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Graduate Program in Biochemistry
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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THE ROLE OF THE ARGININE METHYLTRANSFERASE CARM1 IN GLOBAL TRANSCRIPTIONAL REGULATION

(Thesis format: Integrated Article)

by

Niamh Coughlan

Graduate Program in Biochemistry

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Arginine methylation is a prevalent post-translational modification that is found on many nuclear and cytoplasmic proteins, and has been implicated in the regulation of gene expression. CARM1 is a member of the protein arginine methyltransferase (PRMT) family of proteins, and is a key protein responsible for arginine methylation of a subset of proteins involved in transcription. In this thesis I examine some of the mechanisms through which CARM1 contributes to global transcriptional regulation.

Using a ChIP-DSL approach, we show that the p/CIP/CARM1 complex is recruited to 204 proximal promoters following 17β-estradiol (E2) treatment in MCF-7 cells. Many of the target genes have been previously implicated in signaling pathways related to oncogenesis. JAK2, a member of the Jak/Stat signaling cascade, is one of the direct E2-dependent targets of the p/CIP/CARM1 complex. Following E2-treatment, histone modifications at the JAK2 promoter are reflective of a transcriptionally permissive gene, and we observed modest increases in RNA and protein expression. Notably, E2-induced expression of Jak2 was diminished when p/CIP or CARM1 were depleted, suggesting that the p/CIP/CARM1 complex is required for the observed transcriptional response. Collectively, these results suggest that E2-dependent recruitment of the p/CIP/CARM1 complex causes JAK2 to become 'poised' for transcription, a finding that may be extendable to other target genes and signalling pathways. Furthermore, bioinformatic examination of p/CIP/CARM1 target promoters suggests that transcription factor crosstalk is the favored mechanism of E2-dependent p/CIP/CARM1 complex recruitment.

Using ChIP-Seq, we identified genomic regions to which CARM1 is recruited. Subsequent characterization of binding events suggest a role for CARM1 in transcriptional elongation, and implicate the transcription factor PAX1 as a novel mechanism through which CARM1 can be recruited to the genome. Identification of CARM1-dependent differentially expressed genes revealed that direct recruitment of CARM1 is not essential for the majority of its transcriptional effects in MEFs. However, CARM1 does play a critical role in cellular growth and proliferation, and in the absence of CARM1, the expression of many cell cycle regulators is dramatically affected.

Collectively, this work provides insight into some of the mechanisms through which CARM1 modulates transcription, and highlights its importance in diverse cellular processes.

Keywords

Transcription, transcriptional regulation, chromatin, histone modifications, chromatin immunoprecipitation (ChIP), cancer, estrogen, coregulators, steroid receptor coactivators, arginine methylation, p/CIP, CARM1

Co-Authorship Statement

All chapters of this thesis were written by Niamh Coughlan and edited by Dr. Joseph Torchia.

The data presented in Chapter 2 has been published in BBA Molecular Cell Research as "β-Estradiol-dependent activation of the JAK/STAT pathway requires p/CIP and CARM". The initial ChIP-DSL was performed by Gobi Thillainadesan, and the RNA for the expression microarray was collected by Majdina Isovic. Both of these screens were processed at London Regional Genomics Centre. I performed data analysis and all subsequent experimental work, and participated in study design with Dr. Torchia.

The data presented in Chapter 3 is currently in preparation for publication. The initial ChIP was performed by Gobi Thillainadesan, and sent for sequencing to the University of British Columbia using the Illumina sequencing platform. The RNA for the expression microarray was collected by Majdina Isovic, and processed at London Regional Genomics Centre. I participated in study design with Dr. Torchia, performed all data analysis, and subsequent experimental work, with the exception of the co-immunoprecipitation experiment demonstrating an interaction between CARM1 and Paf1c, which was performed by Bart Kolendowski.

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To the current and former members of the Torchia Lab – you are what kept me coming back in on bad days and staying late on good ones. I have made some great memories in the time we spent together and have learned a great deal from each and every one of you. I must acknowledge my current lab mates – who have watched me slowly go crazy in the process of assembling this thesis. Majdina Isovic has been there from my very first days in the lab, and taught me many many techniques along the way. You should know I've greatly enjoyed annoying you with my 'tomboy' clothes and interests, and smartass comments every now and then. But in all seriousness, you've been a good friend for a lot of years – thank you, and keep the newbies in line! I'm passing the unofficial 'senior student' torch' to the soon-to-be PhD candidate Bart Kolendowski. Thank you for being a calming presence in the lab, for being a friend, and most of all for rolling your eyes every time Majdina is being too Majdina – please continue to do so even in my absence. To our new lab members Matt and Haider, learn every chance you get and from everybody; and know that I am absolutely not limiting that sentiment to science. You will learn more about people and life, and stress and caffeine

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List of Abbreviations

Abbreviation	Meaning
ADMA	Asymmetrically dimethylated arginine
AP-1	Activator Protein 1
C/EBP	CCAAT-enhancer-binding proteins
CARM1	Coactivator-associate Arginine Methyltransferase 1
CBP	CREB-binding protein
CDS	Coding Sequence
ChIP	Chromatin Immunoprecipitation
ChIP-DSL	Chromatin Immunoprecipitation with DNA Selection and Ligation
ChIP-reChIP	Sequential Chromatin Immunoprecipitation
ChIP-Seq	Chromatin Immunoprecipitation Sequencing
CTD	C-terminal domain
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic Acid
E2	17-β Estradiol
ER	Estrogen Receptor
ERE	Estrogen Response Element
FDR	False Discovery Rate
FoxA1	Forkhead box A1
GO	Gene Ontology
GR	Glucocorticoid Receptor
HAT	Histone Acetyltransferase

HDAC	Histone Deacetylase
HRE	Homone Response Element
IGF-1	Insulin-like growth factor 1
IP	Immunoprecipitation
IPA	Ingenuity Pathway Analysis
JAK	Janus Kinase
JMJD6	Jumonji domain-containing protein 6
MEF	Mouse Embryonic Fibroblast
MMA	Monomethylated Arginine
MMTV	Mouse mammary tumor virus
mRNA	messenger RNA
NR	Nuclear Receptor
NRID	Nuclear Receptor Interaction Domain
Oct	Octamer factor
p/CIP	p300/CBP Interacting Protein
Paf1	RNA Polymerase II Associated Factor
Pax1	Paired Box protein 1
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI3K	Phosphoinositide-3 kinase
PPARγ	Peroxisome proliferator-activated receptor gamma
PR	Progesterone Receptor
PRMT	Protein Arginine Methyltransferase

PTM	Post-translational modification
RB	Retinoblastoma protein
RNA	Ribonucleic Acid
RNAPII	RNA Polymerase II
RXR	Retinoid X Receptor
SAM	S-adenosyl Methionine
SDMA	Symmetrically dimethylated arginine
Sp1	Specificity Protein 1
SRC	Steroid Receptor Coactivator
STAT	Signal Transducer and Activator of Transcription
SUMO	Small ubiquitin-related modifier
TDRD3	Tudor domain-containing protein 3
TF	Transcription Factor
TSS	Transcriptional Start Site
VDR	Vitamin D Receptor

Chapter 1

1 General Introduction

1.1 Gene Expression

Gene expression is a general term applied to the process in which the genetic information encoded within DNA becomes processed into a functionally useful form(s). The first step in this process is transcription, wherein RNA Polymerase (RNAP) reads the DNA template strand and produces a primary RNA transcript. The primary transcript is then processed and/or spliced, giving rise to mature mRNA, in addition to the more recently discovered non-coding RNA (ncRNA) [1]. Transport of mRNA from the nucleus to the cytoplasm is essential for protein production, termed translation, which takes place in ribosomes. An overview of the levels at which gene expression can be regulated is shown in Figure 1-1, and can conceptually be broken down into four component sections: transcriptional regulation, post-transcriptional regulation, translational regulation, and post-translational regulation.

While each cell within an organism is encoded with the same genetic information, there are vast differences in the size, shape, function, and lifespan of each cell type. These differences can be largely accounted for by distinct cellular gene expression profiles, with genes being expressed in a tissue/cell-specific and time-dependent manner. Development in particular is characterized by major changes in gene expression corresponding to changes in cell determination, and resulting in the differentiation of cells into distinct lineages. Many cellular processes are common to all cell types, and so all cells will express certain common genes and proteins. It has been determined that a

given human cell expresses between 30-60% of approximately 25,000 genes at any given time [1,2]. Notably, aberrant regulation of transcription is often involved in disease states, including cancer.

1.2 Transcriptional Regulation

The work presented within this thesis is primarily concerned with the regulation of gene expression at the level of transcription. A complex association between cis-acting regulatory DNA sequences, trans-acting DNA-binding proteins, and chromatin compaction modulates transcriptional regulation [3,4]. Regulatory DNA sequences, also known as response elements, generally represent sites within the genome at which protein binding can occur. These elements can occur both proximal to and/or distal from the gene to be transcribed. Proximal regulatory sequences occur within the promoter region, consisting of the core promoter and adjacent regulatory elements, while distal regulatory sequences can exist up to 1Mbp from the transcription start site (TSS). Distal regulatory elements can include enhancers, silencers, insulators, and locus control regions [4]. *Trans*-acting DNA-binding proteins bind to *cis*-acting regulatory sequences and function to either activate or repress transcription.

Transcription classically consists of three stages: initiation, elongation, and termination [1]. Each stage involves multiple factors and a specific sequence of events in order to proceed, with an RNAP common to all stages. There are three different RNA Polymerases in eukaryotes, each responsible for the transcription of a different form of RNA. RNAPI synthesizes ribosomal RNA (rRNA); RNAPII is responsible for the

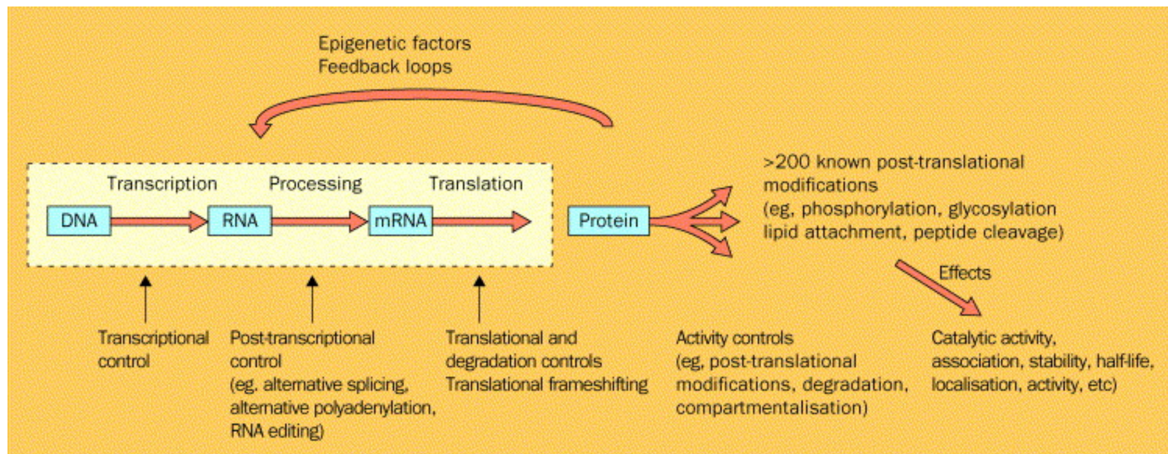

Figure 1-1 Regulation of gene expression

Overview of gene expression, highlighting the various points at which regulatory control is exerted, as well as the some of the mechanisms in regulation. Figure reproduced from Banks *et al.* 2000 [5].

production of messenger RNA (mRNA), as well as several types of noncoding RNAs; and RNAPIII generates primarily transfer RNA (tRNA). RNAPII is responsible for the production of transcripts from protein-coding genes, and its role in transcription has been extensively characterized [6,7].

Prior to initiation, and the addition of the first nucleotide in the mRNA transcript, there are many pre-initiation steps that ultimately make transcriptional initiation a critical regulatory step in the conversion of DNA to RNA. The key factors required for transcription initiation include RNAPII, general and specific transcription factors, Mediator, as well as proteins that possess chromatin-modifying and chromatin-remodeling activities. RNAPII is the enzyme that synthesizes mRNA from the DNA template during transcription. Its recruitment is dependent on a variety of transcription factors, and its activity and processivity can be affected by post-translational modifications [3,8]. General transcription factors help localize RNAPII to the core promoter of the gene to be transcribed, and participate together with RNAPII as part of the transcription initiation complex [3,4].

Specific transcription factors can be defined as sequence-specific DNA binding proteins that become recruited to regulatory regions, such as the proximal promoter or distal enhancer regions of specific populations of genes to be transcribed. Recruitment of specific transcription factors often occurs in response to an extrinsic signal, or in a developmentally-timed manner. The specific TFs can interact with the general transcriptional machinery described above, and drive activation or repression of transcription [3]. Both general and specific transcription factors can also interact with

coregulatory proteins which often act as mediators of their interaction, and modulators of transcription factor activity [3].

Initially, a pre-initiation complex forms on selected promoters upstream of the TSS, involving binding of general transcription factors and RNAPII. Activators and/or repressors that will control the rate of transcription can also participate as part of the pre-initiation complex. Transcriptional initiation progresses through promoter melting, clearance, and escape, until an RNAPII elongation complex is formed and the transcript becomes extended [4]. The C-terminal domain of RNAPII is an important regulatory component, consisting of multiple heptapeptide repeats, which can be extensively post-translationally modified [9]. Phosphorylation of the C-terminal domain of RNAPII is critical for activation, and immediately precedes the onset of transcriptional elongation [3,10,11].

In vivo, the packaging of DNA complicates transcription further, as access to promoter and enhancer regulatory elements can be limited by chromatin condensation. Chromatin-modifying and chromatin-remodeling proteins are therefore critically important during transcriptional initiation, to alter the packaging of DNA such that the regions containing genes to be expressed, and their associated regulatory regions, are accessible to the general transcriptional machinery. A more detailed examination of the role that chromatin plays in transcriptional regulation is the focus of the following section.

1.3 Chromatin and Regulation of Transcription

In order to accommodate the nearly 2m of DNA within a cell's nucleus, a complex system of compaction is employed. Compaction of DNA is facilitated by a group of proteins known as histones, and compacted DNA is referred to as chromatin, the most basic subunit of which is a nucleosome. Nucleosomes are formed when approximately 146bp of DNA is wrapped around an octamer of proteins made up of pairs of the core histones (H2A, H2B, H3 and H4). A fifth histone is also involved in packaging of chromatin; histone H1 acts as a stabilizer, interacting with linker DNA between nucleosomes, and helping to facilitate further coiling into a 30nm fiber [12]. Subsequent looping and compression ultimately result in the final condensed chromosome. An overview of this packaging is shown in Figure 1-2.

Packaging DNA in this manner adds a regulatory component by which gene expression can be controlled. Chromatin can be classified as being in one of two states: heterochromatin or euchromatin [1]. Heterochromatin is the term used to describe tightly packed DNA, which is relatively inaccessible to transcriptional machinery, and is therefore associated with gene silencing. In contrast, euchromatin constitutes more loosely packed DNA, which is generally more gene rich, and accessible to regulatory factors that can transcribe genes. Changes in DNA condensation, and by extension its accessibility, can be a dynamic process, allowing for the expression of specific genes in a temporally- or spatially-dependent manner.

Nucleosome structure adds a further complexity to gene regulation. When DNA is wrapped around the core octamer, the structure allows for the protrusion of the amino (N)-terminal tails of the core histones. These protruding tails are targets for a variety of

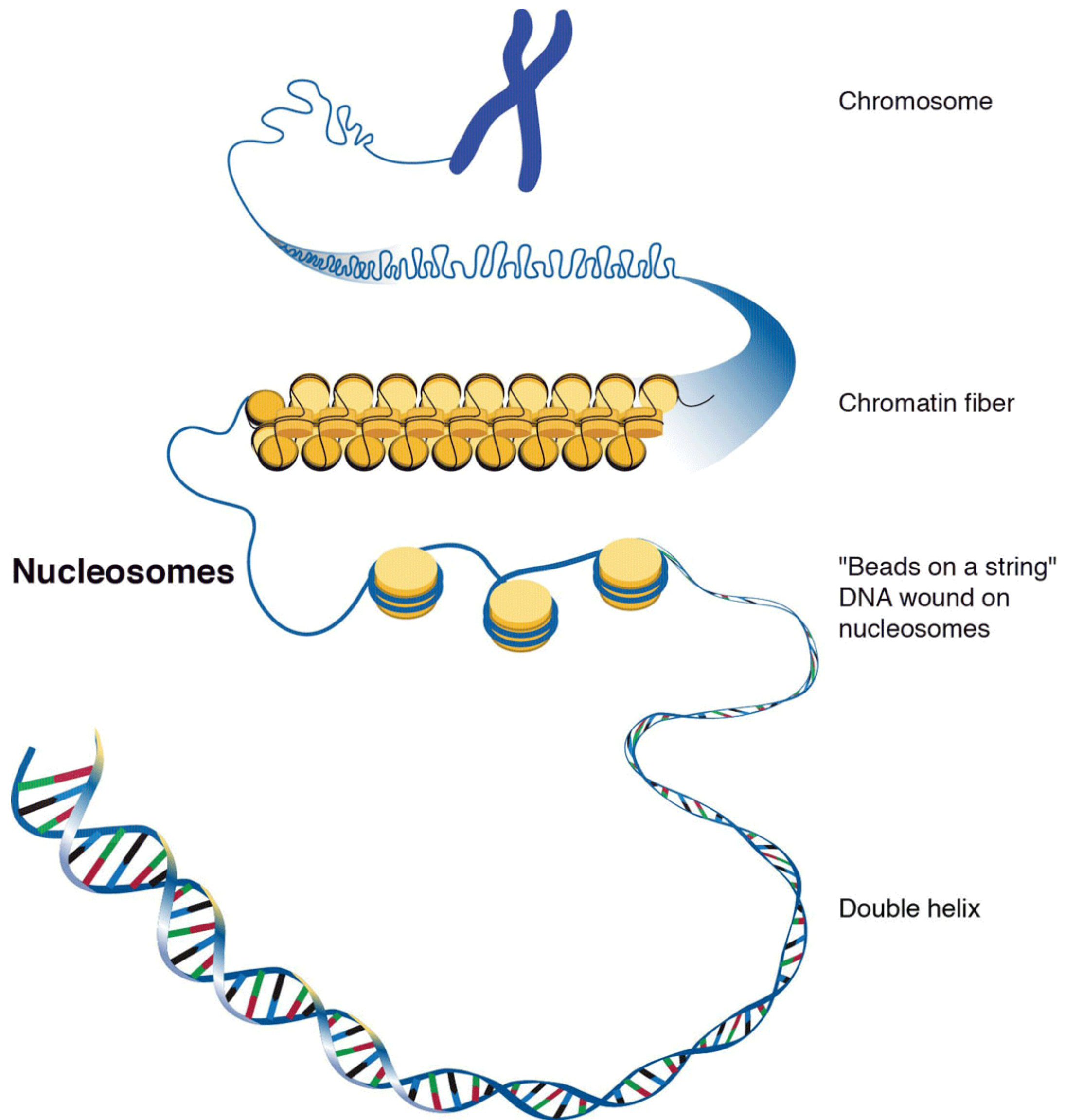

Figure 1-2 DNA packaging - levels of chromatin compaction

DNA is sequentially packaged in cells, such that the double helix is coiled into nucleosomes resembling “beads on a string”, and further packaged into higher order structures, ultimately resulting in a fully condensed chromosome. Heterochromatin refers to tightly packaged chromatin, repressive to transcription. Euchromatin refers to a more open chromatin state, more conducive to gene expression.
Credit: Darryl Leja/National Human Genome Research Institute

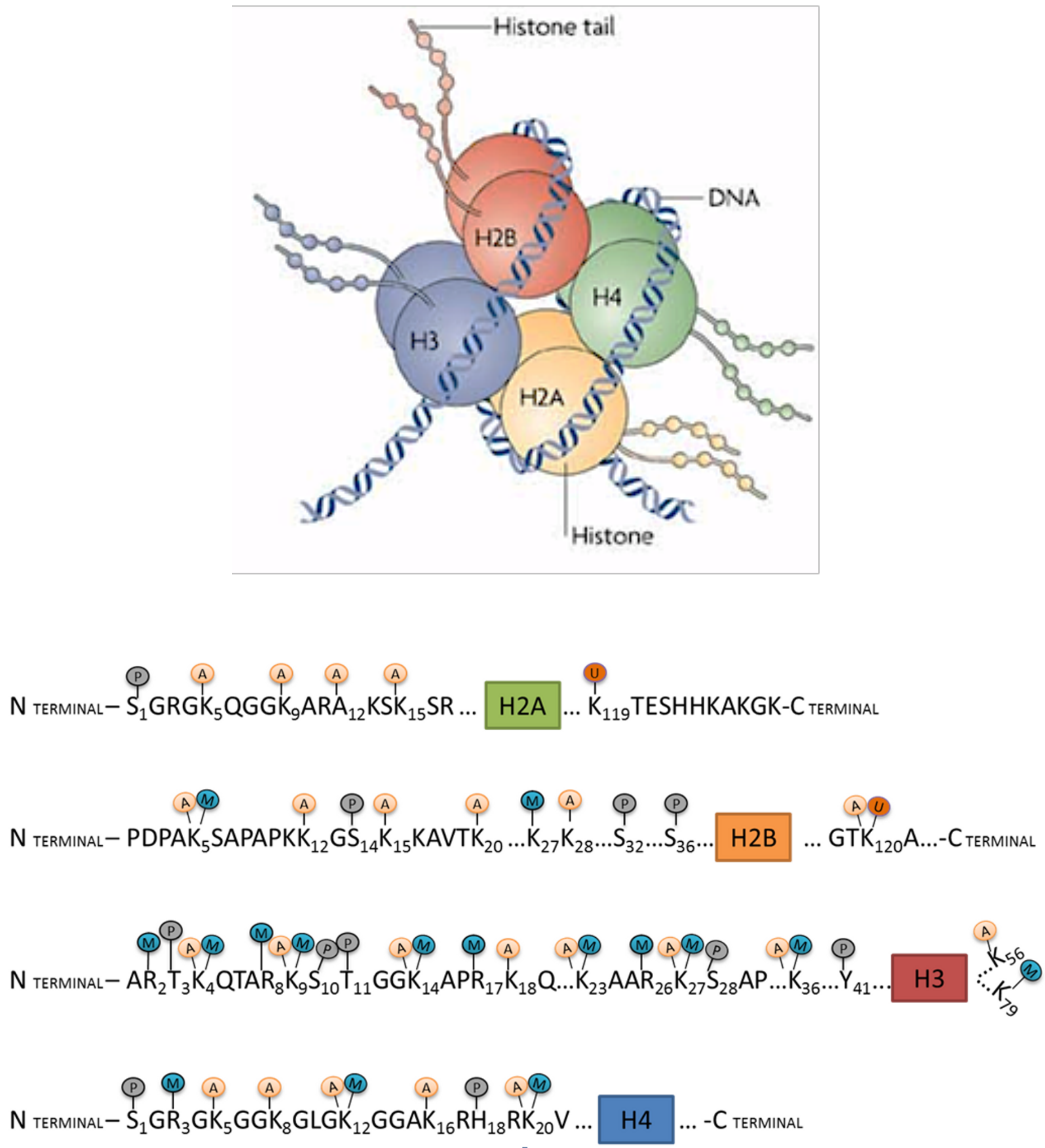

Figure 1-3 Nucleosome Structure & post-translational modifications of histone tails

Top: Representative image of nucleosome structure; comprised of an octamer of histone proteins around which DNA is wrapped. Evident are the N-terminal histone tails protruding outward from the nucleosome. Bottom: Overview of known covalent histone tail modifications. Figure reproduced from New York Academy of Science eBriefing 2011 and Xu 2013 [13].

post-translational modifications that can affect gene expression, either by altering the chromatin structure directly, or by bringing histone tails [and surrounding DNA] into close proximity with regulatory factors thereby facilitating their functional interaction (Figue 1-3).

