly involved in the regulation of H3.3 incorporation into chromatin (Salomoni, 2013). While the evidence that PML regulates the histone chaperone activity of DAXX is preliminary, the link between the PML-dependant regulation of DAXXs nuclear localization and apoptosis and transcription repressor functions is well documented. Whether the accumulation of DAXX at chromocentres in myotubes is: (1) a default due to the loss of PML NBs, or (2) a developmentally regulated process remains to be elucidated. I will discuss both possibilities in the following sections.

During myogenesis, I showed DAXX relocalized from PML NBs in myoblasts to chromocentres in myotubes (Figure 3.4). The addition of TSA to the differentiation culture media (Figure 3.8) or under low calcium conditions (Figure 3.6) impaired the formation of multinucleated myotubes. And, interestingly, I observed both an increase in the number of PML NBs and an increase in DAXX enrichment at PML NBs. Moreover, a 24 hour interferon treatment resulted in a dose-dependent induction of PML expression, increase in PML NBs, and a subsequent relocalization of a fraction of DAXX to the re-established PML NBs (Figure 3.5). These data, together with previous reports demonstrating a chromocentre localization of DAXX in the absence of PML (Li et al., 2000a) support a PML NB-dependent localization of DAXX. In
support of the developmentally-regulated hypothesis, however, are my findings that DAXX is sequestered to chromocentres despite the presence of PML NBs in myotubes (Figure 3.11). One explanation for this possible discrepancy is that the ectopic PML in myotubes is not modified by SUMO. While SUMO-modified PML is essential for the formation of PML NBs (Zhong et al., 2000a), I did not investigate if these bodies are indeed bona fide, functional PML NBs or just aggregates of PML protein. The latter seems more likely as SUMO-modified PML is required to recruit DAXX to PML NBs (Ishov et al., 1999). To address the requirement for SUMOylation of PML in nuclear body formation, Zhong et al. generated a PML mutant (3M-PML) that cannot be SUMOylated and, when expressed in PML null keratinocytes, found it accumulated in aberrant nuclear aggregates (2000). While never observed in vivo, linear PML rods and PML rosette-like structures not enriched in either SUMO or DAXX have been observed in cultured human ES cell (Butler et al., 2009). Therefore, while it is possible for endogenous DAXX to sequester outside of PML NBs in the presence of PML, I think a more plausible scenario is that the enrichment of DAXX at chromocentres occurs in response to differentiation cues.

3.4.2 Both the N-terminus and the SIM contribute to DAXX recruitment to chromocentres

The C-terminus of DAXX containing the functional SIM is required for both its interaction with the PML protein and its localization at PML NBs (Ishov et al., 1999; Lin et al., 2006). The mechanism responsible for the sequestration of DAXX to chromocentres, however, has yet to be elucidated. DAXX cooperates with ATRX to direct H3.3 deposition to pericentric and telomeric heterochromatin (Drane et al., 2010; Goldberg et al., 2010). While DAXX itself is sufficient to bind (H3.3-H4) dimers (Lewis et al., 2010), mutation and immunoprecipitation studies demonstrated that DAXX interacts with ATRX via its N-terminal domain (Tang et al., 2004). The ATR-X syndrome associated mutations are found in the N-terminal ADD and the C-terminal ATP-dependent chromatin-remodeling domains (Gibbons et al., 2008). Interestingly, ATRX interacts directly with histone H3 through the ADD domain and the presence of H3K9 methylation augments this interaction. Furthermore, using double knockout MEFs lacking the H3K9me3 histone methyltransferases Suv39h1 and Suv39h2, it was shown that the presence of H3K9me3 is essential for tethering ATRX to chromocentres (Iwase et al., 2011). The recruitment of ATRX to heterochromatin is more complex than just the ADD domain-H3 interaction, it requires an elaborate network of interactions including a more direct interaction with HP1 via a
C-terminal HP1 interaction motif (Eustermann et al., 2011). What is interesting is it appears that the recruitment of DAXX to chromocentres also requires a combination of interacting partners and binding motifs. I showed that both DAXX and ATRX are enriched in chromocentres in myotubes (Figures 3.4, 3.12) and that the N-terminus of DAXX mediates this localization (Figure 3.13). It is therefore possible that the N-terminus of DAXX, through its interaction with ATRX, enables the recruitment of DAXX to chromocentres. I also showed the SIM located at the C-terminus is required for DAXX to localize to chromocentres as the carboxyl ΔSIM mutant failed to localize to chromocentres (Figure 3.12), and as such, the recruitment of DAXX to chromocentres cannot be solely explained by its interaction with ATRX. It has been shown that SUMOylation of HP1α promotes its targeting to pericentric heterochromatin domains, even in the absence of H3K9me3 (Maison et al., 2011). It could therefore be that the combination of the interaction with ATRX and SUMOylated factors such as HP1 drive the relocalization of DAXX to chromocentres.

### 3.4.3 Developmentally-regulated loss of PML NBs

PML null mice are born with expected Mendelian frequency. However, the terminal differentiation capacity of their myeloid cells is impaired (Wang et al., 1998). PML has also been implicated in the regulation of cell fate specification in the developing neocortex by regulating the size of the cerebral cortex and controlling the proliferative potential of neuronal precursor cells (NPCs) (Regad et al., 2009). It has been shown that PML expression is restricted to NPCs (Regad et al., 2009) and that the number of PML NBs decrease in post mitotic neurons (Aoto et al., 2006). It is an intriguing possibility that a loss of PML NBs is characteristic of terminally differentiated cells.

The product of the retinoblastoma tumor suppressor gene, pRB, is a nuclear protein that connects the progression of the cell cycle with transcription machinery by controlling E2Fs (Dyson, 1998; Weinberg, 1995). E2Fs are a family of transcription factors that regulate the expression of genes involved in many cellular processes including proliferation and differentiation (Muller et al., 2001). To achieve terminal differentiation and myotube formation, proliferating myoblasts must permanently exit the cell cycle (Perry and Rudnick, 2000) and pRB is necessary for this process (De Falco et al., 2006). Newborn mice lacking pRB exhibit extensive skeletal muscle defects and in vitro differentiated RB-deficient myoblasts fail to form multinucleated myotubes (Huh et al.,
It has been shown that early in G1, unphosphorylated pRB binds to and inhibits E2F, and as cells enter S-phase, pRB becomes phosphorylated and releases E2F (Weinberg, 1995). In contrast, terminally differentiated myotubes express hypophosphorylated pRB (Corbeil et al., 1995) suggesting that, during myogenesis, pRB may play a functionally distinct role from the conventional repression of E2F activity (Huh et al., 2004). Indeed, pRB has been shown to associate with endogenous Suv39h1 and this interaction promotes the methylation of H3K9, providing a binding site for HP1 and the subsequent repression of pRB target genes (Nielsen et al., 2001). Furthermore, pRB, through the recruitment of HP1, targets E2F-dependent genes to heterochromatin in adult cardiac myocytes contributing to the molecular basis of the postmitotic phenotype (Sdek et al., 2011). Along these lines, maintaining the stability of the senescent state occurs, in part, through the pRB-directed acquisition of heterochromatin features on E2F target genes (Narita et al., 2003). Interestingly, it has been reported that a fraction of pRB localizes to PML NBs in human myeloid leukemia and cord blood cells, and PML coprecipitated with hypophosphorylated pRB (Alcalay et al., 1998). It should be noted, that soluble, nuclear diffuse unmodified PML is contained in the soluble fraction of nuclear lysates whereas SUMO-modified PML is found in the insoluble fraction containing the nuclear matrix and PML NBs (Muller et al., 1998). Therefore, whether the hypophosphorylated form of pRB can interact with SUMO-PML and localize to PML NBs remains unclear. As well, ectopic expression of PML can down-regulate E2F target genes and relocalize pRB/E2F-containing complexes to PML NBs during the establishment of senescence (Vernier et al., 2011). It would be interesting to investigate the possibility that the downregulation of PML and concomitant loss of PML NBs are key steps in establishing the postmitotic state of terminally differentiated cells possibly by controlling the nuclear localization and functions of pRB during myogenesis.