1.3.1 The Language of Covalent Histone Modifications

Specific patterns of covalent histone modifications can be predictive of distinct biological outcomes; in particular, specific combinations of modifications can serve as a signal for transcriptional activation or repression. Allis and colleagues coined the phrase 'the histone code' to describe the observations that chromatin modifications could cooperate with, or antagonize, one another to trigger different functional responses [14,15]. Histone modifications such as acetylation, phosphorylation, methylation, ubiquitination, and/or sumoylation, can alter transcriptional activity by changing the compaction of the chromatin, thus altering the accessibility of transcriptional machinery to DNA. Alternately, modifications can act as recognition elements for other factors that will alter structure and/or regulate transcription [16,17]. Broadly, three classes of proteins can be associated with the histone code: 'writers', which deposit the modification(s), 'erasers' remove the modification(s), and 'readers' or 'effectors' that recognize the modified N-terminal residue(s) and mediate transcriptional consequences.

Acetylation is the best characterized among the histone post-translational modifications, with a connection between histone acetylation and transcription first documented in 1964 [18]. Generally, an increase in histone acetylation is predictive of

transcriptional activation. Acetylation of histones occurs on lysine residues, and the modification effectively neutralizes the positively charged residue, disrupting the ability of the histone to interact with negatively charged DNA [19-21]. Histone Acetyl Transferases (HATs) are the enzymes responsible for acetylation of histones [22,23], with Histone Deacetylases (HDACs) acting in the reverse capacity [24,25], to remove the acetyl group(s) and allow the chromatin to return to a more transcriptionally repressive heterochromatic state. Acetylated lysines can also act as an interaction surface recognized by specific modules found within regulatory proteins such as bromodomains [26,27], or tandem PHD (plant homeodomain) fingers [28,29]. Both of these domains recognize acetylated lysines with hydrophobic binding pockets, and facilitate the interaction by means of hydrogen bonding [29].

Histone phosphorylation occurs primarily on serine and threonine residues, and has been associated with both transcriptional activation and repression. In addition, phosphorylation has been implicated in DNA damage repair [30-32], and in chromosome condensation during mitosis and meiosis [33-35]. In a transcriptional context, the addition of the negatively charged phosphate group to the positively charged histone tail would be expected to cause decondensation of chromatin. Consistent with this expectation, phosphorylation of histone H3 at serines 10 and 28, and at threonine 11 has been associated with transcriptional activation, as well as with histone acetylation [36-38]. Crosstalk with other modifications is a key component when interpreting histone phosphorylation, with specific phosphate groups affecting the ability of nearby residues to become acetylated [36-38] and/or methylated [39,40].

Understanding the functional effects of methylation of lysine and arginine resides within histones has proved to be a difficult endeavor. Multiple methyl groups can be added to the same residue, complicating the interpretation of transcriptional consequences; lysines can be mono-, di-, and tri-methylated, while arginines can be mono-, and di-methylated (asymmetrically or symmetrically) [41,42]. Methylation of lysine and arginine residues has been implicated in both activation and repression of transcription [42]. Addition of methyl groups does not alter the charge of lysine or arginine residues, and therefore does not drastically affect electrostatic interaction of the positive histone tails with the negatively charged DNA. Instead, methylation of lysine or arginine has been shown to provide interaction surfaces for effector proteins, which can impact the chromatin compaction. Methylation of lysine groups is predominantly achieved by a group of enzymes containing a SET domain [43], using S-adenosyl methionine (SAM) as a methyl donor. The process is reversible, with two protein families implicated in demethylating lysine residues: Lysine-Specific Demethylases (LSD) that belong to the larger collection of amine oxidases [44], and the Jumonji-C (JmjC)-domain-containing family of demethylases [45]. The number of protein domains found to interact with methylated lysine continues to grow, and currently includes chromodomains, PHD fingers, Tudor domains, WD-40 domains, and MBT domains [17,46]. Some of these domains only recognize a specific histone lysine residue, and/or residues that have been modified in a particular way, while others are more promiscuous in their binding. For example, the Tudor domain of 53BP1 exclusively interacts with dimethylated lysine 4 or 9 on histone H3 (H3K4me2, H3K9me2) and dimethylated lysine

20 on histone H4 (H4K20me2), while the chromodomains of HP1 and CDY1 recognize mono-, di-, and tri-methylated lysine 9 on histone H3 (H3K9) [46].

The Protein Arginine Methyltransferase (PRMT) family is responsible for histone arginine methylation, and also uses SAM as a donor. In 2007, Chang *et al*. suggested that Jumonji domain-containing protein 6 (JMJD6) could demethylate histone H3 at arginine 2 (H3R2) and histone H4 arginine 3 (H4R3) [47]. However, this finding was not reproducible, and it could be argued that a 'true' arginine demethylase has yet to be identified. However, the modification is not irreversible; peptidylarginine deiminase (PADI) enzymes can convert methylated [and unmethylated] arginine residues to citrulline [48,49]. Readers of methylated arginine residues have also been discovered, with the PHD motif-containing ADD domain of DNMT3A shown to interact with a symmetrically dimethylated arginine residue on histone H4 (H4R3me2s) [50]. In addition, Tudor domains have been found to recognize asymmetrically dimethylated arginines. For example, Tudor domain-containing protein 3 (TDRD3) [51] has been shown to interact with the arginine methylated C-terminal domain of RNAPII, and this association is important for transcriptional activation by the estrogen receptor (ER) at some genes. Understanding of the dynamic regulation of histone lysine and arginine methylation and demethylation, the factors involved in the deposition and removal of the methyl groups, and the transcriptional and functional roles of these modifications, continues to develop.

Finally, ubiquitination or sumoylation can also occur on lysine residues. Ubiquitination of histones generally allows chromatin decondensation, access for transcriptional enzymes, and increased transcription [52]. Ubiquitination of histones

primarily occurs on histone H2A and H2B. Histone H2A ubiquitination has been implicated in transcriptional repression [53], while modification of histone H2B has been associated with activation and has been shown to promote other activating histone modifications in both yeast and mammalian systems [54-57]. Addition of small ubiquitin-related modifier (SUMO) groups to histones and transcription factors is related to gene silencing, as deposition of this modification has been shown to recruit transcriptional repressors [58,59].

Interpretation of the vast combinations of covalent histone modifications is an ongoing and highly active area of research, with the application of high throughput, genome-wide technologies such as ChIP-Seq greatly increasing our ability to identify patterns of histone modifications. The existence of multiple recognition motifs for individual marks, as in the case of lysine methylation in particular, attests to the complexity of the 'code'. Furthermore, the binding affinity of a reader for a specific modification can be enhanced or inhibited by adjacent marks, with many chromatin regulatory proteins containing more than one 'reader' domain [17]. Ultimately, while any single histone tail modification can suggest a particular chromatin state and transcriptional consequence, it is the combinatorial makeup of the modifications as a whole, and the cross-talk between them that ultimately regulated gene expression.

1.4 Transcription Factors

Transcription factor is a general term for any protein directly involved in regulating transcriptional initiation. These regulatory proteins typically recognize and

interact with DNA in a sequence-specific manner. DNA-protein interaction can be attributed to several known DNA-binding structural motifs. Transcription factors can be grouped based on the motif(s) they contain, the most common of which include helix-turn-helix, zinc finger, leucine zipper, and helix-loop-helix.

The first DNA binding domain to be identified was the helix-turn-helix [60]. This motif consists of two α helices connected by a short chain of amino acids that comprise the turn. The two helices are held at a fixed angle, allowing for the interaction of the C-terminal helix with the major groove of the DNA [1,61,62]. This interaction is sequence specific, and the variability in amino acid sequence both within the helix-turn-helix region, and in the overall protein allow for variability in binding specificity. Helix-turn-helix-containing transcription factors bind as symmetric dimers to symmetrically arranged half-sites within the DNA sequence [1,63].

DNA-binding motifs that include zinc ions as structural components are known as zinc fingers. A combination of cysteine and histidine residues within the amino acid sequence is used to coordinate the zinc ion(s). Zinc finger-containing proteins are numerous and are generally grouped based on their structure, with the first type of zinc finger protein discovered containing an α helix and two-stranded β sheet held together by zinc, coordinated by two cysteine and two histidine residues [1,62,64]. This type of zinc finger can occur in clusters, with repeating patterns of α helices/ β sheets evident. The α helix of each finger interacts with the major groove of the DNA, and the repeating pattern of zinc finger elements ensures a strong DNA-protein interaction [62]. The number of repeating 'fingers' can determine the specificity of this type of zinc finger-containing protein [1]. Another example of a zinc finger DNA binding domain involves two α

helices held together by two cysteine-coordinated zinc atoms, similar in structure to the helix-turn-helix motif previously described. Like helix-turn-helix containing proteins, this type of zinc finger protein typically functions as a dimer, with one of the α helices interacting with the major groove. Notably, this type of zinc finger domain is found within the DNA binding domains of nuclear hormone receptors [1,62]. Many other structural subclasses of zinc fingers exist [64], but their properties are beyond the scope of this thesis.

Leucine zippers constitute a third class of DNA binding domain. The leucine zipper motif is made up of an α helix, with adjacent regions for dimerization and DNA interaction [62]. Dimerization is facilitated by hydrophobic, luecine-rich regions within each monomer, which come together to form a coiled-coil. Beyond the dimerization region, the helices of each monomer are separated, with the resulting structure resembles a Y-shape, and allowing for interaction with the major groove of DNA [1,65]. Heterodimerization of DNA binding proteins, such as leucine zippers, can also occur, thereby expanding the number of possible DNA-binding specificities.

Related to the leucine zipper is the helix-turn-helix motif, which consists of a short α helix connected by a loop to a second, longer, α helix. The loop is quite flexible, allowing the helix-loop-helix protein to fold on itself, thereby facilitating both dimerization and DNA interaction [1,65]. Helix-loop-helix proteins can also form either homo- or hetero-dimers.

1.4.1 Nuclear Receptors

Nuclear receptors (NRs) are a class of proteins that are responsible for sensing and responding to the presence of a variety of hydrophobic ligands, including steroids, retinoic acid, thyroid hormones, and vitamin A and D derivatives [3]. Groups who used tritiated estradiol to isolate and characterize constituents that bind estrogen in the rat uterus [66,67] first suggested the existence of protein receptors for steroid hormones in the 1960s. Advancing technology, specifically recombinant DNA technology in the 70s and 80s allowed for specific receptors to be cloned and sequenced. In 1985, the glucocorticoid receptor became the first nuclear hormone receptor cloned [68], with estrogen receptor alpha (ERα) identified shortly thereafter [69]. The NR superfamily now consists of 48 known members in humans, and has documented roles in processes such as metabolism, differentiation, and development [74].

Collectively, the NR transcription factors share a common domain architecture, consisting of six regions [3,70-72]. The most amino (N)-terminal domains contain the ligand (hormone)-independent activation function 1 (AF-1). This region allows for some transcriptional activity from the NR in the absence of ligand. Next, is the DNA-binding domain (DBD); a region characterized by a pair of zinc fingers, which bind to specific palindromic recognition elements within the DNA, termed hormone response elements (HREs). A hinge region provides flexibility and has been found to play a role in nuclear localization, DNA binding, and coactivator recruitment. The ligand-binding domain (LBD) contains the ligand-dependent activation function 2 (AF-2) and consists of 12 α-helices arranged in three layers, forming an antiparallel “α-helix sandwich”. This region

contains interaction surfaces for coactivator and corepressor proteins. Lastly, the carboxy (C)-terminal domain, which is highly variable, facilitates receptor dimerization.

The NR superfamily can be categorized into several subgroups, including steroid hormone receptors, retinoid X receptor (RXR) heterodimers, as well as two classes of orphan receptors [for which there are no known ligands], which function as either heterodimers with other NRs, or as monomeric receptors, binding HRE half-sites [70,73]. Steroid receptors typically reside in the cytoplasm, and translocate to the nucleus upon ligand binding; whereas, RXR heterodimers are usually retained in the nucleus regardless of ligand binding status [74].

In the canonical mechanism of NR action, the ligand binds its cognate receptor in the cytoplasm or nucleus of the cell, depending on the receptor type, causing a change in receptor conformation that facilitates dimerization. Ligand-bound receptors then translocate to the nucleus if necessary and interact via the zinc fingers of the DBD with specific DNA sequences known as hormone response elements (HREs). HREs were originally thought to be found within the 5’ proximal regulatory region of genes, but are now known to also be found at distal enhancers [71,75]. A pair of palindromic six nucleotide sequences separated by three nucleotides makes up the typical HRE, for example ERα recognizes the sequence AGGTCAnnnTGACCT [76]. Once bound to the HRE, subsequent recruitment of coactivators leads to the decondensation of chromatin through active remodeling and histone modification. Receptor-coactivator complexes recruit general transcription factors and RNAPII, and maintain an open chromatin conformation to allow for transcription initiation and elongation. It is important to note that NR recruitment can also lead to transcriptional repression; both by actively

repressing expression in the absence of ligand through interaction with corepressor proteins [71,77], and/or by antagonistically inhibiting the activity of other transcription factors [71,78].

The classical view of NR action, based primarily on biochemical studies at selected genes, does not provide a complete understanding of their functionality. Evolving technology, specifically chromatin immunoprecipitation coupled to genomic microarray (ChIP-chip) or high throughput sequencing (ChIP-Seq), has allowed for the evaluation of NR recruitment genome wide. The first NR to be mapped in this manner was ERα. Surprisingly, this analysis revealed a predominance of ERα binding occurring at regions far distal from the TSS of genes [79]. Subsequent studies have confirmed that the majority of ERα binding occurs more than 10 kb from the nearest TSS [80,81]; an observation that has been extended to other NRs, including glucocorticoid receptor (GR), peroxisome proliferator-activated receptor gamma (PPARγ), and vitamin D receptor (VDR) [82-84]. The requirement for sequence specific binding motifs (HREs) has also been called into question as NRs have been found to interact with regions that do not contain HREs [79,81], and analysis of NR cistromes has shown that NRs can bind at sites containing HRE half-sites as well as other noncanonical recognition sequences [85].

1.4.2 The Estrogen Receptor

Estrogens are a family of steroid hormones, which are involved in regulating numerous processes ranging from development, to sexual maturation and behavior. On a cellular level, estrogens, most notably E2, are essential mediators of regulatory pathways

responsible for the normal proliferation and differentiation of cells [86,87]. E2 has been epidemiologically and clinically linked to breast cancer incidence, and unsurprisingly, the deregulation of its downstream signaling cascades is a common feature of carcinogenesis [88].

The cellular effects of E2 are mediated through the estrogen receptor (ERα), which is primarily expressed in breast, uterus, ovary, testes, epididymis, bones, and brain [72]. In the classical mechanism of estrogen signalling, as in the canonical NR response previously described, ligand (E2) binding triggers receptor dimerization and interaction with estrogen response elements (EREs), specific palindromic DNA sequences within the regulatory regions of target genes [76,89-91]. ER activation can also promote gene expression in the absence of direct DNA binding, through interactions with other DNA-bound transcription factors such as activator protein-1 (AP-1) and specificity protein-1 (Sp1) [92-94]. Additionally, a Forkhead binding motif has been found to be enriched adjacent to a percentage of ER binding sites [79], suggesting that the DNA-bound ER cooperates with Forkhead transcription factors on many targets.

ERα activity is regulated at many levels [72]; the most relevant of which for the purposes of this thesis is through its E2-dependent interaction with coregulator proteins. Importantly, E2 binding to the ER induces a conformational change that repositions the C-terminus and creates an adaptor surface that facilitates the recruitment of coregulator proteins. Coregulators [both coactivators and corepressors] that bind the ER can alter the chromatin architecture directly [95], function as adaptors/bridging factors which recruit additional coregulator proteins to target genes [96,97], or interact directly with components of the core transcriptional machinery [98,99].

1.5 Transcriptional Coregulators

Transcriptional coregulators are protein factors that can interact with transcription factors and modulate their activity. Many of these proteins contain a variety of enzymatic activities and can exert functional control over transcription [100,101]. In addition, coregulators can act as bridging proteins, interacting with transcription factors and allowing for the assembly of large, multicomponent enzymatic complexes [102]. Coregulators generally fall either into the categories of coactivators, which enhance gene expression, or corepressors, which are suppressive. Coactivators can be further classified as primary coactivators, which come into direct contact with the transcription factors, and secondary coactivators, which participate in the coregulator complexes but do not directly bind TFs [102,103]. To date, almost 350 coregulators have been identified based on these criteria (www.NURSA.org). Collectively, these proteins play a critical role in cellular processes, and provide a level of diverse, dynamic, and adjustably-responsive control specifically for transcription.

1.5.1 Steroid Receptor Coactivators

As discussed in section 1.4.1, NRs are ligand-dependent transcription factors that regulate gene expression and affect various physiological functions. It was initially believed that NRs alone allowed RNAPII and general transcription factors to assemble at promoters and control transcription. However, when it was shown that overexpression of one NR could indirectly inhibit the activity of another, and that *in vitro* assays involving

only NRs and general transcriptional machinery produced at best modest transcriptional responses, it was suggested that NRs may share a common set of coregulatory effector proteins required for full transcriptional response. [104-106]

The first such coregulatory protein, steroid receptor coactivator 1 (SRC1/ NCOA1), was identified by Onate et al. in 1995 [107]. SRC1 was found to interact with NRs in a ligand-dependent manner, robustly activating transcription. Two other homologous proteins were cloned soon after, completing the p160 SRC family: SRC2 (also known as NCOA2, TIF2, GRIP1) [108,109], and SRC3 (also known as p/CIP, RAC3, AIB1, ACTR, TRAM1, NCOA3) [110-114].

The steroid receptor coactivator (SRC) proteins are each approximately 160kDa in size, share 50-55% sequence similarity [115], and have three conserved structural domains (Figure 1-4). The N-terminus contains a basic helix-loop-helix-Per/ARNT/Sim (bHLH-PAS) domain. This region is the most highly conserved among family members and is required for protein-protein interactions important for transcriptional activation, including direct interaction with transcription factors such as myogenin and myocyte-specific enhancer factor 2C (MEF2C), and with enhancer proteins [116,117]. Evidence also suggests that in the case of some family members, this domain can be implicated in nuclear localization [118,119] and in proteasome-dependent turnover [119]. The central nuclear receptor interaction domain (NRID) contains three LXXLL motifs that form amphipathic α-helices, and are responsible for direct contact with NRs. Sequences adjacent to this domain contribute to the specificity of NR binding [120]. Finally, the C-terminus contains two activation domains (AD1 and AD2), which allow for SRCs to interact with secondary coregulators. AD1 interacts with CBP (CREB-binding protein)

and the histone acetyltransferase (HAT) p300. AD2 interacts with the arginine methyltransferases coactivator-associated arginine methyltransferase 1 (CARM1) and protein arginine methyltransferase 1 (PRMT1). The C-terminal region of SRC1 and p/CIP contain minimal intrinsic HAT activity. However, specific substrates have not been well characterized, and this activity is of much lesser importance than the bridging role of SRCs in assembling complexes of transcriptionally active components.

The series of events involved in NR- and coactivator-mediated transcriptional activation has been extensively characterized. In 2003, Métivier *et al*. used a comprehensive chromatin immunoprecipitation (ChIP)-based approach to provide evidence of the ordered, cyclical, and combinatorial recruitment of cofactors in response to E2 in MCF-7 cells [97]. This study demonstrated that at least 30 different proteins become engaged on the pS2 promoter in the presence of E2, and elucidated the kinetics of cofactor recruitment over time; revealing three cycles of protein interaction. SRC1 or p/CIP was found to associate with pS2 in the two transcriptionally productive cycles post E2 treatment [97]. Specifically, SRC1 and/or p/CIP were found to cycle on an off the promoter every 50 min after E2 treatment, initially found to interact 45 min after treatment [97]. Recruitment of these SRC proteins corresponds with the presence of ERα, and in fact, involves their interaction with ERα [97]. There are limitations with this study, including the fact that it focuses only on a single E2-responsive promoter, and that cycling rate in a population of cells is not necessarily reflective of physiological conditions. Nevertheless, it is clear that NR signaling in response to E2 is dynamic and includes a level of specificity and redundancy among coregulators, which ultimately work together to achieve the required transctiptional outcome. Once bound to the NR,

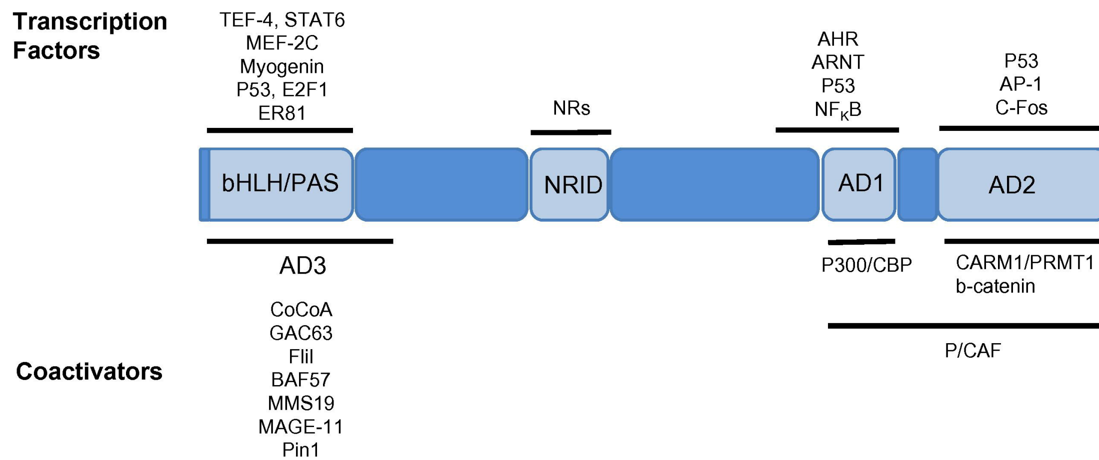

Figure 1-4 SRC structural domains and interacting proteins

SRC proteins consist of three structural domains. The N-terminal basic helix-loop-helix-Per/ARNT/Sim (bHLH-PAS) domain, the central nuclear receptor interaction domain (NRID), and the C-terminal domain, which consists of two activation domains (AD1 and AD2). SRCs coactivate nuclear receptors (NRs), as well as numerous transcription factors, and coordinate the recruitment of a variety of secondary coregulatory proteins. This is a representation of some of the SRC-interacting proteins, mapped to the region with which they make contact. Figure is reproduced from Johnson et al 2011 [121].