3.4.4 Is DAXX contributing to the changing nuclear landscape during myogenesis?

As cells differentiate, they become increasingly restricted in their developmental potential, and as they commit to specific lineage programs, the competence of a particular subset of genes to be expressed is established. Concomitant with the genome-wide epigenetic changes that occur as cells pass through differentiation programs are changes in the morphological appearance of heterochromatin domains. For example, the majority of chromatin is homogeneous and decondensed in ESCs and iPS cells, in contrast to the more heterogeneous appearance of
chromatin in committed NPCs and MEFs (Efroni et al., 2008; Fussner et al., 2011b). Many of these changes can occur on a much smaller scale as well. In response to retinoic acid (RA), which drives ES cells down a neuronal lineage, Hoxb1 and Hoxb2 loop out of the chromosome territory following their temporal program of gene expression (Chambeyron and Bickmore, 2004). It is therefore not surprising that dramatic changes in global chromatin organization accompany muscle differentiation. I speculate that the heterogeneous chromatin of lineage committed precursor cells, such as myoblasts and NPCs, and the more open nature of their corresponding differentiation products are characteristic of terminal differentiation programs. Indeed, ESI micrographs of NPCs reveals that the organization of chromatin is more similar to myoblasts than to MEFs. The ultrastructure analysis of fully differentiated neuronal chromatin, however, and whether it exhibits similar patterns to myotubes remains to be elucidated. Despite the lack of data on neuronal chromatin, my findings in myotubes raise the intriguing question of what chromatin remains in the open compartment following terminal differentiation?

Insight into addressing this can be taken from studies on the human β-globin locus. The human β-globin locus consists of five genes arranged in the order in which they are expressed. Upstream of the gene cluster is the locus control region (LCR). In erythroid cells, β-globin genes are transcriptionally active, whereas in a naturally occurring deletion of the LCR, the Hispanic thalassemia locus, they are transcriptionally inactive (Fraser and Grosveld, 1998). Interestingly, wild type alleles are located away from centromeric heterochromatin whereas the Hispanic alleles are more closely associated to heterochromatin domains (Schubeler et al., 2000). This suggests that transcriptionally active and silenced loci can be found in distinct nuclear compartments. In support of this argument, it was shown that inactive pRB was closely associated with centromeres in undifferentiated human leukemic promyelocytic cells as compared to terminally differentiated granulocytes where active pRB is located further from heterochromatic regions (Bartova et al., 2002). Therefore, it is possible that the majority of chromatin in myotubes is sequestered into compact domains while genes required to maintain homeostasis and the post mitotic state, loop out into the open euchromatic regions. Is DAXX contributing to this changing chromatin landscape by mediating the structural organization of pericentric heterochromatin-containing domains, possibly via the deposition of H3.3?

The activation of differentiation-specific genes depends on the appropriate regulation of local and global chromatin structures and on the binding of lineage-determining transcription factors.
To accomplish this, the genome must be marked in some way such that the gene expression profile of the differentiated cell is reflected in the chromatin structure of the undifferentiated cell. The particular mechanisms responsible for establishing tissue-specific chromatin marks, however, are still not entirely clear. It is well established that histone post translational modifications help define chromatin environments and influence gene expression by influencing higher-order chromatin structures and through the recruitment of nonhistone protein complexes (Kouzarides, 2007). It has also been shown that histone variants can distinguish active and inactive chromatin (Hake and Allis, 2006). For example, H3.3 is predominantly incorporated in the regulatory regions of active genes whereas H3.2 enrichment coincides with the H3K27 and H3K9 repressive methylation (Hake and Allis, 2006). While it is clear that histone variants are indeed critical determinants of selective gene expression, their role in establishing tissue-specific gene patterning remains elusive. Recently, however, the chromodomain helicase DNA-binding domain 2 (Chd2) chromatin remodeling enzyme was identified as a myogenic cell fate determinant by specifically mediating the incorporation of H3.3 into the promoters of myogenic genes (Harada et al., 2012). Interestingly, the depletion of H3.3 impaired myotube formation. It has been shown that DAXX can reside at pericentric heterochromatin and is responsible for the deposition of H3.3 into major satellite repeats (Drane et al., 2010; Ishov et al., 1999). Therefore, I speculate that DAXX is being recruited to chromocentres during muscle differentiation to mediate the massive heterochromatin reorganization events that mark myogenesis. While DAXX-dependent H3.3 deposition patterns remain to be determined, particularly in the context of tissue differentiation, the possibility that DAXX establishes heterochromatin boundaries by marking repetitive regions of the genome with H3.3 and these boundaries are responsible for guiding tissue-specific heterochromatin organization is an intriguing possibility. If such a scenario were true, it would explain why the loss of DAXX has such devastating consequences in development.
Chapter 4