SRC family members act as scaffolding proteins and recruit many secondary coregulators, including the acetyltransferase p300 and methyltransferase CARM1 [122,123]. CBP/p300 and CARM1 can then both modify the histones, promoting transcriptional activation (Figure 1-5) [124].

SRC functional effects are ubiquitous, primarily due to the ability of the family members to interact with and modulate the activities of a wide range of transcription factors, in addition to NRs (Figure 1-4), such as NFκB [125], SMADs [126], E2F1 [127], STATs [128], RB [129], and p53 [130]. Extending the number of cellular process that are impacted by SRCs, interactions have also been shown with a variety of oncogenes, tumor suppressors, kinases and phosphatases, ubiquitin and SUMO (small ubiquitin-related modifier) ligases, as well as with other coregulators.

1.5.2 Post-translational Modifications of SRCs

Numerous studies have demonstrated that SRCs undergo post-translational modifications (PTMs) in response to numerous stimuli. These PTMs include phosphorylation, ubiquitination, sumoylation, acetylation, and methylation. These modifications can affect protein stability, interaction with TFs and secondary coregulators, in addition to transcriptional activity of SRCs (Figure 1-6).

Phosphorylation of SRCs can change their affinity for specific NRs and/or affect NR-dependent gene expression. SRC1 can be phosphorylated at Thr1179 and/or Ser1185 in response to epidermal growth factor (EGF) or interleukin 6 (IL-6). This results in enhanced association with p300 and CBP and increases NR-dependent

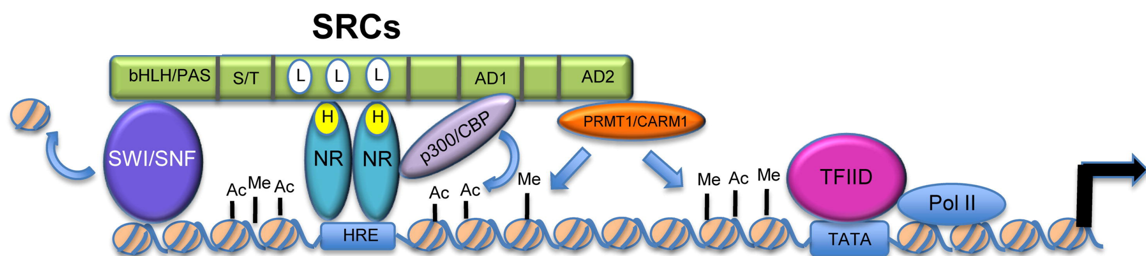

Figure 1-5 SRC-mediated coactivation of NRs

SRC proteins are recruited to liganded NRs, interacting through their LXXLL motifs in the central nuclear receptor interacting domain (NRID). Secondary coregulatory proteins and chromatin remodeling complexes such as SWI/SNF are then recruited through their- basic helix-loop-helix-Per/ARNT/Sim (bHLH-PAS) or C-terminal AD1 and AD2 domains, respectively. As shown, p300/CBP interacts with SRCs via the AD1 domain, while CARM1 contacts AD2. These secondary coactivators modify the chromatin and bridge the NR complex with the general transcription machinery to elicit transcriptional activation. Figure reproduced from Johnson et al 2011 [121].

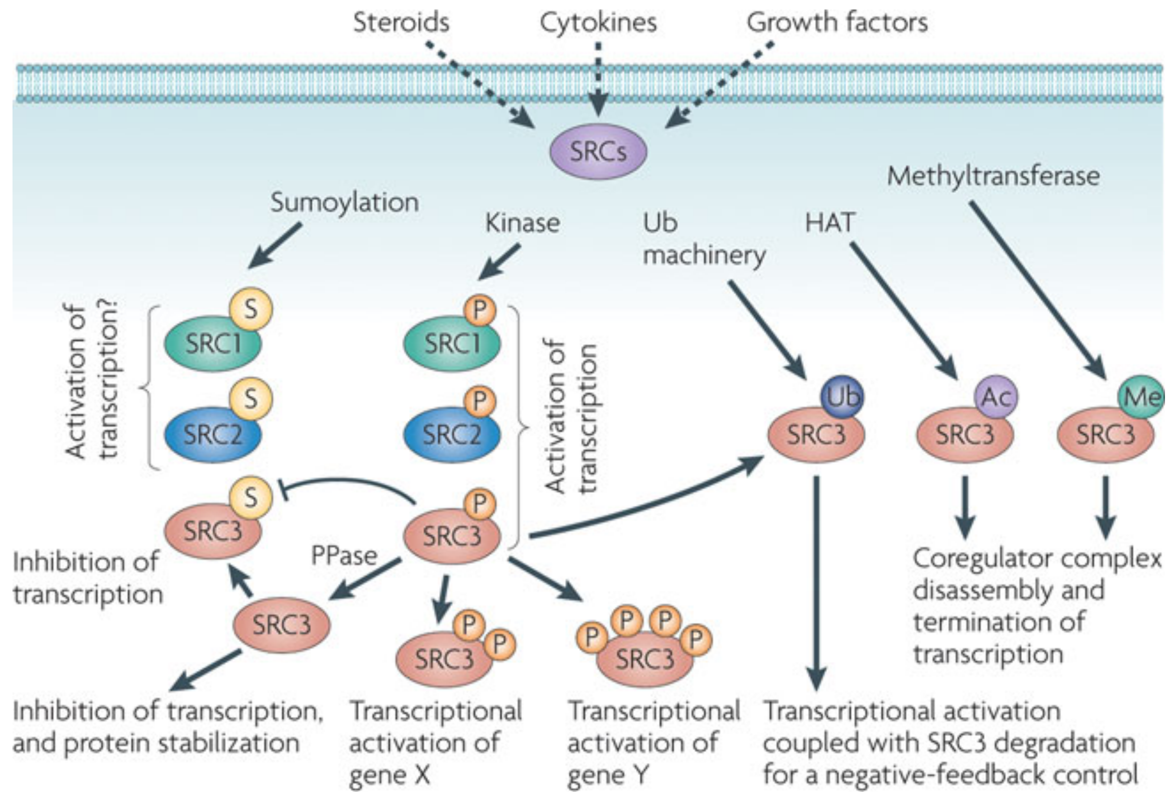

Figure 1-6 Functional consequences of SRC post-translational modifications

In response to a variety of signals SRC proteins can be post-translationally modified, thereby affecting intracellular concentration, activity, and specificity. Documented modifications include phosphorylation, ubiquitination, sumoylation, acetyaltion, and methylation. Figure reproduced from Xu et al 2009 [141].

transcription, [131,132]. p/CIP (SRC3) contains nine sites (Thr24, Ser101, Ser 102, Ser505, Ser543, Ser857, Ser860, Ser867, Tyr1357) that can be phosphorylated by a variety of different kinases, including but not limited to MAPK, IKK, and AKT [133-136]. The ability to accept signals from multiple pathways, and the number of different kinases that can contribute to regulation p/CIP highlight the central nature of p/CIP, and other SRCs as integrators of many different signalling pathways. As an example of the functional consequences of p/CIP, phosphorylation of Tyr1357 increases its binding to p300 and transcription factors, thereby promoting ERα-, PR-, and NFκB-dependent transcription [136]. In addition, p/CIP phosphorylation can lead to its redistribution to either the nucleus or to the cytoplasm, altering its availability to regulate different transcriptional effects [118,137,138].

Two sites of ubiquitination have been identified within the NRID of p/CIP [139]. Polyubiquitination at these sites occurs following phosphorylation events, linking transcriptional activation with p/CIP degradation and transcriptional turnover [139,140]. Sumoylation, in contrast, can protect SRCs against degradation, by targeting common lysine residues and preventing ubiquitination. Sumoylation can also cause SRCs to adopt a transcriptionally inert conformation [141,142].

Acetylation and methylation of SRCs can also occur, affecting their affinity for interacting proteins. For example, p/CIP can be acetylated by p300 and CBP, which results in disassembly of the coactivator complex and terminates transcription [123]. Methylation of p/CIP by CARM1 also causes complex dissociation [143,144], and leads to p/CIP degradation [144].

Collectively, post-translational modification(s) of SRC proteins occur as the result of many different signalling pathways, and combine to fine-tune the coactivator potency, cellular concentration, and selectivity of the SRCs, ultimately allowing for precise regulation of gene expression programs [145].

1.5.3 p/CIP

p/CIP (p300/CBP interacting protein) was originally identified in a region of chromosome 20 (20q13) that is frequently amplified in breast cancer, and was consequently named amplified in breast cancer-1 (AIB1) [110]. This member of the p160 family of steroid receptor coactivators was independently cloned by several other groups and assigned a different name by each: steroid receptor coactivator-3 (SRC3) [146], activator of thyroid hormone and retinoid receptor (ACTR) [111], receptor associated co-activator-3 (RAC-3) [112], thyroid hormone receptor activating molecule (TRAM) [113], and p300/CBP interacting protein (p/CIP) [114]. p/CIP is generally the name given to the mouse homolog of SRC3, however we use this name interchangeably when referring to either the mouse or human protein. These assigned names are quite revealing of function, as p/CIP has been shown to interact with and enhance the activity of several nuclear receptors, including ERα, retinoic acid receptor (RAR), PR, and thyroid hormone receptor (TR) [95,112-114]. In addition, p/CIP interacts with a variety of coregulator proteins, including CBP and CARM1 [123,146].

p/CIP expression is detectable in a multitude of tissue types, including muscle, heart, lung, placenta, pancreas, kidney, brain, liver, uterus, pituitary, testis, and mammary

gland [111,113,126,146,147]. Mouse models in which p/CIP is knocked out exhibit growth retardation and a reduced adult body size, which appears to be in part due to reduced IGF-1 levels [147,148]. While male reproductive function is slightly reduced, there are significant defects in female reproduction. In p/CIP null female mice, sexual maturation is delayed, mammary gland ductal growth is reduced, and reproductive potential is diminished [147]. Estrogen levels were lower in p/CIP knockout mice when compared to age-matched wild type littermates, corresponding to the observed reproductive phenotype [147].

Additive severity of SRC knockouts was observed when double-knockout mouse models were generated, suggesting that there exist cooperative functions among family members. When SRC1 and p/CIP are simultaneously knocked out, most mice die before birth [149]. Those who survive exhibit defects in metabolism; specifically, compromised regulation of genes involved in adipogenesis and mitochondrial uncoupling. Leptin levels are increased in these mice, and a defect in adaptive thermogenesis coupled with developmental arrest in intercapsular brown fat means they are resistant to obesity due to a high basal metabolic rate.

1.5.4 p/CIP in Breast Cancer

Each of the SRC family members has been found to be overexpressed in human cancers [145], with roles in promoting cancer initiation, progression, and metastasis through a variety of pathways. p/CIP is the most frequently amplified SRC in cancer, with a notable association in hormone-promoted breast and prostate cancers [145]. While

in normal breast tissue, p/CIP levels are typically low, in breast cancer, p/CIP mRNA is overexpressed in 13-64% [110,150-152] of different tumor cohorts, and p/CIP protein is overproduced in 10-25% [153-155]. Overexpression of p/CIP correlates with increased tumor size and grade, tamoxifen resistance, and poor disease-free survival [110,150,154,155].

Several mouse models have been used to elucidate the exact role(s) of p/CIP in breast cancer initiation and progression. MMTV-driven overexpression of p/CIP in the mouse resulted in the formation of spontaneous mammary tumors, thereby solidifying the role of p/CIP as an oncogene [156]. However, tumors were also observed in the uterus and pituitary in this model, the result of nonspecific MMTV-dependent expression. In addition, corresponding to observations from p/CIP knockout mouse models, p/CIP-overexpressing mice have altered [hyperactive] IGF-1 signalling [156]. In another model, p/CIP null mice harbouring the MMTV-*v-ras* transgene breast tumour incidence was decreased [157]. This mouse model also displayed altered IGF-1 signaling; lack of p/CIP resulted in partial resistance to IGF-1, partly causing the suppression of mammary tumorigenesis. In the HER2/*neu*-induced mammary tumorigenesis model, p/CIP knockout also offered some protection from tumor development; with tumor formation delayed in $HER2^{+/+}$;p/CIP $^{+/-}$ mice and completely suppressed in $HER2^{+/+}$;$p/CIP^{-/-}$ mice [158]. Furthermore, compared with MMTV-HER2 control mice, MMTV-HER2 mice heterozygous for p/CIP showed decreased phosphorylation of HER2, cyclinD1, and cyclin E, and reduced activity of AKT, JUN N-terminal kinase and had a reduced rate of proliferation [158]. Genetic ablation of p/CIP in MMTV-PyMT mice protected against lung metastasis, as compared to WT/PyMT mice [159]. The role of p/CIP in breast cancer

metastasis is attributed to its regulation of matrix metalloproteinases MMP2 and MMP9, and their ability to mediate epithelial-mesenchymal transition (EMT) and cellular invasiveness in this model system [159].

ER status can be used as an indicator to assess prognosis and determine treatment strategies in breast cancer [160]. Since p/CIP acts as a coregulator for ERα, a great deal of research has focused on the ability of p/CIP to mediate ERα-dependent gene expression in the context of breast cancer. In the MCF-7 breast cancer cell line, p/CIP depletion results in reduced recruitment of ERα to its target gene promoters, corresponding with loss of transcription of these genes [161]. Furthermore, the absence of p/CIP corresponds to decreased E2-mediated proliferation, survival, and soft-agar colony formation [123]. Despite these observations, the literature remains quite conflicted as to the significance of p/CIP in ER-dependent oncogenesis due to contradicting clinical data correlating p/CIP expression with ER status [150,162].

In addition to its involvement in E2-responsive breast cencer, the role of p/CIP in hormone-independent breast cancer has also been characterized. ER-negative breast cancers are more aggressive and unresponsive to anti-estrogens [163]. p/CIP promotes cellular growth of ER-negative cell lines, by coactivating E2F1-dependent transcription of genes including E2F1, cyclin E, and cyclin-dependent kinase 2 (CDK-2) [127]. Growth and survival of breast cancer cells can also be modulated by p/CIP through its interaction with AP-1 [164], and its coactivating role in NFkB-dependent transcription has been linked with the expression of inflammation, immune response, and cell survival [165]. In addition there is evidence of a link between p/CIP overexpression and activation of the insulin-like growth factor (IGF)/PI3K/Akt signalling [156,157]. This pathway is

related to cell growth, survival, and migration and is often found constitutively activated in cancer [166].

There is extensive evidence linking p/CIP with breast cancer, and as discussed it participates in the regulation of a wide variety of pathways and processes important for oncogenesis. p/CIP expression is generally an indicator of poor prognosis, and can affect responsiveness to treatments(s) [145,155]. Continued characterization of p/CIP-regulated genes will provide greater insight into the molecular mechanisms it uses to promote cancer.

1.6 Protein Arginine Methylation

Arginine methylation is a post-translational modification found on both nuclear and cytoplasmic proteins, which has been implicated in many different cellular processes, including, but not limited to, transcriptional regulation, signal transduction, RNA processing, and DNA repair. Defects in arginine methylation are known to be involved in several disease processes, including cardiovascular disease, spinal muscular atrophy, and cancer [167,168].

Early reports assert that approximately 2% of arginine residues in rat liver nuclei are methylated, suggesting that this is a commonly occurring post-translational modification [169]. Arginine methylation involves the covalent addition of one or more methyl groups to the guanidino nitrogen of an arginine residue, adding bulkiness but not affecting charge. There exist three known forms of methylated arginine: monomethylated arginine (MMA), asymmetrically dimethylated arginine (ADMA), and symmetrically

dimethylated arginine (SDMA) [170]. The generation of these modifications can be attributed to a family of proteins collectively known as Protein Arginine Methyltransferases (PRMTs).

1.6.1 The Protein Arginine Methyltransferase Family

To date, there have been 11 members of the protein arginine methyltransferase (PRMT) family identified [171]. This protein family shares four conserved motifs that constitute the catalytic methyltransferase domain, with additional domains conferring substrate specificity (Figure 1-7). Members of the PRMT family can be subcategorized based on their structure, or more commonly, on their specific methyltransferase activity. Using S-adenosyl-L-methionine as a donor molecule, Type I methyltransferases form ADMA, while Type II form SDMA, on the guanidino nitrogen(s) of arginine residues, both through a monomethylated intermediate (Figure 1-8) [172]. A third type of PRMT (Type III) has more recently been suggested to only generate MMA. Type I is the most common class of methyltransferases, and includes PRMT1, PRMT2, PRMT3, PRMT4 (CARM1), PRMT6, and PRMT8. In comparison, only two family members are consistently categorized as Type II (PRMT5 and PRMT9). PRMT7 has been shown to act either as Type II or Type III, in a substrate-dependent manner. The final two members of the family (PRMT10 and PRMT11) have yet to be enzymatically classified [171].

Most PRMTs methylate glycine- and arginine-rich patches (GAR motifs) within their substrates [173], although specific recognition sites for each of the family members are not well characterized. PRMT4, more commonly known as CARM1, is unusual

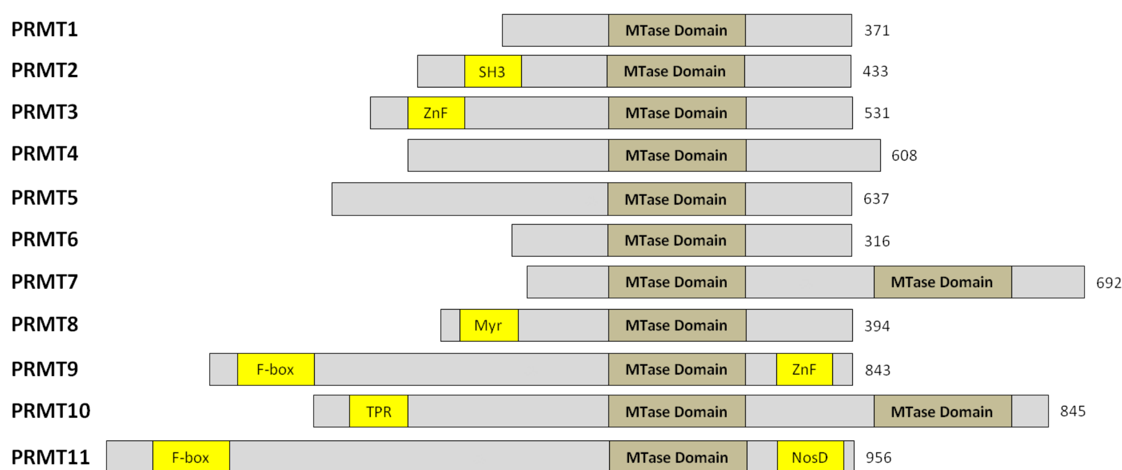

Figure 1-7 The Protein Arginine Methyltransferase Family

Schematic representation of the 11 members of the protein arginine methyltransferase (PRMT) family. Each member of the family contain at least one conserved methyltransferase domain with signature motifs I, post-I, II, and III and a THW loop. Unique domains are thought to contribute to substrate specificity, and are shown in yellow. CARM1 is identified herein as PRMT4. Figure reproduced from Esse 2012 [167].

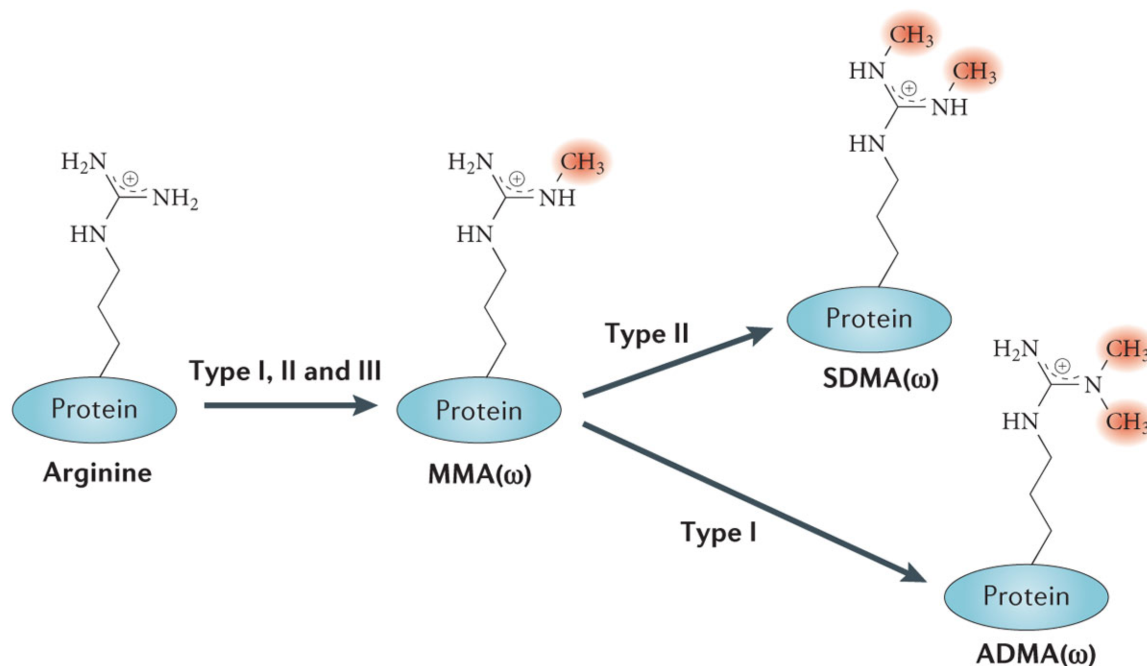

Figure 1-8 Types of methylation on arginine residues

Type I, II, and III PRMTs generate monomethylated arginine (MMA) on one of the guanidino nitrogen atoms. Subsequent generation of asymmetrically dimethylated arginine (ADMA) is catalyzed by Type I PRMTs (PRMT1, PRMT2, PRMT3, PRMT4/CARM1, PRMT6, PRMT8), while Type II PRMTs (PRMT5, PRMT9) produce symmetrically dimethylated arginine residues (SDMA). PRMT7 can act as either a Type II or III PRMT, and PRMTs 10 and 11 have not yet been enzymatically classified. Figure reproduced from Yang et al 2013 [166].

among PRMTs since its substrates do not contain this GAR motif. CARM1 has been found instead to methylate proline-, glycine-, methionine-rich (PGM) regions [174].