4 Future directions

In this thesis, I have shown that DAXX is a key factor in nuclear organization by maintaining the structural organization of heterochromatin domains and the integrity of the nucleolus (Figures 2.4, 2.5). I proposed a model where the DAXX-dependent selective methylation of H3.3K9me3 establishes essential pericentric heterochromatin genomic boundaries required for the proper structural organization of heterochromatin and heterochromatin-dependent domains (Figure 2.8). In addition, I showed that DAXX is sequestered in chromocentres of fully differentiated myotubes (Figure 3.4) and speculated that via the deposition of H3.3 into specific repetitive regions of the genome, DAXX guides tissue-specific heterochromatin organization. Determining DAXX-dependent chromatin landscapes and how these boundaries contribute to differentiation pathways are the focus of future experiments.

4.1 Determining DAXX-dependent chromatin landscapes

I showed that in the absence of DAXX, H3K9me3 can be uncoupled from both compact heterochromatin domains and major satellite repeats (Figures 2.4-2.6). Two possibilities exist that describe these observations. The first is that major satellites are losing H3K9me3-enrichment yet are still packaged as compact chromatin, and the second, major satellites are enriched in H3K9me3 and non-heterochromatin sequences are being structurally heterochromatinized. While determining the underlying sequences comprising compact heterochromatin would require a specialized experiment that physically removes and/or isolates these domains, such as the nano-dissection technique recently developed in our lab to identify the DNA in the vicinity of a particular PML NB (Chen et al., 2014), ChIP-Seq can be used to identify genomic regions enriched in the H3K9me3 repressive histone modification (Bulut-Karslioglu et al., 2012). Therefore, to identify the exact genomic locations of H3K9me3-enrichment, ChIP-Seq can be performed in wild type and DAXX null MEFs to determine the DAXX-dependent global pattern of H3K9me3 distribution. While non-repeat accumulations can be compared relatively easily, mapping differences in major satellite repeats will be difficult because the sequence identity of the repeats given the large arrays of pericentric heterochromatin defy annotation (Bulut-Karslioglu et al., 2012). There are, however, intergenic major satellite sequences interspersed in the mouse genome that, due to specific single nucleotide
polymorphisms, can be appropriately annotated (Robinson et al., 2011). Therefore, these intergenic major satellite sequences could be used to identify DAXX-dependent changes in H3K9me3-enriched pericentric heterochromatin domains. Since total H3K9me3 levels are similar between the two MEF cell lines, I predict that the H3K9me3 peak pattern in DAXX null cells will be more spread out and dispersed throughout the genome, in particular at non-repeat regions, which would explain the unusual chromatin compactions seen by ESI. Due to the technical requirements of FISH, confirming that aberrant accumulations of H3K9me3 are indeed packaged into compact heterochromatin is not possible using ESI-based techniques.