PRMTs are ubiquitously expressed, and affect a variety of important cellular processes, including RNA processing, DNA repair, signal transduction, and transcriptional regulation [170,171]. Of particular interest for the purposes of this thesis is the role of PRMTs in transcription. Protein arginine methylation was initially detected on histones [169], and occurs on the N-terminal tails of histone H3, H4, and H2A [2,171,175]. Notably, there are several arginine residues within histone H3 that become methylated (H3R2, H3R8, H3R17 and H3R26) [42,171]. Asymmetric dimethylation of histone H3 generally correlates with activation of transcription [171,176].

Methylation of arginine residues also affects a wide array of non-histone transcriptional regulatory proteins, including transcription factors, coregulators, elongation factors, and RNAPII. Participation of PRMTs as components of coregulatory complexes are well known to modulate the activity of NRs [98,177], as well as other TFs [178]. In addition, arginine methylation of coregulatory proteins such as p/CIP and CBP can affect their ability to form complexes, and their stability [143,144]. The PRMT family members PRMT1 and PRMT5 have been shown to methylate the transcriptional elongation factor SPT5 in its RNAPII-binding domain, resulting in transcriptional pausing, wherein the engaged RNAPII accumulates just downstream of the promoter region [179]. Furthermore, several components of the transcriptional elongation-associated Paf1c complex has been shown to interact with H3R17me2, thereby linking an additional PRMT, CARM1, with elongation [180]. Finally, the C-terminal domain of RNAPII can be methylated by CARM1, and contributes to transcriptional activation, in

part by creating a docking site for the arginine methylation effector proteins such as TDRD3 [181].

1.6.2 CARM1

Coactivator Arginine Methyltransferase 1 (CARM1), also known as PRMT4, is a Type I PRMT originally identified based on its ability to interact with GRIP-1 (SRC-2), a member of the p160 family of nuclear receptor coactivators [123]. CARM1 has since been shown to interact with the two other members of the p160 family, SRC1 and SRC3 (more commonly referred to in this thesis as p/CIP). CARM1 was the first of the PRMTs to be shown to regulate transcription [123]. Its role as a secondary coregulator of transcription, synergistically enhancing gene expression of steroid-hormone-regulated genes has been extensively studied [117].

CARM1 methylates substrates that can be broadly classified as possessing RNA-binding properties (PABP1, HuR, HuD, and splicing factors) [182-184], and substrates that are involved in transcriptional regulation, including several residues of histone H3 (H3R17, H3R26) [185], p/CIP [143,144], and CBP/p300 [186,187]. Notably, the methyltransferase activity of CARM1 is essential for its transcriptional effects [177].

CARM1 deletion in the mouse model revealed embryos that were small in size, and die late in development or shortly after birth [188]. An elevated rate of lethality was also noted for heterozygotes between birth and weaning. CARM1 knockout mice were found to have insufficient lung development, blocked T-cell development in the thymus, and altered lipid metabolism. CARM1 null mouse embryonic fibroblasts (MEFs), isolated

from E12.5 embryos, do not support the methylation of CARM1 substrates, including histone H3, p300 and PABP1. Furthermore, CARM1$^{-/-}$ MEFs were found to be defective in E2-dependent signalling and in NFkB signalling pathways [188].

1.6.3 CARM1 in Cancer

CARM1 is overexpressed in breast, prostate, and colorectal cancer [189-191]. Recently, several large-scale clinical studies have confirmed that CARM1 levels are elevated in aggressive breast tumors [192,193]. Furthermore, the expression of CARM1 in breast cancer is a predictor of diminished survival, and of poor disease-free survival [192,193]. Notably, CARM1 expression plays a significant role in ERα-induced proliferation and differentiation of the MCF-7 breast cancer cell line [194]. In fact, ERα-regulated gene expression has been shown to rely on the presence of both p/CIP and CARM1 [195]. Since, p/CIP itself has minimal intrinsic enzymatic activity, and primarily acts to recruit additional coregulators, it follows that CARM1 is likely an important mediator of the oncogenic effects attributed to these ERα-dependent genes.

CARM1-dependent methylation has been implicated in many additional pathways and processes commonly deregulated in cancer [170], including the DNA damage response [196], regulation of the cell cycle [195], and WNT signalling [197]. In addition, CARM1 is involved in promoting a favorable microenvironment for tumor growth and metastasis [198,199]. Collectively, accumulating evidence alludes to the importance of CARM1 and regulation of protein arginine methylation in oncogenesis.

1.7 Thesis Overview

The work presented within this thesis aims at extending our current understanding of the role that the arginine methyltransferase CARM1 plays in global transcriptional regulation. To this end we took a top-down approach in two quite different model systems, using large-scale ChIP-on-chip and ChIP-Seq approaches in conjunction with expression microarray analysis to initially identify global effects. We subsequently refined our observations to specific critical pathways and/or target genes regulated by CARM1.

Chapter 2 details our assessment of CARM1 as part of a coregulatory complex with the steroid receptor coactivator p/CIP in an estrogen-dependent transcriptional context. In this study, we show, using a ChIP-on-chip approach, that in response to stimulation with E2, a p/CIP/CARM1 complex is recruited to a subset of responsive promoters in MCF-7 breast cancer cells. Following extensive bioinformatic characterization, we ultimately focused on Jak2, a member of the JAK/STAT signalling pathway, and one of the direct E2-dependent targets of p/CIP/CARM1. E2-dependent histone modifications at the Jak2 promoter reflected recruitment of a functional p/CIP/CARM1 complex, and were generally transcriptionally permissive. Modest increases in Jak2 expression were observed, leading us to theorize that an additional factor(s) may be required for a more substantial transcriptional response. Notably, however, E2-induced expression of Jak2 was diminished when p/CIP or CARM1 were depleted, suggesting that the p/CIP/CARM1 complex is required for the observed transcriptional response. Collectively, the results of this study led us to suggest that E2-dependent recruitment of the p/CIP/CARM1 complex causes JAK2 to become 'poised'

for transcription, a finding that may be extendable to other target genes and signalling pathways.

The work we present in Chapter 3 takes a novel approach to characterizing CARM1-dependent transcriptional regulation. Previous assessment of the role of CARM1 in gene expression has been primarily focused on its ability to interact with, methylate, and modulate the function of the transcription factors and transcriptional regulators with which it interacts. No consideration had been given to the independent recruitment of CARM1 on a genome-wide scale, and little to the ability of CARM1 to affect expression by directly modifying the chromatin. For this study, we utilized a mouse embryonic fibroblast (MEF) model system in which CARM1-dependent gene regulation could be assessed without limiting its functionality to specialized cellular programs or particular disease states. Again beginning with a genome-wide approach [this time using ChIP-Seq], we identified genomic regions to which CARM1 is recruited. Subsequent characterization of binding events suggests a role for CARM1 in transcriptional elongation, and implicates the transcription factor PAX1 as a potential mediator of CARM1 genomic recruitment. When we identified genes that are differentially expressed when CARM1 is absent, we found that direct recruitment of CARM1 was not essential for its transcriptional effects. Functional analysis focused us on a critical role for CARM1 in cellular growth and proliferation, and we showed that in the absence of CARM1, the expression of many cell cycle regulators is dramatically altered. When subjected to further replicative stress, cell cycle-compromised, CARM1-null cells did not survive.

Collectively, the work presented herein highlights a few of the mechanisms through which CARM1 can affect global transcriptional regulation.

1.8 References

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Chapter 2

2 β-Estradiol-dependent activation of the JAK/STAT pathway requires p/CIP and CARM

2.1 Introduction

Transcriptional activation is a highly dynamic process that involves a large and diverse class of proteins known as coactivators. Coactivators mediate specific transcriptional responses by utilizing several interrelated mechanisms involving chromatin remodeling and covalent modification of histones. These mechanisms often work cooperatively to alter the structural restrictions imposed by packaging of DNA into chromatin. Additionally, many coactivators function as adaptors/bridging factors to recruit additional coactivator proteins to target genes [1]. Detailed genome-wide chromatin immunoprecipitation (ChIP) studies of binding sites for various transcription factors, such as the estrogen receptor (ER), have provided significant insight into the dynamics of coregulator activity at selected targets [2]. For example, the ER undergoes a cyclic pattern of association and dissociation at selected ER targets [2,12], and its association with DNA often coincides with the recruitment of several ERα-interacting complexes. These complexes consist of various combinations of coregulators, the basal transcriptional machinery, as well as RNA polymerase II [3]. The p/300 CBP interacting protein (p/CIP), also known as SRC3/AIB1/ACTR/RAC3, [4–9] belongs to a family of **s**teroid **r**eceptor **c**oactivator (SRC) proteins containing two additional family members (SRC1 and SRC2). p/CIP interacts directly with the liganded ER, and functions primarily as a bridging factor that binds to hormone-bound nuclear receptors to promote coactivator complex assembly [10,11]. ChIP assays have established that p/CIP associates with many

endogenous ER target genes in response to 17β-estradiol (E2), including pS2 [3], cathepsin D [12] and cyclin D1 [13]. Additionally, p/CIP interacts with other liganded nuclear receptors [5,7–9] and other classes of transcription factors such as E2F [14] and NFkB [15].

Several studies have shown that p/CIP undergoes a variety of posttranslational modifications in response to extracellular signals such as phosphorylation, acetylation, methylation, ubiquitination, and sumoylation [10,16–20]. These modifications provide an important regulatory mechanism that defines the combinatorial associations with additional coactivators, resulting in the formation of diverse multimeric complexes, which generate distinct gene expression programs. The coactivator-associated arginine methyltransferase protein (CARM1) is one such coactivating partner that interacts with the carboxy terminus of p/CIP as well as other SRC proteins. CARM1 has been shown to methylate proteins involved in RNA processing as well as specific arginines at positions 17 and 26 on histone H3, suggesting that CARM1 plays a direct role in gene transcription [21,22]. Furthermore, several studies have demonstrated a correlation between recruitment of CARM1, methylation of histone H3, and activation of several steroid responsive genes [23–26]. Sequential ChIP analysis has identified a complex consisting of p/CIP and CARM1 on several estrogen responsive genes [16,27,28], and CARM1 synergizes with p/CIP to activate NR-dependent transcription [29–31]. Collectively, these studies suggest that direct recruitment of CARM1 by p/CIP represents an essential activating step for ER-dependent transcription. In the present study, we have used sequential ChIP–reChIP assays in conjunction with genome-wide microarray screening to identify E2-dependent gene promoter targets of the p/CIP/CARM1 complex. Importantly,

our studies identify the JAK2 promoter as a novel target for the p/CIP/CARM1 complex in response to E2, indicating a novel interplay between ER signaling and the JAK/STAT pathway at the level of transcription, which may have implications in ER positive breast cancers where the JAK/STAT signaling pathway is constitutively active.

2.2 Results

2.2.1 Identification of genes directly targeted by the p/CIP/CARM1 complex in response to β-estradiol (E2)

To identify p/CIP/CARM1 target genes which play a role in E2-dependent signaling we embarked on a genome-wide chromatin immunoprecipitation assay approach based on DNA Selection and Ligation (ChIP-DSL) [32]. Briefly, MCF-7 cells were treated with E2 for 45 min and a standard ChIP assay was performed using an antibody against p/CIP. The immunoprecipitated material was then reChIPed using a CARM1-specific antibody. The resulting p/CIP/CARM1-enriched and input DNA was then purified, biotinylated, and combined with 40,000 unique predesigned oligonucleotides. After annealing, the biotinylated DNA was selected using streptavidin Sepharose and hybridized to a 20,000 gene promoter array (Figure 2-1A). The identification of E2-dependent p/CIP/CARM1 targets was based on the following criteria: first, we established a list of genes displaying a statistically significant enrichment relative to input ($p<0.05$) regardless of the treatment. From this list, genes that displayed a 2-fold or greater enrichment in the E2-treated cells relative to control were identified (i.e. a twofold or greater enrichment was observed in cells treated with E2 as compared to the untreated cells). The experiment was performed in triplicate and based on these

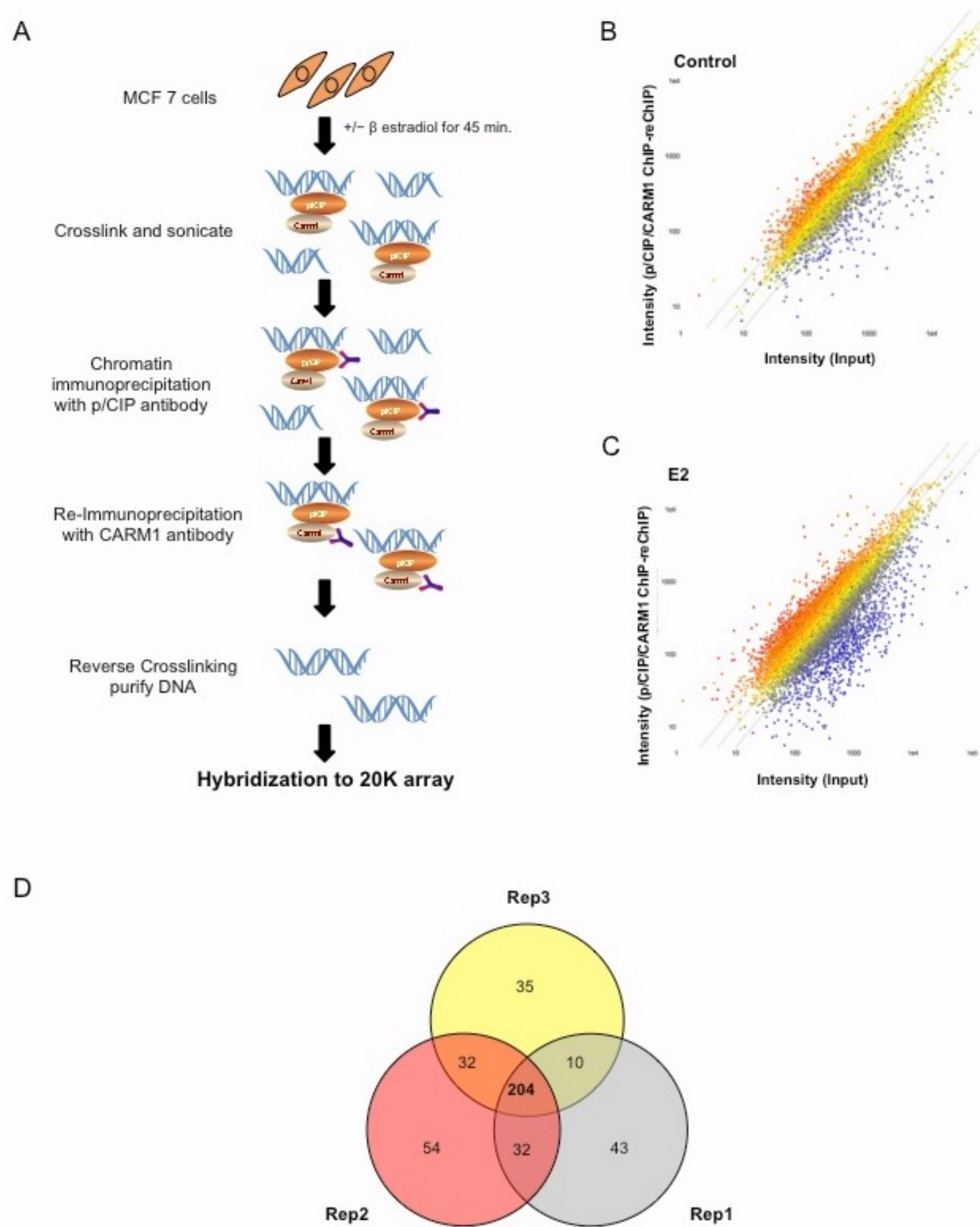

Figure 2-1 ChIP-DSL analysis of p/CIP/CARM1 target genes in MCF-7 cells.

(A) Sequential ChIP-reChIP coupled to DNA Selection and Ligation (ChIP-DSL) was used to assess global promoter occupancy by the p/CIP/CARM1 complex. Sequential ChIP-reChIP was performed using either IgG or anti-p/CIP followed by anti-CARM1 antibodies in control and 17β-estradiol stimulated MCF-7 cells. Total input and antibody-enriched DNA were biotinylated and annealed to a 40mer oligonucleotide pool. Annealed oligonucleotides were selected for with streptavidin-coated metal beads, and appropriate 40mers are ligated to form an 80mer which is then labelled and hybridized to the Hu20K array, which contains sequences from 20,000 unique human promoters. Scatter plots of (B) control and (C) 17β-estradiol stimulated p/CIP/CARM1 ChIP (y axis) versus input (x axis) from three independent biological replicates, demonstrating a normal cluster distribution. (D) Venn diagram depicting the overlap in genes enriched from three independent ChIP-DSL experiments (Rep 1 to 3).

criteria we identified 204 gene promoters that become co-occupied by p/CIP and CARM1 in response to E2 treatment for 45 min (Figure 2-1B-C & Table B-1). To validate the ChIP-DSL analysis, we performed independent ChIP–reChIP experiments for a random set of target genes identified (Figure 2-2). In the majority of cases tested, treatment with E2 resulted in the simultaneous recruitment of p/CIP and CARM1, indicating that the false positive rate was extremely low. It should be emphasized that in our experimental protocol we have identified gene targets for p/CIP/CARM1 as a consequence of E2 treatment; which may include genes directly regulated by the ER, as well as genes indirectly regulated by the ER through its association with other transcription factors, or via a nongenomic pathway.

2.2.2 The ER interacts with a fraction of p/CIP/CARM1 target gene promoters

To better validate our analysis, we compared our results with two previous studies examining direct E2-dependent ER binding on a genome-wide scale [33,34]. Carroll et al. discerned 3665 unique E2- dependent ER binding sites using a ChIP–chip Affymetrix Human tiling array approach [33], while Welboren et al. used a ChIP-Seq approach to identify 10,205 genome-wide ER-interaction sites [34]. Using the published chromosomal locations for ER-binding targets identified in these studies, we distinguished known genes present within and/or adjacent to these genomic sites. Next, we conducted direct gene-by-gene comparisons of p/CIP/CARM1 targets in our study with the ER targets identified in each of the previous studies. An important consideration

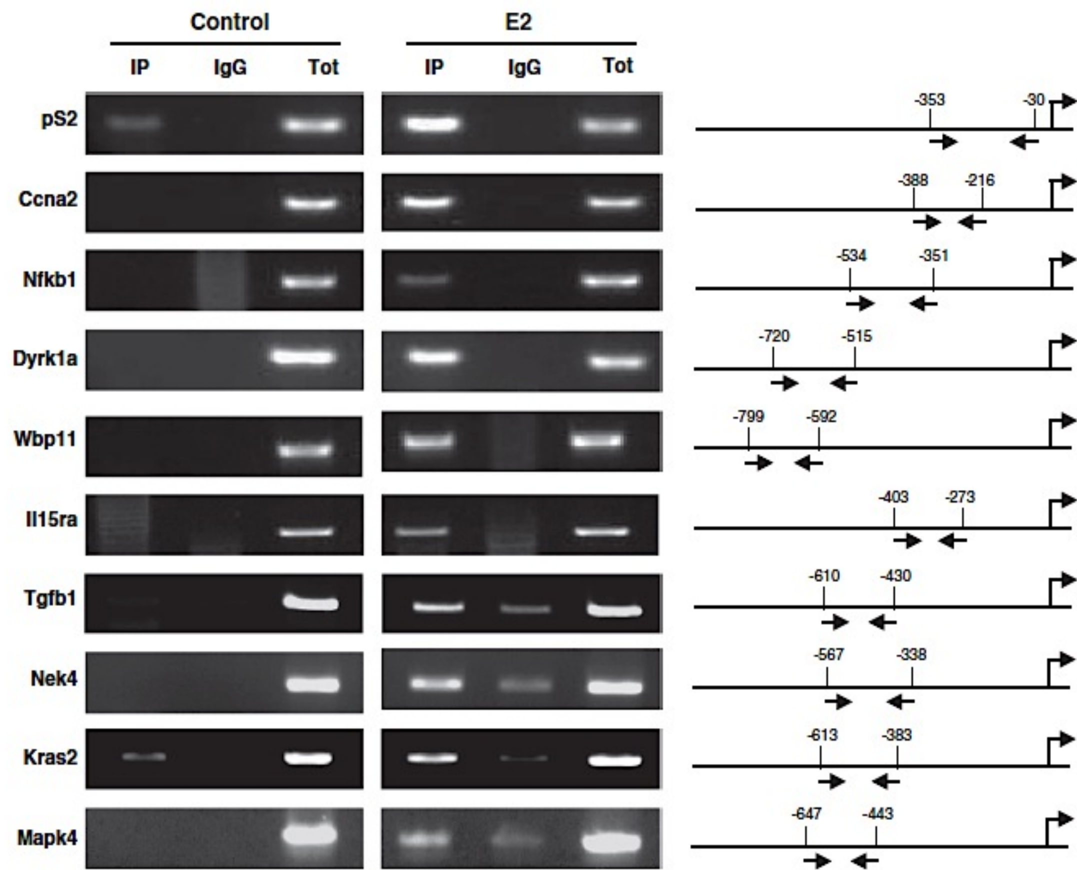

Figure 2-2 ChIP analysis of selected direct p/CIP/CARM1 target genes

Control and 17β-estradiol stimulated MCF-7 cells were cross-linked with 1% formaldehyde, and sequential ChIP-reChIP was performed using either IgG or anti-p/CIP followed by anti-CARM1 antibodies. Recovered DNA was assayed by PCR using primers corresponding to the promoter regions indicated. Selected target genes shown are pS2, CCNA2, NFKB1, DYRK1A, WBP11, IL15RA, TGFB1, NEK4, KRAS2, and MAPK4

for the purposes of this comparison is that while our analysis was restricted to the 1 kb proximal promoter regions of genes, the studies conducted by both the comparison groups encompassed binding sites throughout the entire genome. Notably, in each of studies used for comparative analysis, only a small proportion (4–7%) of ER interaction sites were located within promoter regions. Nevertheless, our analysis indicated that 65/204 (32%) [33] and 118/204 (58%) [34] (Figure 2-3A) of identified p/CIP/CARM1 complex targets have previously been shown to display ER binding following E2 treatment. Moreover, 59/204 (29%) p/CIP/CARM1 complex targets were ER binding targets common to both studies, and were therefore considered to be high confidence ER interactors (Figure 2-3B, Table 2-1).