Previous ChIP-Seq experiments revealed that H3.3 is preferentially enriched in the transcription start sites of active genes and this deposition is driven by the chaperone HIRA (Goldberg et al., 2010). In ES cells, a fraction of H3.3 is deposited at telomeres and it is this deposition that requires the DAXX-ATRX chaperone complex (Goldberg et al., 2010). Interestingly, upon depletion of the H3.1 chaperone CAF-1, H3.3 is deposited at replication sites by the HIRA complex rather than DAXX (Ray-Gallet et al., 2011). These data demonstrate that compensatory mechanisms exist between histone chaperone deposition pathways to maintain chromatin integrity. However, genome-wide studies investigating H3.3 deposition at repetitive regions of the genome in MEFs as well as the consequences of the loss of DAXX on H3.3 deposition patterns have yet to be determined. I did show by IF that a fraction of chromocentres are indeed enriched in H3.3 though not to the extent that directed ChIP experiments have predicted (Drane et al., 2010). Therefore, to determine the DAXX-dependent global H3.3 deposition pattern, ChIP-Seq needs to be performed in wild type and DAXX null MEFs to address the following questions addressed: (1) What is the deposition pattern of H3.3 in major satellite sequences, and (2) Does DAXX facilitate deposition into different types of repeats? If the deposition pattern is similar between the two genomes, then either DAXX is not responsible for driving the deposition of H3.3 into repetitive elements or an alternate H3.3-specific chaperone such as HIRA is acting in a compensatory manner to ensure H3.3 deposition. I expect, however, that there will be subtle changes in the deposition patterns between the two cell types and these changes will provide insight into the histone chaperone activity of DAXX.

A limitation of ChIP-Seq is that it provides binding patterns or modification profiles of a single epitope. To determine DAXX-dependent H3.3-defined heterochromatin boundaries and the selective methylation of H3.3K9me3, the histone variant composition and modification status of
the same genomic location is required. Therefore, a ChIP-reChIP-Seq experiment, rather than a traditional ChIP-Seq experiment is required (Furlan-Magaril et al., 2009). To accomplish this, wild type and DAXX null MEFs can be subject to H3.3-ChIP, reChIP for H3K9me3, followed by whole-genome sequencing. The cohabitation of the variant and the specific modification will need to be investigated at both repeat and non-repeat regions of the genome with a focus on major satellite repeat sequences. First, one would quantify the number of H3.3 peaks enriched in H3K9me3 in wild type cells and compare the results to those obtained from DAXX null cells. If DAXX does indeed deposit H3.3 into major satellite regions, in wild type cells I expect them to contain the H3K9me3 modification. It will be interesting to determine the H3K9me3 profile of H3.3-enriched chromatin in the absence of DAXX. For example, will those regions continue to be enriched in H3K9me3 or will they lose this mark? As well, if H3.3 is being deposited into alternative locations via a different deposition complex, do these alternate sites contain H3K9me3? I expect the H3.3K9me3 patterns between the two cell lines to be quite different, reflecting the dramatic structural abnormalities of heterochromatin domains observed by ESI and FISH in the absence of DAXX.

4.2 Nuclear organization of terminally differentiated cells

I described the organization of chromatin during myogenesis (Figure 3.14) and speculated that the open chromatin of the myotubes and the clumpy chromatin of myoblasts could be characteristic of terminally differentiated cells and their precursors, respectively. To investigate this further, the chromatin organization of another differentiation cell type, such as the ND7 neuronal cell line, could be characterized. The ND7 cell line was generated by fusing nondividing rat dorsal root ganglion cells with the N18 mouse neuroblastoma cell line. The resulting cells proliferate indefinitely in culture and, similar to C2C12 cells, can be induced to differentiate into mature neuronal cells by transfer to serum-free media (Howard et al., 1993; Suburo et al., 1992; Wood et al., 1990). As a prelude to determining the structural organization of heterochromatin domain in ND7 differentiation, DAXX protein levels and localization will be monitored in the precursor as well as terminally differentiated neurons by Western blot analysis and IF, respectively. It has been shown that PML NBs decrease in post mitotic neurons (Aoto et al., 2006). Therefore I expect a similar decrease in body number in the differentiated ND7 cells and a concomitant sequestration of DAXX to chromocentres. To determine if the ultrastructure of chromatin of ND7 cells is similar to C2C12, ND7 cells will be differentiated and prepared for
ESI, together with cycling ND7 cells. ESI images can be used to examine both the organization of heterochromatin domains as well as the structure of the background chromatin.

In the discussion of the changing chromatin landscapes during differentiation (Section 3.4.4), I proposed that the open chromatin of a fully differentiated myotube contains genes involved in homeostasis and maintaining the post-mitotic state while all other chromatin is sequestered into compact domains. To test this hypothesis, myoblasts and myotubes can be prepared for LM/ESI with gold conjugated H3K9me3 antibodies. This technique can distinguish the chromatin within the large compact domains characteristic of myotubes. I anticipate the result to be similar to that of mouse lymphocytes chromocentres that are characterized by H3K9me3-enriched core of compact chromatin surrounding by a rim of heterochromatin devoid of this mark (Figure 1.4). A similar experiment will be repeated using the undifferentiated and differentiated ND7 cells. Together, these experiments can be used to determine if the chromatin landscapes seen in myogenesis are indeed characteristic of terminally differentiated cells or are unique to myogenic differentiation. Furthermore, they will provide insight into why the terminally differentiated cells is organized in such a manner.

4.3 DAXX-dependent chromatin organization during differentiation

Recently DAXX has been identified as a histone chaperone and is responsible for deposition of H3.3 into repetitive regions of the genome (Drane et al., 2010; Goldberg et al., 2010). This, together with my observations that DAXX is required to maintain the structural organization of heterochromatin domains led to my hypothesis that DAXX-dependent H3.3 deposition establishes the boundaries for the H3K9me3 modification and the ensuing compaction of the targeted chromatin. Furthermore, I discussed the possibility that the embryonic lethality observed in mice lacking DAXX could be a result of a failure to properly establish tissue-specific heterochromatin domains essential for development. To explore the requirement for DAXX in myogenesis, DAXX expression can be stably suppressed by introducing microRNAs (miRNA) that target DAXX in C2C12 cells (C2C12^miDAXX). It has been established that up to 30% of myoblasts undergo apoptosis during differentiation (Wang and Walsh, 1996). This, together with studies demonstrating that the silencing of DAXX by siRNA results in increased levels of apoptosis and the extensive apoptosis in the embryos devoid of DAXX (Chen and Chen, 2003;
Michaelson et al., 1999; Michaelson and Leder, 2003), leads me to believe that the
differentiation program of C2C12\textsuperscript{miDAXX} cells will be characterized by increased levels of
apoptosis and might fail to form fully differentiated multinucleated myotubes. Therefore, prior to
ESI experiments, C2C12\textsuperscript{miDAXX} differentiation can be characterized to determine if there are
phenotypes related to the formation of myotubes and to the ensuing chromocentre
reorganization. Once characterized, control C2C12 and C2C12\textsuperscript{miDAXX} cells will be differentiated
and the myoblasts as well as the corresponding differentiation products can be prepared for
LM/ESI and H3K9me3-enriched chromatin as well as surrounding euchromatin can be
compared. I predict that if differentiation can indeed proceed, the resulting myotubes will be
marked by aberrant accumulations of compact domains as well as regions of compaction that are
uncoupled from H3K9me3. As a parallel experiment, the same miRNAs can be used to establish
DAXX-deficient ND7 cells (ND7\textsuperscript{miDAXX}). Similarly, as described for C2C12\textsuperscript{miDAXX} myogenesis,
ND7\textsuperscript{miDAXX} differentiation can be characterized and an ESI-based analysis of chromatin can be
performed. In addition to comparing ND7\textsuperscript{miDAXX} differentiation products to those from control
ND7 cells, the chromatin organization of ND7\textsuperscript{miDAXX} can be compared to C2C12\textsuperscript{miDAXX} to gain
insight into differentiation-induced DAXX-dependent chromatin organization. Together these
data and the expected failure to properly establish chromatin organizations typical of terminally
differentiated cells may explain why mice lacking DAXX fail to form appropriately-defined
tissues and cannot complete organogenesis.