2.2.3 Binding site enrichment in the promoters of p/CIP/CARM target gene promoters

Since our p/CIP/CARM1 target genes may include genes directly and/or indirectly regulated by the ER, we examined 1 kb upstream promoter sequences of the gene targets for transcription factor binding site enrichment, to discern potential mechanisms for E2-dependent p/CIP/CARM1 binding. Using a candidate scanning approach, we conducted a search for enriched motifs within our target gene promoter sequences. Previous studies have shown that in addition to directly binding ER-binding elements (EREs) in response to ligand, the ER associates with C/EBP [35], and can also be targeted to the DNA via interaction with Oct [36,37], Sp1 [38,39] and/or AP-1 [40,41]. Forkhead motifs were also of interest, as evidence has linked the presence of the

Table 2-1 p/CIP/CARM1 target genes bind the ER.

Gene ID	Accession Number	Description
TLR3	NM_003265	Transmembrane receptor
DBC1	NM_014618	Peptidase
LAP3	NM_015907	Peptidase
LDN12	NM_012129	Calcium-independent cell-cell adhesion
SLC4A5	NM_021196	Solute carrier family 4, sodium bicarbonate cotransporter, member 5
PTER	NM_030664	Phosphotriesterase related
TRAF3	NM_003300	TNF receptor-associated factor 3/apoptosis
LETM1	NM_012318	Leucine zipper-EF-hand containing transmembrane protein 1
GSF4/CADM1	NM_014333	Cell adhesion molecule 1/tumour suppressor
OCA2	NM_000275	Oculocutaneous albinism II
SLC26A1	NM_022042	Solute carrier family 26 (sulfate transporter), member 1
FRMD1	NM_024919	FERM domain containing 1
4BP1	NM_153029	NEDD4 binding protein 1
CTNND1	NM_001331	Catenin (cadherin-associated protein), delta 1
CDH7	NM_033646	Cadherin 7, type 2
RAD9B	NM_152442	RAD9 homolog B (S. cerevisiae)/DNA replication
ECT2	NM_018098	Epithelial cell transforming sequence 2 oncogene
PCSK5	NM_006200	Proprotein convertase subtilisin/kexin type 5
GATM	NM_001482	Glycine amidinotransferase (L-arginine:glycine amidinotransferase)
TUBGCP6	NM_020461	Tubulin, gamma complex associated protein 6
KCTD16	XM_098368	Potassium channel tetramerisation domain containing 16
TGFB1	NM_000660	Growth factor
NEK4	NM_003157	Enzyme
RPS4X	NM_001007	Ribosomal protein S4, X-linked
STARD4	NM_139164	StAR-related lipid transfer (START) domain containing 4
HRASLS	NM_020386	HRAS-like suppressor
PKP4	NM_003628	Plakophilin 4/cell adhesion
SMARCAL1	NM_014140	SWI/SNF related, matrix associated, regulator of chromatin, a-like 1

CD9	NM_001769	CD9 molecule/cell adhesion
C9orf95	NM_017881	Orf
TMEM16F	XM_113743	Transmembrane protein 16F
IVNS1ABP	NM_006469	Influenza virus NS1A binding protein
DDX54	NM_024072	DEAD (Asp-Glu-Ala-Asp) box polypeptide 54/tranascriptional regulator
ITPKB	NM_002221	Inositol 1,4,5-trisphosphate 3-kinase B
POLR2F	NM_021974	Polymerase (RNA) II (DNA directed) polypeptide F
LAMA1	NM_005559	Laminin, alpha 1/cell adhesion protein
TSHB	NM_000549	Thyroid stimulating hormone, beta
RDH10	NM_172037	Retinol dehydrogenase 10 (all-trans)
KCNQ1	NM_000218	Potassium voltage-gated channel, KQT-like subfamily, member 1
KCTD3	NM_016121	Potassium channel tetramerisation domain containing 3
PCP4	NM_006198	Purkinje cell protein 4
LRFN4	NM_024036	Leucine rich repeat and fibronectin type III domain containing 4
ABAT	NM_000663	4-aminobutyrate aminotransferase
PTPRJ	NM_002843	Protein tyrosine phosphatase, receptor type, J
JAK2	NM_004972	Janus kinase 2 (a protein tyrosine kinase)
GPR132	NM_013345	G protein-coupled receptor 132
HARS	NM_002109	Histidyl-tRNA synthetase
MDS009	NM_020234	DTWD1 DTW domain containing 1
POLG	NM_002693	Polymerase (DNA directed), gamma
HSD17B12	NM_016142	Hydroxysteroid (17-beta) dehydrogenase 12
AKAP13	NM_006738	A kinase (PRKA) anchor protein 13
DPYS	NM_001385	Dihydropyrimidinase
DYRK1A	NM_101395	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A
EDC3	NM_025083	Homo sapiens enhancer of mRNA decapping 3 homolog (S. cerevisiae)
SLC39A10	XM_047707	Solute carrier family 39 (zinc transporter), member 10
SAMD3	NM_152552	Sterile alpha motif domain containing 3
FLRT2	NM_013231	Fibronectin leucine rich transmembrane protein 2/cell adhesion
MGC14156	NM_032906	PIGY phosphatidylinositol glycan anchor biosynthesis, class Y
MXD3	NM_031300	MAX dimerization protein 3

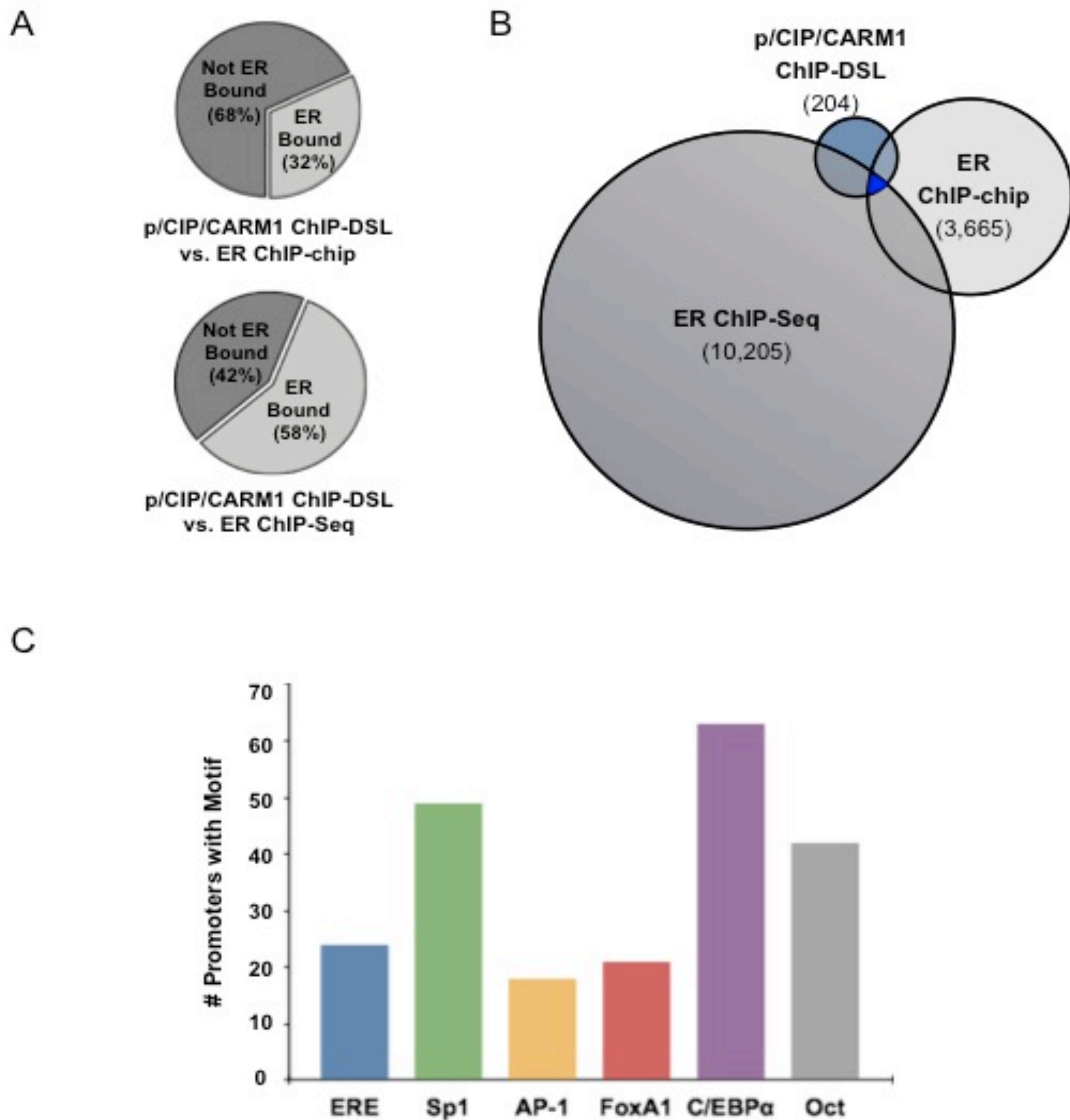

Figure 2-3 Binding site enrichment analysis of p/CIP/CARM1 target promoter sequences

(A) Pie charts indicating the proportion of p/CIP/CARM1 target genes that are known to bind the ER based on comparison with ChIP-chip analysis performed by Carroll et al. (top) and ChIP-Seq analysis performed by Welboren et al. (bottom). (B) Venn diagram showing overlap of p/CIP/CARM1 direct target genes (highlighted in blue) with ER-binding sites identified by ChIP-chip reported by Caroll et al. and identified by ChIP-Seq reported by Welboren et al. 59 p/CIP/CARM1 target genes were common to both the Carroll et al. and Welboren et al. analysis, and were considered to be high confidence ER-binding targets. (C) Bar graph showing

FoxA1/ HNF3α motif to ER recruitment [33,42]. When p/CIP/CARM1 target gene promoter sequences were compared to a background control set of human housekeeping gene promoters, many enriched putative binding motifs were identified (Table B-2). While EREs were shown as being significantly enriched among p/CIP/CARM1 complex promoters, it was not the most prevalent motif when ranked either by p-value or by enrichment score. Sp1, C/EBP, Oct, FoxA1, and AP-1 motifs were all found to be more significantly enriched than EREs among the p/CIP/ CARM1 target gene promoters we identified. Evaluation of these consensus sites within the individual promoter sequences confirmed the presence and relative abundance of the motifs, with approximately 12% of target promoters containing one or more EREs, 31% C/EBPα motifs, 24% Sp1, 20% Oct, 10% FoxA1, and 9% contain putative AP-1 sites (Figure 2-3C). Several of the promoters contain consensus sites for more than one of the considered transcription factors.

EREs occurred simultaneously more often with C/EBP, Oct, and Sp1 motifs rather than with FoxA1 or AP-1 motifs (Figure A-1). While a negative correlation between ERE and AP-1 elements has previously been observed [33], our findings are contrary to previous reports [33,42] in that FoxA1 motifs did not consistently coincide with the presence of EREs. This discordance is likely due to our exclusive focus on the proximal promoter, since Kwon et al. also noted limited association of FoxA1 sites with proximal promoter ER binding events as identified by ChIP-DSL [43]. The overall enrichment pattern of putative transcription factor motifs did not change for the 59 p/CIP/CARM1 target genes that are considered to be high confidence ER binding targets (Figure B-2a) or for targets transcriptionally upregulated following E2 treatment (Figure B-2b).

Collectively, this analysis demonstrates a consistent pattern of enriched motifs among p/CIP/CARM1 complex target promoters, and suggests that transcription factor crosstalk is likely the favored mechanism of E2-dependent p/CIP/CARM1 recruitment at the proximal promoters of target genes, regardless of ER binding status or transcriptional state. The presence of EREs suggests that the classical response with direct binding of the ER is also involved, albeit to a lesser extent.

2.2.4 A proportion of p/CIP/CARM1 target genes are directly regulated by E2

To correlate p/CIP/CARM1 binding data with the E2-dependent transcriptional response, we performed gene expression profiling using MCF-7 cells treated with 10 nM E2 for 12 h. RNA was isolated, reverse transcribed and hybridized to Affymetrix microarrays. A list of differentially expressed genes from three independent experiments was generated ($p\text{-value}<0.05$ was used as the cutoff). Based on these criteria, we identified 396 and 231 genes that were significantly upregulated or downregulated, respectively, following E2-treatment (Figure 2-4A), consistent with previous expression profiling studies [43,44]. Comparison of the microarray expression data with the ChIP-DSL results determined that 33 (16.2%) targets proximally bound by the p/CIP/CARM1 complex are significantly upregulated, and 8 (3.9%) p/CIP/CARM1 target genes were downregulated following E2 treatment (Table 2-2). The reliability of this analysis was confirmed by quantitative real-time PCR (qPCR) analysis of selected genes (Figure 2-4B). These findings suggests that proximal binding may be more relevant for

transcriptionally upregulated genes, and shows that while the p/CIP/CARM1 complex may have a preferential role in transcriptional activation, it also plays a direct role in the repression of specific genes.

2.2.5 The JAK/STAT signaling pathway is a central target for p/CIP/CARM1

Using Ingenuity Pathway Analysis we were able to identify a number of networks consisting of target genes that are functionally or biochemically linked. Several of the genes play a role in disease (Figure 2-5A), and the molecular and cellular functions most associated with p/CIP/CARM1 target genes have been linked to the initiation and progression of cancer (Figure 2-5B). We found that the network containing one of the highest number of functionally linked targets is the canonical JAK/STAT signaling pathway. Several interconnected genes within this pathway are direct targets for p/CIP and CARM1, including janus tyrosine kinase 2 (Jak2), the interleukin 15 receptor (Il15ra), and Pias2-a sumo ligase, which functions as a coregulator for STAT proteins (Figure 2-4C, colored targets). Adding to the implied relevance of p/CIP/CARM1-mediated regulation of this pathway, Stat3 and Stat5 (Figure 2-4C, gray targets) were also identified as E2-dependent p/CIP/CARM1 binding targets, however, they were not included on the final list as they did not meet the applied statistical criteria. We focused our initial analysis on the Jak2 gene. JAK2 plays a central role in JAK/ STAT signaling and mammary gland development, and hyperactivation of this protein is associated with

cancer initiation. Importantly, functional ablation of JAK2 in mice protects against the onset of mammary tumorigenesis [45].

2.2.6 E2-dependent regulation of JAK2

JAK2 expression was assessed using qPCR following E2 treatment of MCF-7 cells for various time periods. We observed an initial decrease in Jak2 expression 1 h following E2 treatment, and a gradual increase in expression thereafter, such that at 12 and 24 h the expression levels are consistent with preliminary expression array data, with an approximately 1.5-fold increase in expression when compared to the untreated control ($p<0.05$) (Figure 2-4B). Analysis of JAK2 protein levels exhibit a corresponding trend, with a modest increase (~1.3 fold) in protein expression after 12 and 24 h of E2 stimulation, followed by a decrease to control unstimulated expression levels at 72 h (Figure 2-4D).

To validate recruitment of p/CIP and CARM1 to specific regions of the Jak2 promoter we performed ChIP assays. For the purposes of this analysis, we used a region of the promoter that corresponds to a region 787 to 950 bp upstream of the transcriptional start site (TSS) (Figure 2-6A). An ERE-Sp1 half-site was identified within this portion of the Jak2 promoter.

Cells were treated with E2 for 45 min and promoter occupancy was assessed by sequential ChIP assay using specific antibodies recognizing p/CIP and CARM1. In addition, ChIP assays were performed using an antibody against the ER to assess a

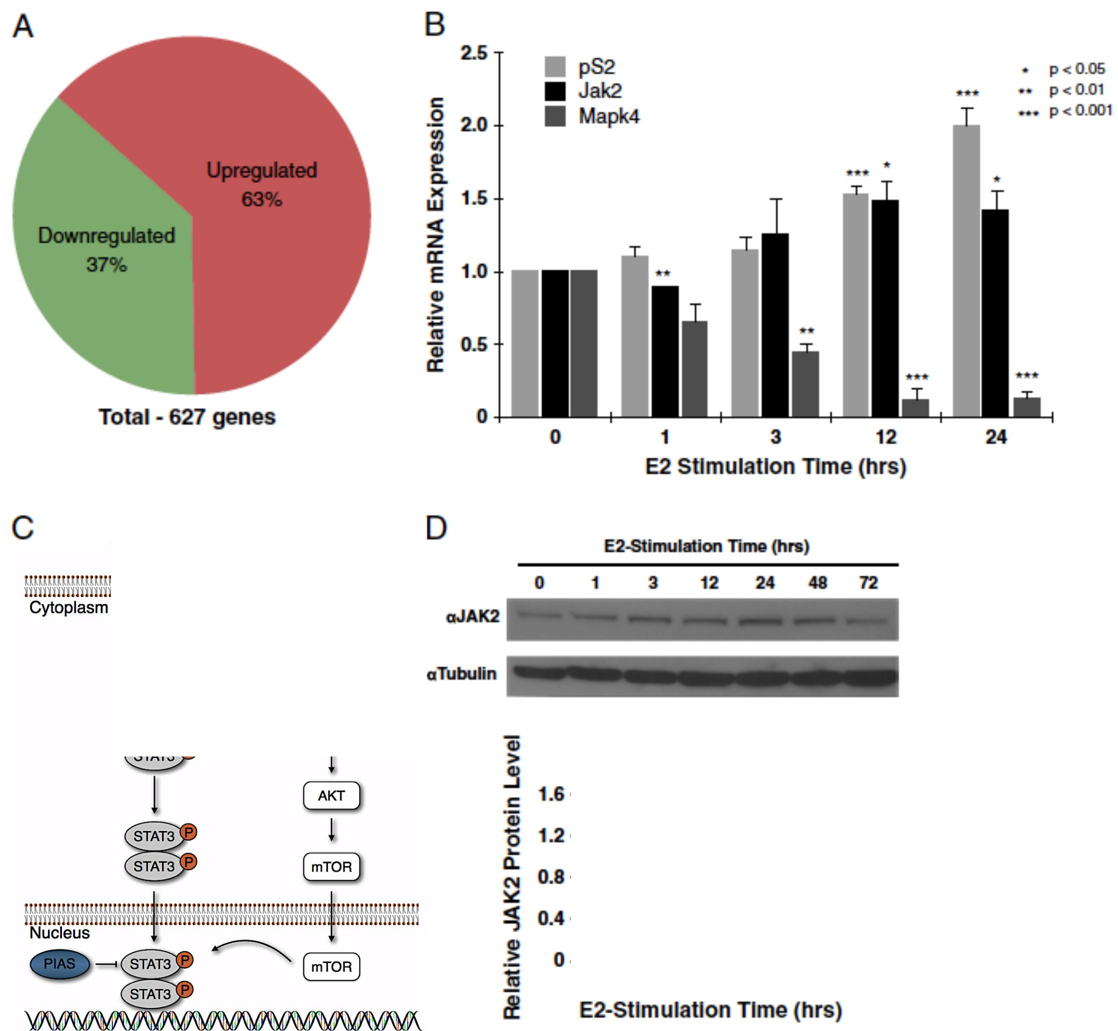

Figure 2-4 p/CIP/CARM1 target genes are directly regulated by E2.

(A) Differentially expressed genes after E2-stimulation relative to unstimulated control cells. (B) Realtime RT-PCR analysis of pS2, JAK2, and MAPK4 following stimulation of MCF-7 cells with 17β-estradiol for 1hr, 3hr, 12hr, and 24hrs. Data is expressed as means and standard error of the mean from repeated experiments, performed independently (pS2 n=7, JAK2 n=7, MAPK4 n=3). Paired student's t-test was performed and statistically significant changes from untreated samples are indicated. (C) Canonical pathway diagram, highlighting statistically significant p/CIP/CARM1 complex direct targets in color. Targets in grey are bound by p/CIP/CARM1 but do not meet statistical criteria. **(**D) Western blot showing JAK2 levels in MCF7 cells following 17β-estradiol stimulation of MCF7 cells for 1, 3, 12, 24, 48, and 72 hrs. Relative abundance of protein levels at 12, 24, 48, and 72hr time points was quantified by densitometry.

Table 2-2 Genes directly regulated by the p/CIP/CARM1 complex [a]

Gene ID	Accession Number	Description
Genes Activated by E2		
NFKB1	NM_003998	Nuclear factor of kappa light polypeptide gene enhancer
MOCOS	NM_017947	Enzyme
JAK2	NM_004972	Janus kinase 2 (a protein tyrosine kinase)
STARD4	NM_139164	StAR-related lipid transfer (START) domain containing 4
HARS	NM_002109	Histidyl-tRNA synthetase
IGSF4/CADM1	NM_014333	Cell adhesion molecule 1/tumour suppressor
KCTD3	NM_016121	Potassium channel tetramerisation domain containing 3
GTF2E2	NM_002095	General transcription factor IIE, polypeptide 2, beta 34kDa
ZIM3	NM_052882	Zinc finger, imprinted 3/transcription factor
PDCD8	NM_004208	Enzyme/cell death
WBP11	NM_016312	WW domain binding protein 11
PRKRIR	NM_004705	Protein-kinase, interferon-inducible RNA dependent inhibitor
ZNF567	NM_152603	Transcription factor
IVNS1ABP	NM_006469	Influenza virus NS1A binding protein
BMI1	NM_005180	BMI1 polycomb ring finger oncogene
M6PR	NM_002355	Mannose-6-phosphate receptor (cation dependent)
HSD17B12	NM_016142	Hydroxysteroid (17-beta) dehydrogenase 12
TM4SF8	NM_005724	Tetraspanin 3
CCNA2	NM_001237	Cyclin A2/cell cycle
LAP3	NM_015907	Peptidase
POLR2F	NM_021974	Polymerase (RNA) II (DNA directed) polypeptide F
RAD9B	NM_152442	RAD9 homolog B (S. cerevisiae)/DNA replication
DYRK1A	NM_101395	Dual-specificity tyrosine(Y)-phosphorylation regulated kinase 1A
PCNA	NM_002592	Proliferating cell nuclear antigen
PCP4	NM_006198	Purkinje cell protein 4
ZNF800	NM_176814	Unkown
PELO	NM_015946	Pelota homolog (Drosophila)
DPH2L2	NM_001384	DPH2 homolog (S. cerevisiae)
RFXAP	NM_000538	Regulatory factor X-associated protein
CLDN12	NM_012129	Calcium-independent cell-cell adhesion
PIAS2	NM_004671	Protein inhibitor of activated STAT, 2
ELAC1	NM_018696	ElaC homolog 1 (E. coli)/trna processing
RDH10	NM_172037	Retinol dehydrogenase 10 (all-trans)
Genes Repressed by E2		
PXMP4	NM_007238	Peroxisomal membrane protein 4, 24kDa
HBD	NM_000519	Hemoglobin, beta /// hemoglobin, delta
MAPK4	NM_002747	Mitogen-activated protein kinase 4
ITPKB	NM_002221	Inositol 1,4,5-trisphosphate 3-kinase B
MXD3	NM_031300	MAX dimerization protein 3
MGC15882	NM_032884	C1orf94
LMOD1	NM_012134	Leiomodin 1 (smooth muscle)
CD9	NM_001769	CD9 molecule/cell adhesion

[a] Comparative analysis of ChIP-chip data and expression analysis following 12hr E2-stimulation of MCF-7 cells allowed for the identification of p/CIP/CARM1 regulated genes.

possible mechanism of recruitment for the p/CIP/CARM1 complex to the Jak2 promoter. We found that the p/CIP/CARM1 complex binds in a ligand-dependent manner to the Jak2 promoter (Figure 2-6B). Importantly, we also determined that the ER binds Jak2 in response to ligand (Figure 2-6B). The presence of both the p/CIP/CARM1complex and the ER at the same region of the promoter suggests that E2-dependent complex recruitment to the Jak2 promoter is mediated through its interaction with the ERE-associated ER. This finding is consistent with the putative status of Jak2 as one of the 59 high-confidence ER targets.

To determine whether the E2-dependent assembly of a p/CIP/CARM1 complex impacts the chromatin marks at the Jak2 promoter, ChIP analysis was performed using antibodies corresponding to histone modifications associated with transcriptional status; histone H3 lysine 4 trimethylation (H3K4me3) and acetylation of histone H3 at Lysines 9 and 14 (H3Ac) are modifications generally indicative of transcriptionally active chromatin structure [46,47]. In addition, CARM1 is known to asymmetrically dimethylate arginine 17 on histone H3 (H3R17me2), a mark that is also associated with transcriptional activation [48].

E2-dependent changes in histone modifications were observed at the Jak2 promoter. In response to treatment, there was a modest increase in acetylation of H3K9 and H3K14 (H3K9/14ac) (Figure 2-6C) and a statistically significant enrichment in dimethylation of H3R17 (Figure 2-6D), consistent with recruitment of a functional p/CIP/CARM1 complex. Trimethylation of H3K4 remained unchanged by E2 treatment (Figure 2-6C), perhaps suggestive of a transcriptionally permissive but not fully active gene state, in

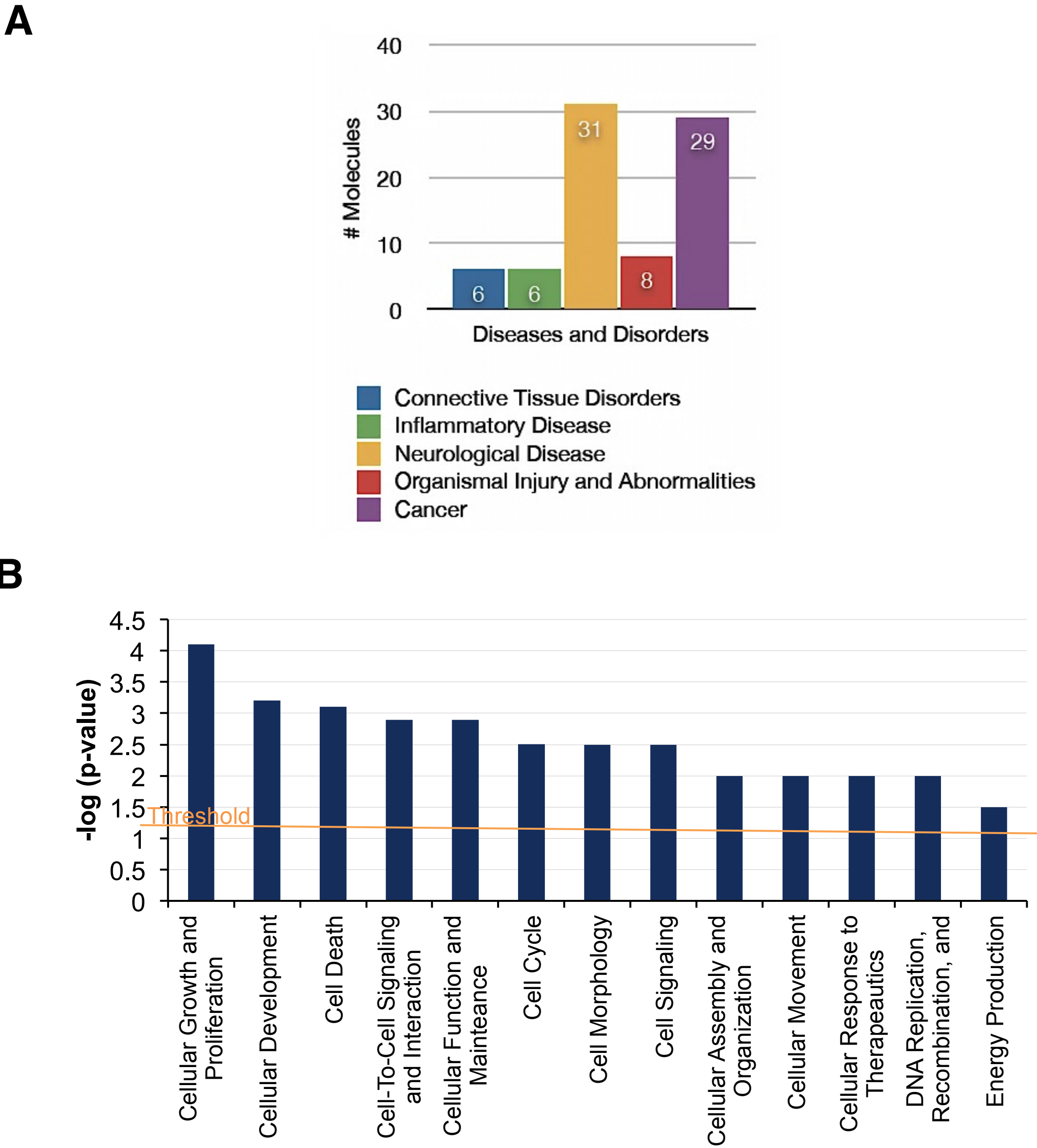

Figure 2-5 Functional classification of p/CIP/CARM1 target gene promoters.

(A) Schematic Ingenuity pathway analysis indicates (A) that cancer is one of the diseases most commonly associated with the p/CIP/CARM1 target genes and (B) that molecular and cellular functions associated with the gene set highlights cellular growth and proliferation as predominating. Adapted from © 2000-2011 Ingenuity Systems, Inc. All rights reserved.

agreement with the modest E2-dependent increases in mRNA observed (Figure 2-4B). To determine if the changes in histone modifications are dependent on the presence of p/CIP, quantitative ChIP analysis was performed on the Jak2 promoter following siRNA-mediated gene silencing of p/CIP. p/CIP was present minimally on the promoter despite p/CIP depletion (Figure 2-7B), likely due to incomplete knockdown (Figure 2-7A). The level of p/CIP present on the promoter was increased in response to hormone (Figure 2-7B). The functional effect of p/CIP depletion was determined by ChIP assay evaluating the presence of the CARM1-dependent histone modification H3R17me2 in response to E2. As a chromatin mark associated with transcriptional activation, in the absence of p/CIP [and complex] recruitment, E2-dependent dimethylation of R17 on histone H3 was reduced (Figure 2-7B), indicating that p/CIP is likely required for recruitment of CARM1, and its subsequent methyltransferase activity.

To further our understanding of the role that the p/CIP/CARM1 complex plays in regulating Jak2, we assessed its E2-dependent expression in the absence of p/CIP or CARM1. When a control siRNA was used, we saw a statistically significant induction of Jak2 expression, based on realtime PCR, after 12 and 24 h of E2 treatment. However, when p/CIP was downregulated using siRNA, this effect was diminished (Figure 2-7C). A similar experiment was performed following CARM1 knockdown and, although the knockdown was not complete, there was a more dramatic loss of E2-dependent Jak2 expression as compared to p/CIP knockdown (Figure 2-7D). Interestingly, when MCF7 cells were treated with E2, we also observed an increase in Stat3 phosphorylation, indicative of E2-dependent JAK/STAT pathway activation. However, when either p/CIP or CARM1 was depleted, this effect was lost (Figure 2-7C & D). Collectively, these data

suggests that p/CIP and CARM1 are in part required for the E2-dependent regulation of Jak2 transcription and activation of the JAK/STAT signaling pathway in MCF7 cells.

2.3 Discussion

In the present study, we identified E2-dependent target genes for the p/CIP/CARM1 coregulatory complex. We then determined the transcriptional status of those genes following 12 h E2 treatment, and compared the lists with known ER binding sites in an attempt to clarify a mechanism for complex recruitment and subsequent gene regulation.

Global characterization of p/CIP (SRC-3) binding sites conducted by Lanz et al. identified 12,294 E2-dependent targets using ChIP-Seq, 5512 of which were distinct from those found using vehicle stimulation [49]. Many of these sites overlap with previously identified ER binding regions, and, in accordance with accumulating genomic analysis indicating that the majority of ER binding sites are located in regions far upstream of the TSS, only a minority (~3%) of E2-dependent p/CIP binding is suggested to occur within 500 bp of the TSS. The level of CARM1 activity (as assayed by its methylation of H3R17 and/or p/CIP) across the genome also appears to cluster predominantly at a distance from promoters [50]. These observations of a limited set of proximal promoter binding sites for p/CIP, and minimal CARM1 methylation activity at promoter elements correspond with the relatively small number of promoters we identified as interacting with the p/CIP/CARM1 complex, and is also consistent with proximal ER binding events previously identified using the 1 kb promoter-specific ChIP-DSL approach [43]. Although now largely superseded by ChiP-Seq, ChIP-DSL is a highly sensitive assay

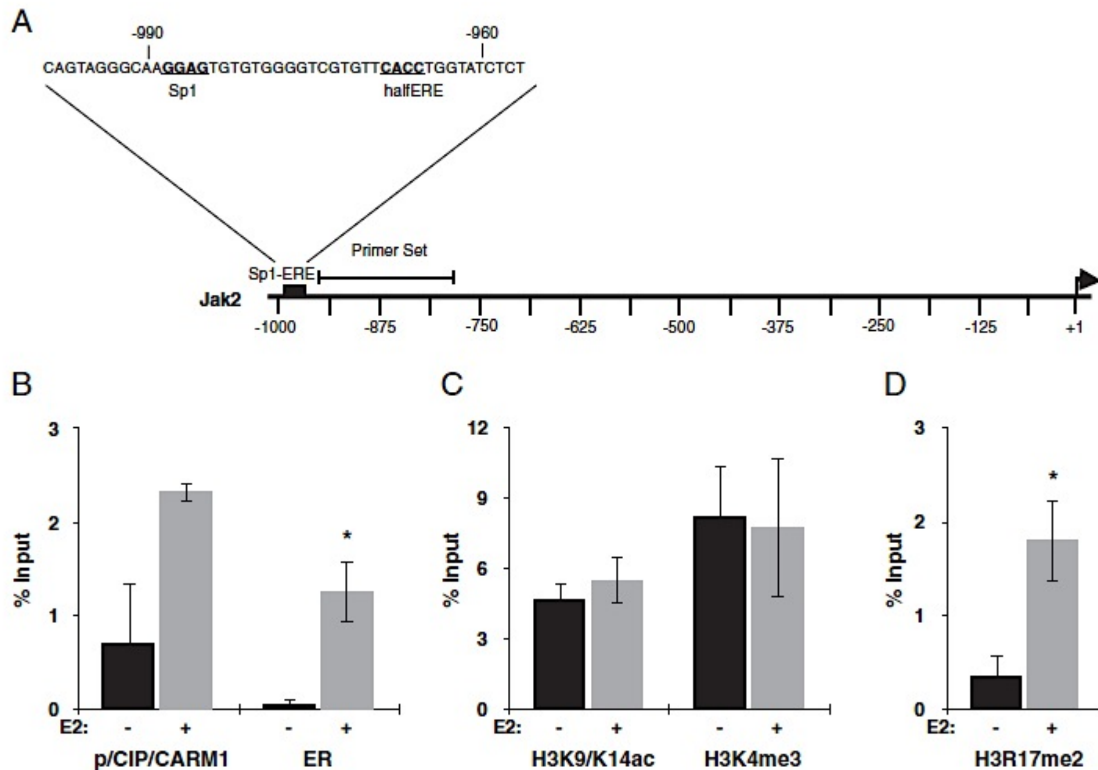

Figure 2-6 Characterizing E2-dependent histone modifications to JAK2 proximal promoter

(A) Schematic representation of the JAK2 1kb proximal promoter, highlighting region used for ChIP analysis. (B) Control and 17β-estradiol stimulated MCF-7 cells were cross-linked with 1% formaldehyde, and ChIP or sequential ChIP-reChIP was performed using the indicated antibodies, followed by qPCR. ChIPs were performed in triplicate, standardized to IgG, and shown as percentage of Input. Statistical significance was determined using Student's t-test and is indicated by * ($p<0.05$). (C-D) ChIP-qPCR analysis of JAK2 promoter following E2 treatment. ChIPs were performed in duplicate (H3K9/k14ac, H3K4me3) or triplicate (H3R17me2) using indicated antibodies, quantitated with real-time PCR, standardized to IgG control and shown as percentage of Input. Statistical significance was determined using Student's t-test and is indicated by * ($p<0.05$).

which eliminates some of the biases introduced by more conventional ChIP-on-chip whole-genome approaches. In ChIP-DSL, the immunoprecipitated genomic DNA is used only as a template to mediate annealing and ligation of aligned oligonucleotide pairs. After annealing, the biotinylated DNA is then selected using streptavidin Sepharose and Taq ligase is used to ligate oligonucleotides positioned directly adjacent to each other, creating complete amplicons, which are hybridized to a 20,000 gene promoter array. A limitation of this approach is that analysis is restricted to those promoters found on the array, which contain 1 kb of upstream regulatory sequence. Studies have shown that the majority of ER binding sites are found more distal than the proximal promoter. Nevertheless, promoter proximal interactions do occur in response to E2 and this interaction can have transcriptional consequences. Furthermore, our study does not examine ER binding directly, but rather focuses on occupancy of p/CIP/CARM1 in response to E2. Thus, it would theoretically include those targets that are targeted to promoter regions by E2 independent of direct DNA binding, and possibly via enhancer–promoter interactions.

A major advantage of this approach is that we identified 204 promoter-proximal interaction sites for the p/CIP/CARM1 complex, eliminating the complicated process of assigning responsive genes to distant binding sites, and instead were able to directly correlate complex interaction with transcriptional effect. Previous microarray studies with E2-stimulation can be broadly categorized based on the length of hormone treatment. There is an observable difference in the pattern of expression change, such that at early time points (<6 h) more genes are upregulated and more variation is evident between time points, while at later time points (>12 h) there is a more stable pattern of expression

change and the majority of genes are downregulated [33,44]. We showed that after 12 h of stimulation with E2, approximately 20% of p/CIP/CARM1 complex target genes identified by ChIP-DSL were transcriptionally altered (16.2% upregulated and 3.9% downregulated). The relatively small number of transcriptionally changed target genes after 12 h implies that proximal recruitment of the p/CIP/CARM1 complex is not predictive of E2-dependent gene expression at this late time point. However, among those targets that are transcriptionally altered, the complex plays a preferential role in activation, consistent with the role of p/CIP as a coactivator. While the changes in target gene expression that we observed were modest, we do observe notable changes in chromatin modifications, suggesting that E2 may facilitate crosstalk.

Direct comparison of target gene promoters in our study with known genomic ER binding sites in MCF-7 cells [under equivalent E2- stimulation conditions] [33,34] identified 59/204 (29%) p/CIP/CARM1 complex targets as putative high-confidence ER binding targets. Therefore, it seems likely that the p/CIP/CARM1 complex is recruited to this subset of E2-responsive promoters via the ER. We also identified a trend in which binding motifs for transcription factors known to facilitate ER recruitment were enriched among p/CIP/CARM1 complex target promoter sequences. While Sp1, C/EBP, Oct, FoxA1, and AP-1 motifs have previously been shown to be enriched, in addition to EREs, in the sequences surrounding ER binding events on a genome-wide scale [33,42,50], there has not previously been much discussion related to the proximal promoter region or when considering recruitment of an E2-regulated complex. Neither the predicted ER-binding state nor the transcriptional status of complex target genes after 12 h E2 treatment was predictive of enrichment of transcription factor binding motifs

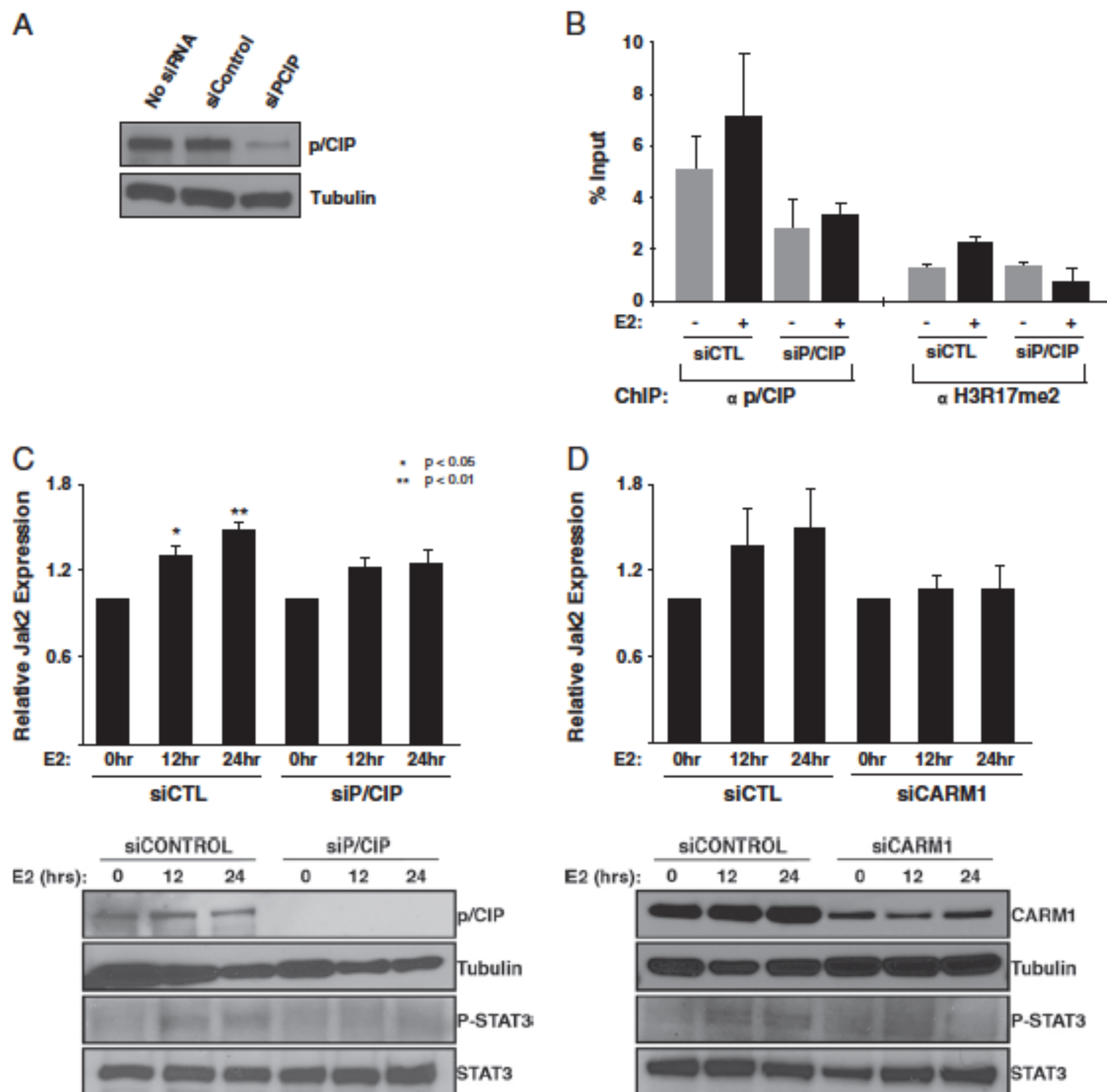

Figure 2-7 p/CIP/CARM1 mediated E2-dependent regulation of JAK2

(A) Representative Western blot showing 72hr siRNA depletion of p/CIP in MCF7 cells. (B) ChIPs were performed in triplicate at JAK2 promoter regions using indicated antibodies, quantitated by real-time PCR, standardized to IgG control and shown as a percentage of Input. Data is expressed as means and standard error of the mean from triplicate experiments. (C-D) Analysis of E2-dependent JAK2 transcription (top) and STAT3 activation (bottom) with siRNA-mediated depletion of (C) p/CIP or (D) CARM1. (Top) Realtime RT-PCR analysis of JAK2 was performed following 12 and 24hr exposure to hormone, and data is expressed as the mean and standard error of the mean from triplicate experiments, performed independently. Student's t-test was used to compare E2-induced expression changes and statistically significant differences from control samples are indicated. (Bottom) Representative Western blot showing knockdown efficiency and STAT3 activation in MCF7 cells following E2 treatment.

studied, and so, consensus mechanisms for differential transcriptional responses as implied by the recruitment of the ER could not be inferred.