4.4 Elucidating the mechanism of DAXX-dependent chromatin organization

The experiments described above address the role of DAXX in the global organization of
heterochromatin during differentiation. To complete the story, a mechanistic analysis of DAXX
in the context of the structural organization of chromatin is required. To accomplish this, the
FLAG-DAXX expression vectors described in Chapter 3 of this thesis (Figures 3.12, 3.13) can
be transfected into C2C12\textsuperscript{miDAXX} and ND7\textsuperscript{miDAXX} cells and prepared for IF and LM/ESI
analysis. I expect that full length DAXX will rescue differentiation-induced phenotypes and
faithfully recapitulate how chromatin is organized in the control cells. I demonstrated that the N-
terminal DAXX expression vector (1-423) retaining the histone binding domain localizes to
chromocentres in myoblasts and myotubes (Figure 3.13) and speculated that DAXX is recruited
to chromocentres during muscle differentiation to mediate the heterochromatin reorganization
events that mark myogenesis, possibly via the deposition of H3.3. Therefore, I predict that this vector will, at least partially, rescue the null phenotype. The C-terminal vector (490-740) containing the PML-interacting domain, on the other hand, will likely not rescue the null phenotype as the expressed truncated DAXX would not localize to chromocentres in myotubes (Figure 3.13). I showed that the ΔSIM mutant, despite containing the histone binding domain, did not localize to chromocentres in myotubes (Figure 3.12). Therefore, if its expression does not rescue the null phenotype, it can be inferred that the chromocentre localization of DAXX is essential for the organization of chromatin domains. On the other hand, if the ΔSIM mutant can rescue the phenotype, one may conclude that the histone binding domain, rather than the chromocentre localization is required for DAXX to mediate differentiation-induced heterochromatin reorganization. This scenario would be consistent with the idea that the sequestration of DAXX to pericentric heterochromatin in myotubes is indeed a default state due to the absence of PML NBs and that it mediates heterochromatin-related functions from the nucleoplasm rather than structurally defined bodies. These experiments will elucidate which domains of DAXX drive its functions as a key regulator of the structural organization of heterochromatin domains in differentiation.
Summary

The chromatin landscape, defined by the arrangement of euchromatin and heterochromatin domains is at the heart of nuclear processes including gene regulation and cell differentiation. While the structure of the nucleosome, the building block of chromatin is defined, a consensus on higher-order chromatin organization has yet to be elucidated. What is known is that the modification of histone tails, together with the marking of chromatin domains through the deposition of histone variants are essential steps in establishing chromatin organization. The identification of key factors and mechanisms involved in the establishment and maintenance of heterochromatin is thus essential in understanding how epigenetic markers contribute to nuclear organization in general and regulate vital cellular processes. DAXX, recently identified as an H3.3 histone chaperone, is thought to be part of a novel chromatin assembly pathway specific for repetitive regions of the genome (Drane et al., 2010; Goldberg et al., 2010; Lewis et al., 2010). These findings led to my hypothesis that DAXX is involved in structurally organizing the genome. Therefore, defining its function in heterochromatin organization was the focus of this thesis. My studies accomplished this by identifying DAXX as a factor in heterochromatin organization and characterizing the breakdown of the heterochromatin compartment upon its loss. A key finding of this work, was that the loss of DAXX resulted in the uncoupling of an epigenetic mark from the underlying chromatin. Importantly, this is the first body of work that addresses the in vivo consequence of the loss of a histone chaperone on the structure of heterochromatin domains. As well, for the first time, it provides a direct link between the deposition of H3.3 and the structural integrity of the nucleolus. Furthermore, since DAXX null embryos are embryonic lethal and exhibit a loss of identifiable tissue types (Michaelson et al., 1999), I sought to determine a potential cause for the dramatic phenotype by investigating its role in an in vitro model of tissue differentiation. I showed that DAXX is sequestered to pericentric heterochromatin domains in multinucleated myotubes suggesting it might contribute to the unique chromatin landscape of terminally differentiated cells. DAXX, via the deposition of H3.3 in specific regions of the genome, may function in establishing the organization of heterochromatin domains required for tissue differentiation and development. Future experiments will further elucidate the mechanism by which DAXX establishes these essential chromatin-based boundaries that define nuclear organization.
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Copyright Acknowledgements

Contents of Chapter 1 (1.4) and Chapter 3 (3.1) have been published in the journal Micron:
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