Several of the targets identified are components of the canonical JAK/STAT signaling pathway. This pathway mediates the activity of a wide variety of cytokines and growth factors [51]. JAK2 is a central component of the pathway and is responsible for phosphorylation and activation of the STAT family of proteins, which normally reside in the cytoplasm and, upon activation, translocate to the nucleus and bind to specific target genes involved in cell proliferation and survival [51]. JAK2 or STAT5 null mice display phenotypes remarkably similar to the ERα and p/CIP knockout animals including defects in mammary gland cell proliferation and apoptosis [52,53]. Importantly, overexpression or constitutive activation of STAT3 and 5 proteins has been described in many types of cancers [54] and promote the occurrence of sporadic mammary cancers in mice [45,55–57]. In proliferating ER-positive tumor cells, E2 is known to stimulate phosphorylation and activation of STAT3 and 5, although the mechanism has not been fully elucidated [58–60]. The PIAS family, most notably recognized for their role as coregulators for STAT proteins, is also of interest as PIAS1 has been shown to sumoylate p/CIP [and other SRCs], affecting activity and stability in steroid-receptor signaling pathways in MCF-7 cells [20]. Collectively, these findings suggest that ER signaling and the JAK/STAT pathway may cooperate in the regulation of mechanisms implicated in mammary cancers. This cooperation may be mediated, at least in part, through the p/CIP/CARM1 complex.

We observed recruitment of the p/CIP/CARM1 complex to the 1 kb promoter of Jak2 in response to E2, with a transcriptional upregulation after 12 and 24 h, but no significant

effect on protein levels with E2 stimulation. Analysis of histone modifications to the proximal 1 kb promoter revealed an unclear pattern that was predictive neither of transcriptional activation nor repression. A modest increase in acetylation of histone H3 at K9 and K14 was observed at a region approximately 1 kb upstream of the Jak2 TSS in response to E2, which coincides with p/CIP/CARM1 complex recruitment, suggestive of a transcriptionally active gene. In contrast, there was a lack of discernible change in H3K4me3 in response to E2 at the same promoter region, a modification that would be expected to be present near the TSS of an actively transcribed gene [61,62]. We suggest that these changes reflect a promoter that is not necessarily in line with a fully transcriptionally active state but may instead be permissive, poised for more robust transcriptional activation, in a process that may require additional signals.

Our study of chromatin modifications was focused to a single time point for E2 treatment. This is based on previous studies showing maximal p/CIP/CARM1 recruitment to the pS2 promoter 45 min after the addition of hormone [16]. Importantly, a statistically significant E2- dependent increase in H3R17me2 was evident, corresponding with the region of p/CIP/CARM1 recruitment, suggesting the complex is functional on the Jak2 promoter. Reinforcing this idea, we noted that following depletion of p/CIP, E2-dependent H3R17me2 of the Jak2 promoter was greatly reduced in addition to an observable loss of p/CIP recruitment. Finally, siRNA mediated depletion of p/CIP resulted in a reduction in E2-induced transcription of Jak2, with residual E2-responsiveness likely due to functional redundancy between SRC family members. However, depletion of CARM1 caused a more dramatic loss in E2-dependent Jak2 transcription, suggesting that its enzymatic activity is in fact important for the observed

regulation by the p/CIP/CARM1 complex. CARM1 activity has previously been associated with the regulation of a subset of the ER cistrome [50], and herein we provide a specific example of its E2-dependent recruitment, as part of an active coregulatory complex, to the Jak2 promoter. We also observed an increase in phosphorylated STAT3, indicative of activation of the JAK/ STAT signaling pathway. While this activation is not necessarily dependent on Jak2 transcriptional upregulation, this effect was lost following siRNA-mediated silencing of p/CIP or CARM1.

Collectively, while the changes in E2-dependent H3R17 dimethylation are indicative of active Jak2 transcription, the other chromatin marks we examined, as well as the modest response at the RNA and protein levels, were inconsistent with a fully transcriptionally active promoter. We suggest that an additional signal may be required for a more robust transcriptional response of the Jak2 gene. In addition, despite the changes in expression of select genes, the majority of complex targets did not exhibit a widespread transcriptional response to 12 h E2 treatment, ultimately suggesting that E2 may cause a general "rewiring" of specific signaling pathways, through recruitment of the p/CIP/CARM1 complex, so that many essential genes become "poised" for transcription.

2.4 Materials & Methods

2.4.1 Plasmids, antibodies, and reagents

A complete list of primers used can be found in Table B-3 in Appendix B. Antibodies used in this study are listed in Table B-4 in Appendix B. All of the antibodies used for these studies are commercial antibodies, with the exception of the p/CIP

antibody. The method by which this antibody was generated, purified, and tested has been previously described [4]. 17β-Estradiol (water soluble) was purchased from Wisent. All siRNA used was purchased from Dharmacon.

2.4.2 Western Blotting

Cells were grown in phenol red-free DMEM, supplemented with charcoal stripped FBS and antibiotics, and stimulated with 10^{-7} M E2 for various time periods as indicated. Cells were washed twice in phosphate buffered saline (PBS), harvested and lysed in RIPA lysis buffer (~150 μl/60 mm plate) consisting of 50 mM Tris (pH 8.0), 150 mM NaCl, and 1% NP-40, 0.1% SDS and protease inhibitor cocktail. Extracts were centrifuged for 10 min at 14,000 rpm at 4 °C and the soluble fractions were retained. Samples were normalized for protein content and were separated by SDS-PAGE, transferred to nitrocellulose or PVDF membrane and blocked overnight in PBS containing 0.1% TWEEN-20 and 5% nonfat dried milk. The appropriate antibodies were then diluted in blocking buffer and the membrane was probed for 2 h at room temperature with rocking, followed by incubation with secondary antibody for 1 h. Proteins were detected using ECL according to the manufacturer's recommendations (Amersham).

2.4.3 RNA isolation and real-time PCR

Total cellular RNA was isolated using RNeasy kit (Qiagen). The quality and quantity of RNA were evaluated by measuring OD 260/ 280. For real-time PCR analysis, 0.2 μg of RNA was reverse-transcribed with TaqMan reverse transcriptase (Applied Biosystems) using random hexamers to generate cDNA. All amplicons were detected using the 5′ nuclease (Taqman) assay with 5′ labeled probes. Probes were already

predesigned and quality tested (Applied Biosystems). Reactions were performed according to the manufacturer's recommendations (Applied Biosystems) and were run in replicates of two, in a 96-well format. Each reaction included 18S RNA as a control for normalization, and reactions lacking cDNA served as negative controls. Two independent experiments were performed for each gene following treatment with E2, and a mean value was obtained and compared to the mean expression level of each gene from untreated cells. Applied Biosystems 7500 Real Time PCR System software was used to identify cycle threshold (Ct) for each reaction.

2.4.4 RNA microarray analysis

Total RNA was extracted from MCF-7 cells treated with 10 nM E2 for 12 h and from control, untreated cells. Independent biological triplicates were performed for each treatment, including control samples. cDNA was prepared from control and treated samples, labeled and hybridized to HgU133A+2 human affymetrix DNA microarray. Hybridization, washing, scanning and analysis of genechips were performed at the University of Western Ontario, London Regional Genomics Centre (London, Ontario, Canada). An average intensity of each E2-treated sample was compared to the average intensity for control non-treated samples. Three biological replicates for each array were processed and the data was transformed using Robust Multi-Array normalization and values below 0.01 were set to 0.01. Each measurement was normalized by dividing all measurements in that sample by the 50th percentile. Ratios were then calculated for all samples against the median of the control samples. A student **t**-test statistical analysis

was conducted and false positives were reduced using Benjamini and Hochberg false discovery rate.

2.4.5 Chromatin immunoprecipitation assay

MCF-7 cells were cross-linked with 1% formaldehyde at room temperature for 5 min. Cross-linking was quenched by immediately washing cells twice with ice-cold PBS and harvesting in PBS containing PMSF. Cell pellets were lysed in 0.2 ml of cell lysis buffer (50 mM Tris-HCl [pH 8.1], 10 mM EDTA, 1% SDS, and protease inhibitors) and incubated on ice for 10 min. Lysates were sonicated to yield DNA fragments ranging in size from 300- to 1000-bp. Approximately 450µg of the cross-linked, sheared chromatin solution was used for immunoprecipitation. A small portion of each IP was saved as input DNA (5%). Supernatants were diluted 10-fold in dilution buffer (20 mM Tris-HCl [pH 8.1], 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, and protease inhibitors) and immunoprecipitated using a protein A-Sepharose slurry or with MagnaChIP Protein A magnetic beads.

When using the Protein A-Sepharose slurry, lysates were precleared with 50 µl of 50% slurry protein A-Sepharose containing 2.5 µg of sheared salmon sperm DNA for 2 h at 4°C. Immunoprecipitation was performed overnight at 4°C with 1.5-4 µg of the antibodies. 50 µl of protein A-Sepharose containing 2.5 µg of salmon sperm DNA per ml was added to the solution and incubated for 1 h at 4 °C. Magnetic beads were washed using PBS with 0.1% Tween-20, incubated with the relevant antibodies for 2.5hrs at 4 °C, and rewashed prior to immunoprecipitation overnight at 4°C. The beads were washed one time each with wash buffer I (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM

Tris-HCl, 150mM NaCl), wash buffer II (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, 500mM NaCl), wash buffer III (0.25 M LiCl; 1% NP-40; 1% Na-Deoxycholate; 1 mM EDTA; 10 mM TrisHCl) and twice with TE buffer. Immunoprecipitated material was extracted twice with 150 µl elution buffer (1% SDS-0.1 M $NaHCO_3$). If sequential ChIP was conducted, eluted samples were re-Immunoprecipitated overnight at 4°C with 1.5-4 µg of secondary antibodies. Washes and elution steps were repeated. NaCl was added to the final 150 µl eluate to a concentration of 200 mM and the cross-linking was reversed by heating at 65 °C overnight. DNA was purified using Qiagen PCR purification spin columns.

For analysis by conventional PCR, conditions were as follows: initial denaturing cycle of at 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 sec, ~57 °C for 30 sec and 72 °C for 45sec, and a final elongation step of 72 °C for 10 min. For experiments involving E2-stimulation, MCF-7 cells were plated to approximately 90% confluence and treated with 10^{-7}M E2 for 45 min prior to ChIP analysis.

For some experiments, DNA isolated from ChIP experiments was subjected to quantitation by real time PCR using Brilliant SYBR green master mix (Stratagene; 600548). Primers were identified using the Primer Express program (Stratagene) and tested to establish optimum reaction conditions. Reactions were performed in a 25ul volume according to manufacturer's recommendations. The reaction was carried out and measured using Mx3000P realtime instrument. Standard curves were generated using total input DNA (copy number range: $8X10^5$ to $8X10^1$). The IP and IgG DNA copy number was calculated by extrapolating their respective Ct value from the standard curve. The nonimmune IgG copy number was subtracted from IP DNA copy number. The

resulting IP copy number was initially normalized against the total input DNA by dividing the IP by input. The average copy number with E2-treated IPs were then standardized to untreated control IPs, and recruitment represented as a fold-change with E2-treatment. All measurements were done in duplicate and an average Ct value was used to calculate copy number. Two independent realtime reactions were done for each experiment.

2.4.6 ChIP-DSL assay

Chromatin immunoprecipitation coupled to DNA selection and Ligation (ChIP-DSL) was used to assess global promoter occupancy by p/CIP/CARM1. MCF-7 cells were cross-linked with formaldehyde and subjected to standard sequential ChIP–reChIP assay using affinity purified anti-p/CIP and anti-CARM1 antibodies. The procedure for oligonucleotide annealing, solid phase selection ligation and PCR amplification were performed exactly as described (Aviva Systems Biology; H20K, Cat# AK-0504). The antibody-enriched DNA and the total input were biotinylated followed by annealing to the 40mer oligonucleotide pool. The DNA–oligonucleotide complexes are then selected by binding to streptavidin-conjugated magnetic beads, while the non-annealed oligonucleotides are washed away. Correctly paired 40mers are then ligated to form the corresponding 80mer which is flanked by both universal primer annealing sites (T3 and T7) giving rise to a complete amplicon. A PCR reaction was then conducted on the amplicons using fluorescently labeled T7 and regular T3 primers. Total input DNA was PCR amplified using Cy5 (green) labeled T7 primer and the immunoprecipitated (IP) sample was amplified using Cy3 (red) labeled T7 primer. The PCR products are co-

hybridized to the 80mer array (Hu20K) to derive an enrichment ratio for each target. After hybridization and washing, array slides were scanned on a One Virtek (Bio-Rad) Chip Reader, and the ArrayVision (v6.0) software package (London Regional Genomics Centre, London, Ontario, Canada) was used to quantify fluorescence intensity. The ChIP on chip intensity values were normalized using a Lowess curve, which was fit to the log intensity versus log-ratio plot and 20% of the data was used to calculate the Lowess fit at each point. Following normalization, a two-sided student's t-test was conducted where standard deviation of the replicates was used to calculate a p-value. Fold change was calculated for each gene using a mean value that was calculated from all three biological replicates.

2.4.7 Ingenuity Pathway Analysis

Ingenuity Pathways Systems (http://www.ingenuity.com) analysis was employed to group statistically significant genes. The 204 genes that bound p/CIP/CARM1 directly and were transcriptionally affected by E2 were considered for Functional Analysis to identify the biological functions and/or diseases that were most significant to the data set. A right-tailed Fisher's exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone.

2.4.8 Promoter Enrichment Analysis

Promoter sequences were identified using Gene2Promoter within the Genomatix Suite (www.genomatix.de). Input was in the form of gene accession numbers, and comparison with transcripts that have been mapped to the ElDorado genome yielded

mapped sequence results and extracted promoters. A defined 1000bp upstream of the mapped transcriptional start sites were selected for further transcription factor binding site analysis. To search for enriched consenus motifs within ElDorado extracted promoter sequences the BIOBASE Knowledge Library was used, making use of MATCH software and the Transfac database. Searches were performed with the best supported promoters using the vertebrate non redundant (minFP) profile. Background frequencies were determined using control set of human housekeeping gene promoters. Optimized matrix cutoffs and search window positions were used. Significant matrices were found with p-value<0.001 and Yes/No>1.2.

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Chapter 3

3 Whole Genome Analysis of CARM1 in wildtype and CARM1-knockout Mouse Embryonic Fibroblasts

3.1 Introduction

Arginine methylation is a prevalent post-translational modification, found on both nuclear and cytoplasmic proteins, that has been implicated in the regulation of transcription, signal transduction, RNA processing, and DNA repair [1-4]. Additionally, defects in arginine methylation are known to be involved in several disease processes such as cancer [4-6].

The family of proteins responsible for arginine methylation is collectively known as protein arginine methyltransferases (PRMTs). Coactivator-associated Arginine Methyltransferase 1 (CARM1), also known as PRMT4, is a Type I PRMT that catalyzes the formation of asymmetrically dimethylated arginines (aDMA) through a monomethylated intermediate [7]. CARM1 is unique among the PRMTs in that it appears to recognize substrates containing a PGM motif (proline, glycine, methionine), rather than the more common GAR motif recognized by other PRMT family members [1,2,7,8].

CARM1 was the first of the PRMTs to be shown to function as a transcriptional coactivator [1], based on its ability to interact with the p160 coactivator GRIP-1 (also known as SRC2, NCoA2) [1]. It has since been shown to interact with the two other members of the p160 family, SRC1 and SRC3 (p/CIP) [1]. CARM1 can methylate both histone and non-histone proteins, and its ability to synergistically enhance transcription of

steroid-hormone-regulated genes through its methylation of other coregulators such as p300, p160 family member(s), and of histone H3 has been extensively characterized [9-12].

CARM1 has been shown to regulate a wide variety of transcriptionally relevant target genes, in cell cycle control in addition to those involved in hormone-dependent signaling. For example, in response to DNA damage CARM1 methylates the p300 KIX domain. This promotes recruitment of BRCA1 by p300 to the p53-responsive promoter of p21 and promotes p21 transcription and increased expression [3]. Additionally, CARM1 is recruited to the promoter of cyclin E1 (CCNE1) in an E2F-dependent manner, where it functions as a positive regulator of transcription, by methylating histone H3 at arginines R17 and R26 [13]. Deletion of CARM1 in mice results in embryos that are small in size, and die late in development or shortly after birth [14]. CARM1 null mouse embryonic fibroblasts (MEFs), isolated from E12.5 embryos, do not support the methylation of CARM1 substrates, including histone H3, p300 and PABP1, and are defective in NR-dependent signalling [14]. Additionally, NF-kB dependent gene expression, important for cellular events such as apoptosis, cell proliferation and differentiation, is also impaired in CARM1-null MEFs [14]. CARM1 has been shown to form a complex with p300 and NF-kB *in vivo*, directly interacting with NF-kB, and enhancing its promoter-specific recruitment. CARM1 also interacts with β-catenin and positively regulates β-catenin-mediated gene expression, indicated by H3R17 methylation [15]. CARM1 can also covalently modify components of the core transcriptional machinery. CARM1 methylates the C-terminal domain of RNA Polymerase II at a single arginine residue (R1810), creating a docking site for a tudor-

domain containing effector molecule (TDRD3) and promoting transcription [16]. PAF1c, part of the transcription elongation complex, interacts with the CARM1-specific H3R17me2 mark, and affects transcription of CARM1-regulated, estrogen responsive genes, indicating that PAF1c may act as an arginine methylation effector [17].

CARM1 has also been shown to interact with several oncogenic transcriptional regulators, suggesting that CARM1 plays a role in cancer. CARM1 levels have been found to be elevated in aggressive breast tumours [18,19], and in conjunction with elevated SRC3 plays a role in ERα-dependent breast cancer cell differentiation and proliferation [20,21]. Recent studies using large panels of invasive breast cancer samples suggest that CARM1 has an oncogenic effect in breast cancer, and that its expression is a predictor of diminished survival, and of poor disease-free survival [18,19]. CARM1 is also overexpressed in androgen-independent prostate carcinomas, and in colorectal cancers [18,19,22].

The majority of CARM1 studies have been conducted in cancer cell lines, and evaluation of the role CARM1 plays in gene regulation has been primarily focused on its ability to interact with and regulate the function of specific transcriptional regulators with little consideration given to the recruitment of CARM1 on a genome-wide scale, unbiased by association with binding partners.

In this study, we use a ChIP-Seq approach in conjunction with expression microarray to assess the role of CARM1 in genome occupancy and global gene regulation. We conducted our examination in CARM1 wildtype and knockout mouse embryonic fibroblasts (MEFs), a system unaffected by genomic instability in which the

relevance of direct CARM1-dependent gene regulation can be studied. The absence of any extrinsic signal or disease context is quite novel and allows for a broader understanding of CARM1 action, without limiting its functionality to specialized cellular programs or particular disease states. Our characterization of binding events suggests a role for CARM1 in transcriptional elongation, and implicates the transcription factor PAX1 as potential mechanism through which CARM1 can be recruited to the genome. While our findings suggest that direct genomic recruitment of CARM1 is not critical for its transcriptional and functional effect(s) in an un-induced system, we note a critical regulatory role for CARM1 in cellular growth and proliferation, showing that in the absence of CARM1, the expression of many cell cycle regulators is dramatically altered and cells become sensitized to additional stressors.

3.2 Results

3.2.1 Analyzing genome-wide CARM1 recruitment

To identify global genomic interaction sites for CARM1, we performed ChIP-Seq in CARM1$^{+/+}$ MEFs (Figure 3-1A). Approximately 40 million sequenced reads were obtained and processed to eliminate corrupt, short, and/or highly repetitive sequences, before being aligned to the mouse genome (MM9). Based on this alignment and using an FDR of 0.001, 7022 CARM1-dependent peaks were identified. These peaks were subsequently filtered using the Mann-Whitney (MW) U test as an intra-sample statistic to remove false peaks based on strand separation, thereby refining the list to include only high confidence CARM1-enriched regions. Applying a MW p-value of 0.05, we identified 432 enriched regions. Several of these regions were validated using conventional ChIP and ChIP-qPCR (Figure 3-2 and Figure C-1). We determined that

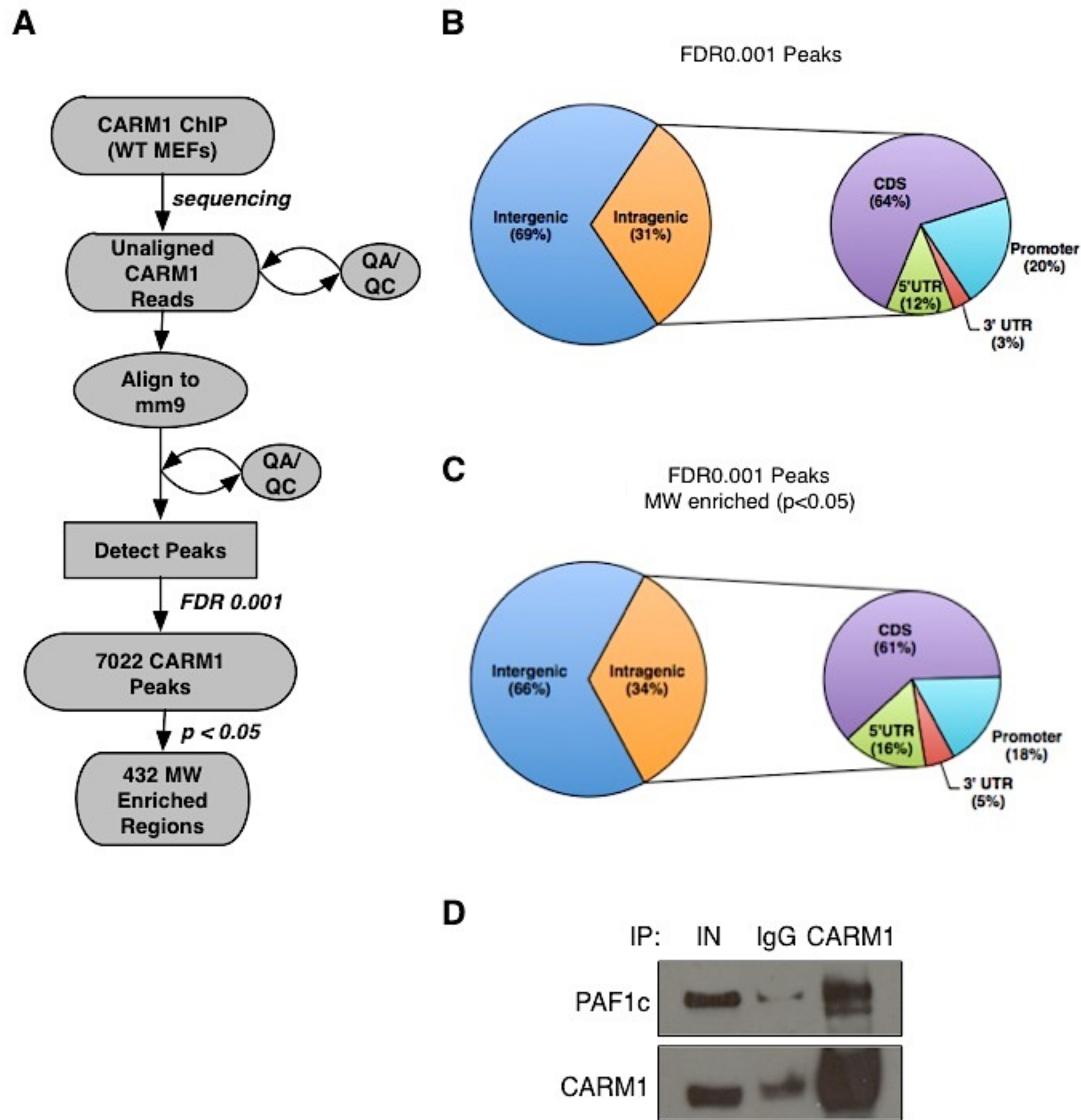


Figure 3-1 Genome-wide identification of CARM1 recruitment sites in MEFs

(A) ChIP-Seq was performed to identify global genomic CARM1 interaction sites. Sequenced reads were aligned to the mouse genome (MM9) and peaks were called using Partek Genomics Suite. (B) Pie chart indicating the genomic distribution of FDR0.001 CARM1 peaks. Promoter was defined as 5kb upstream & 1kb downstream of TSS. (C) Pie chart indicating the genomic distribution of subset of FDR0.001 CARM1 peaks identified as significantly enriched CARM1 binding regions by Mann-Whitney U test ($p < 0.05$). Promoter was defined as 5kb upstream & 1kb downstream of TSS. (D) Association between CARM1 and PAF1c. CARM1 was immunoprecipitated from CARM1$^{+/+}$ MEFs; immunocomplexes were separated by SDS-PAGE and probed with either αPaf1c or αCARM1 antibodies (n=2).

CARM1 binding is fairly frequent and occurs on average every 378 kb in the mouse genome. However, Mann-Whitney enriched regions of CARM1 binding events were more sparse, occurring every 6,174kb. The incidence of CARM1-interacting sites and/or enriched regions showed weak positive correlation with chromosome length (Figure C-1).

When CARM1 enriched regions were annotated to neighboring genes, 153 genes were identified. Interestingly, in some regions, more than one CARM1-binding event can be associated with the same gene (Figure C-1). This pattern is most prevalent on chromosome 8 and X (Figure C-1). However, the functional significance of multiple binding sites is currently unclear.

When we considered the genomic distribution of all 7022 CARM1 binding events, or of the smaller cohort of 432 MW-enriched regions, we found that the pattern of peaks and enriched regions is very similar (Figure 3-1B & C). The majority of binding (66-70%) occurs in intergenic regions, with the remaining 30-34% associated with intragenic elements, which includes the promoter region, 3'- and 5'-UTRs, and coding sequence (CDS). Of the intragenic regions, 17-18% of identified CARM1-interacting sites were found to be promoter-proximal (defined as 5kb upstream and 1kb downstream of TSS), with a further 16% of binding in the 5'UTR, indicative of a regulatory role for CARM recruitment. Contrary to our initial expectations, the majority of CARM1-bound intragenic sites were found to occur within the CDS. These binding events suggest a potential role for CARM1 in alternative splicing, a function in which CARM1 has already been implicated through its methylation of several splicing factors [2]. Alternately, the predominance of CDS binding sites could also be indicative of

participation by CARM1 in transcriptional elongation. To assess this possibility, we performed immunoprecipitation experiments in wildtype MEFs using a CARM1 antibody. Subsequent probing for the elongation-associated RNA polymerase-associated factor 1 complex (Paf1C) revealed that CARM1 can interact directly with Paf1c (Figure 3-1D), suggesting a potential role for CARM1 in transcriptional elongation.

3.2.2 Elucidating the mechanism of CARM1 recruitment

In an attempt to understand the mechanism(s) through which CARM1 is recruited to the chromatin in the absence of an extrinsic signal, enriched transcription factor (TF) binding motifs within all 7022 CARM1 binding events were identified. The most enriched TF consensus motifs were Hunchback (PAX1), ZNF354C, YY1, Gfi, and MZF1 (Figure 3-3A), all of which have been implicated in transcriptional regulation during development and/ or oncogenesis. Further assessment allowed for the identification of an enriched *de novo* motif among peaks (Figure 3-3B). Notably, the motif discovered *de novo* bears striking similarity to the PAX1 consensus motif, which was identified as the most significantly enriched among CARM1 peaks. This concordance suggests that this may be a preferred TF through which CARM1 is recruited to the genome.

Comparison of CARM1 peaks and individual TF motif occurrences across whole chromosomes revealed an expected correlation wherein the top 5 enriched TF motifs frequently coincide with the MW enriched, high-confidence binding regions for CARM1. When specifically comparing CARM1 MW-enriched binding regions with the locations of PAX1 motifs, we observed that high-confidence CARM1 regions corresponded with

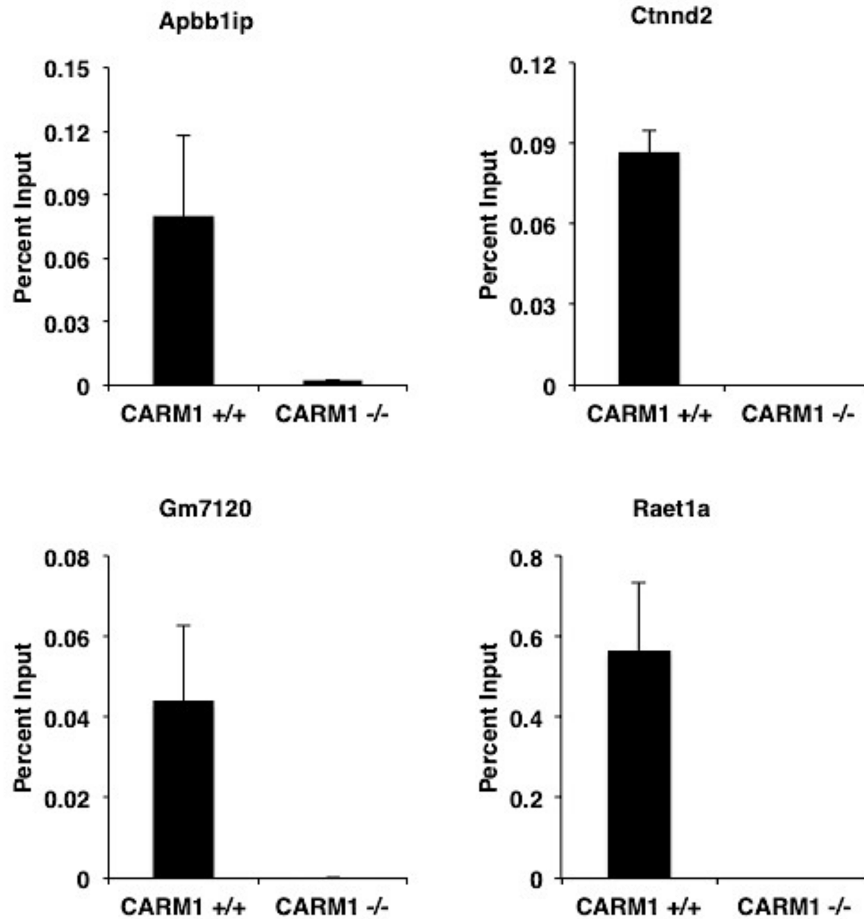


Figure 3-2 Quantitative ChIP analysis validating CARM1 occupancy at sites identified by ChIP-Seq

CARM1 recruitment to Apbb1ip-, Ctnnd2-, Gm7120-, and Raet1d-proximal genomic locations was tested in CARM1$^{+/+}$ MEFs using CARM1$^{-/-}$ MEFs as a negative control. CARM1 ChIPs were standardized to IgG control, and data is represented as mean percent of input and error bars represent standard error of the mean.

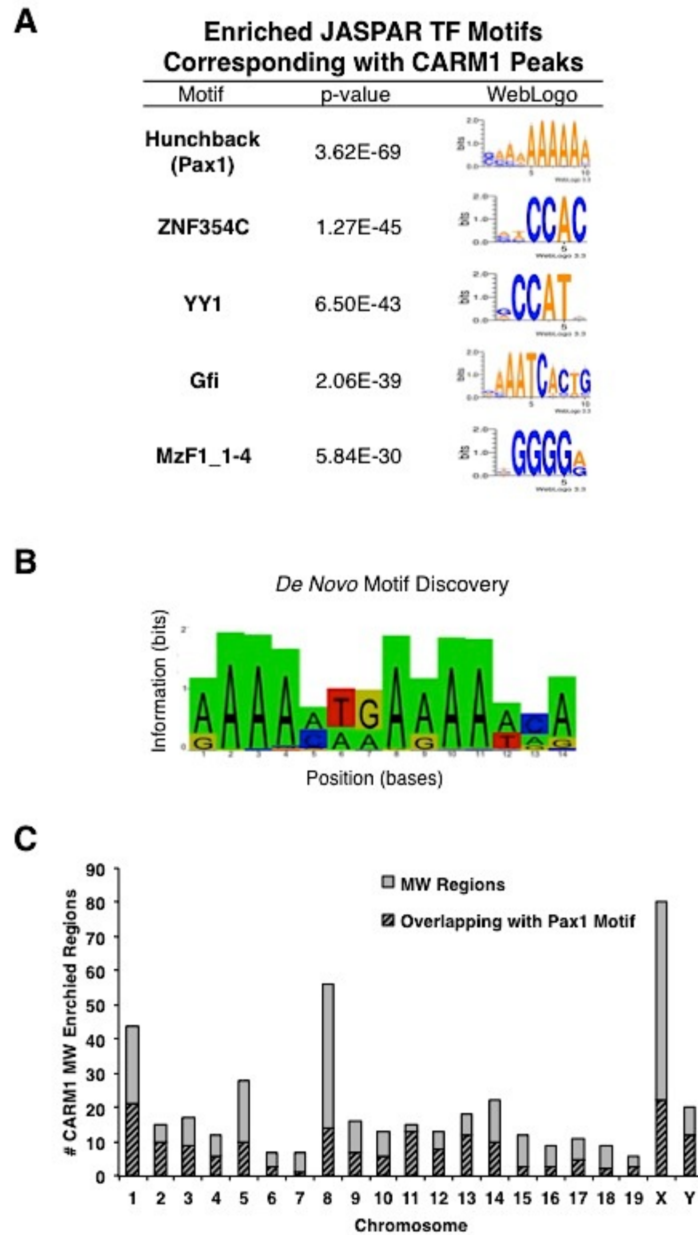


Figure 3-3 Transcription factor motif enrichment within CARM1 peaks identifies PAX1 as possible mechanism of CARM1 recruitment

(A) Enriched JASPAR transcription factor binding motifs found among CARM1 peak sequences identified by ChIP-Seq. (B) 14bp motif predicted using de novo motif discovery in Partek Genomics Suite. Height of each position indicates the importance of a base at a particular location. Different colors were used to represent different nucleic acids: G - yellow; T – red; C - blue; and A - green. (C) Bar graph contrasting the number of MW enriched regions of CARM1 binding on each chromosome (grey) with the number of corresponding PAX1 consensus motifs (hatched).

PAX1 motifs on average 45% of the time (Figure 3-3C). A representative example of this trend on chromosome 14 is shown in Figure C-2.

Several of the CARM1 enriched regions containing putative PAX1 binding sites were selected to assess the role of PAX1 in CARM1 genomic recruitment. Regions were randomly selected from different chromosomes, and in many cases represented different genomic contexts (ex. promoter-associated, within CDS, and/or intergenic). Quantitative ChIP analysis was performed in wildtype and CARM1 null MEFs to validate CARM1 binding at these regions (Figure 3-4A). This analysis was repeated following siRNA-mediated depletion of PAX1 in wildtype MEFs to determine if CARM1 recruitment is dependent on the presence of PAX1. When PAX1 was depleted (Figure C-3), there was a reduction in CARM1 recruitment at analyzed regions (Figure 3-4B), suggesting that PAX1 may facilitate the binding of CARM1 to a subset of genes.

3.2.3 Identifying CARM1-dependent global transcriptional effects

Gene expression profiling by microarray analysis was performed in both wildtype and knockout CARM1 MEFs to assess global transcriptional effects of CARM1. Gene expression profiles for CARM1$^{-/-}$ and CARM1$^{+/+}$ MEFs were compared with an unadjusted p-value cutoff of 0.01. Using this approach, we identified 643 genes which were found to have a 2-fold or greater change in expression; 283 were upregulated, and 360 downregulated in the absence of CARM1 (Figure 3-5A). The findings of the expression microarray were extensively validated, using both Western blotting and real-

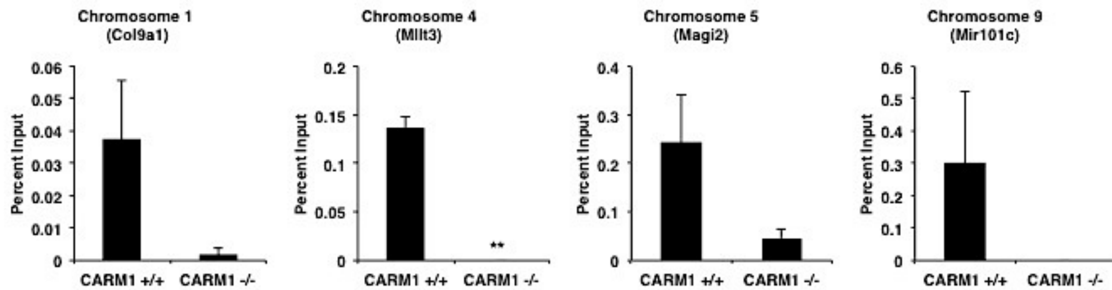
time RT-PCR analysis to confirm the expression of selected genes (data not shown and Figure 3-5B).

In general, the expression changes in the absence of CARM1 were modest, with most up- or down-regulation falling within ±5-fold. The 10 genes showing the greatest increase and 10 genes showing the greatest decrease in expression in CARM1$^{-/-}$ MEFs as compared to CARM1$^{+/+}$ MEFs are given in Table 3-1. Several of these genes have previously been implicated in a variety of cancers and other diseases, affecting cellular programs such as growth, proliferation, p53-mediated apoptosis, cellular migration, attachment, cell signalling, and drug metabolism. However, the range of distinct processes impacted by these CARM1-regulated genes implies that functional importance of CARM1 lies in its ability to act as a master regulator of numerous physiological pathways that can be coopted in disease states.

3.2.4 Changes in gene expression regulated by genomic CARM1 recruitment

To discern targets that may be directly regulated by CARM1 recruitment, expression microarray data was compared with the ChIP-Seq, so that CARM1 peaks could be associated with differentially expressed (DE) genes. Differentially expressed genes were also compared with the smaller list of MW enriched CARM1-binding regions. CARM1-interacting sites within varying distances of the TSS for differentially expressed genes were identified (Table 3-2). As previously discussed, more than one CARM1 peak can be associated with the same gene. This was notably evident when identifying genes directly-regulated by CARM1, and while 147 differentially expressed

A



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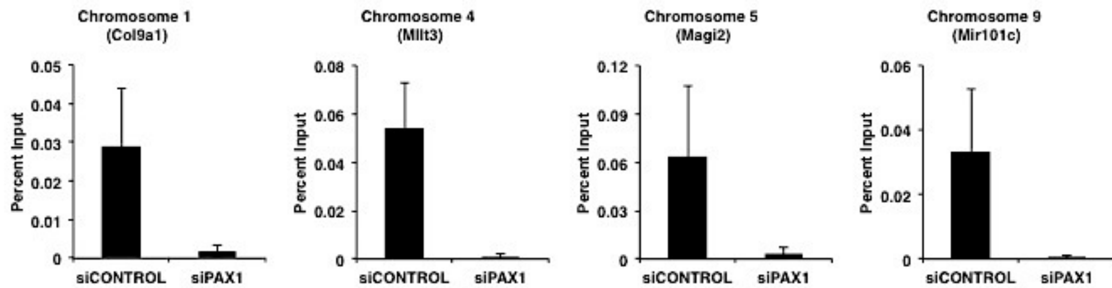


Figure 3-4 PAX1-dependent CARM1 recruitment

Quantitative ChIP analysis testing CARM1 occupancy at sites identified by ChIP-Seq that contain PAX1 TF motif. (A) Validation of CARM1 recruitment to noted genomic locations was tested in $CARM1^{+/+}$ MEFs, using $CARM1^{-/-}$ MEFs as a negative control. (B) $CARM1^{+/+}$ MEFs were transfected using either control siRNA, or an siRNA directed against PAX1, and qChIP analysis at the indicated regions was performed. CARM1 ChIPs were standardized to IgG control, and data is represented as mean percent of input and standard error of the mean.

Table 3-1 Most differentially expressed genes in CARM1$^{-/-}$ MEFs.

Gene Symbol	RefSeq ID	Fold Change
Aspn	NM_025711	-136.352
Postn	NM_015784	-118.104
Thbs2	NM_011581	-40.107
Fam129a	NM_022018	-33.4576
Mmp3	NM_010809	-32.6422
AW551984	NM_178737	-30.314
Gas6	NM_019521	-27.245
Dpt	NM_019759	-26.9105
Arhgdib	NM_007486	-24.9759
Plxdc2	ENSMUST00000028081	-24.1199
Pappa2	NM_001085376	16.9961
Cp	NM_001042611	20.1459
Tmem176b	NM_023056	20.1924
Tmem176a	NM_025326	21.6729
Anpep	NM_008486	22.1114
2610305D13Rik	NM_145078	25.2344
Prl2c3	NM_011118	25.6564
Cyp1b1	NM_009994	25.7046
Pde3b	NM_011055	28.3394
Peg3	NM_008817	34.6941

Table 3-2 Differentially expressed CARM1-associated genes.

	# CARM1-associated genes		% CARM1-dependent expression	
	Total*	**Unique**	**Up-regulated**	**Down-regulated**
1kb	92	52	43	57
5kb	112	58	46	54
10kb	128	63	49	51
50kb	340	147	43	57

*Total # genes counts the same gene(s) more than once if multiple CARM1 peaks are within the noted distance

genes were found to have one or more CARM1 peak(s) within 50kb, a total of 340 peaks could be associated with these genes. However, the presence of more than one CARM1 binding event did not affect the degree to which genes were up- or down-regulated. Of the 147 unique DE genes, 43% were transcriptionally upregulated, and 57% downregulated (Figure 3-6A), proportions that are consistent with the overall trend of transcriptional changes following CARM1 loss (Figure 3-5A).

Only 5% of peaks were associated with differentially expressed genes when CARM1 binding occurred within 50kb of the TSS, and less than 2% if binding was more proximal [5kb to TSS], suggesting that direct genomic recruitment of CARM1 is not critical to its transcriptional effects in the absence of extrinsic signal.

3.2.5 Functional consequences of CARM1-dependent global transcriptional changes

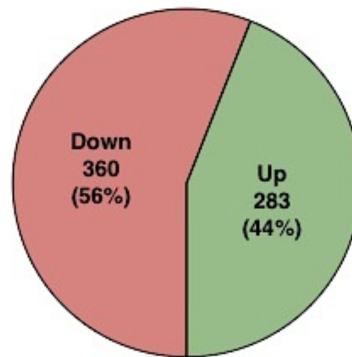
Gene ontology (GO) analysis was performed to more fully characterize the relevance of CARM1-dependent global transcriptional changes. Enriched functional groups were reported if they had more than 2 genes represented and GO terms were grouped according to their relevance in biological processes, molecular functions, or with respect to cellular component. When GO analysis was conducted on CARM1-dependent differentially expressed genes, 2454 GO terms were identified. By comparison, when GO analysis was conducted on the subset of DE genes that had a peak or MW enriched region within 50kb of the TSS, the number of corresponding terms was substantially reduced (Figure 3-6B). This observation reinforces our suggestion that CARM1 genomic binding

events are most likely not required for CARM1-dependent transcriptional or functional consequences.

Terms were initially ranked based on attribute score, with a higher score indicating processes that may be of particular importance to the data set, ostensibly those processes that are influenced most by CARM1. As expected, protein arginine methylation is notably affected by loss of CARM1, with associated methyltransferase processes corresponding to 7 of the 10 highest ranked GO terms. Steroid hormone signalling, and acetylation of histones were also ranked highly; corresponding with the most extensively characterized cellular functions of CARM1. Both of these functional changes have been extensively characterized in the literature, and were independently validated in our study [data not shown].

Taking an alternate approach, enrichment score was used to rank GO terms. A greater enrichment score suggested that genes in a given functional group were overrepresented among CARM1-regulated genes. The most prevalent molecular functions associated with the differentially regulated genes were transcription factor activity and protein binding, again corresponding with the known roles of CARM1. Among the associated biological processes, metabolic processes and growth were identified as two of the most represented functional categories (Figure 3-7A). Growth characteristics that were affected by the loss of CARM1 included cellular proliferation, cell cycle, cell shape and adhesion. Based on these findings we assessed differences in cellular proliferation between wildtype and CARM1 null MEFs. CARM1 null MEFs proliferated at a reduced rate when compared to wildtype (Figure 3-7B) and were also

A



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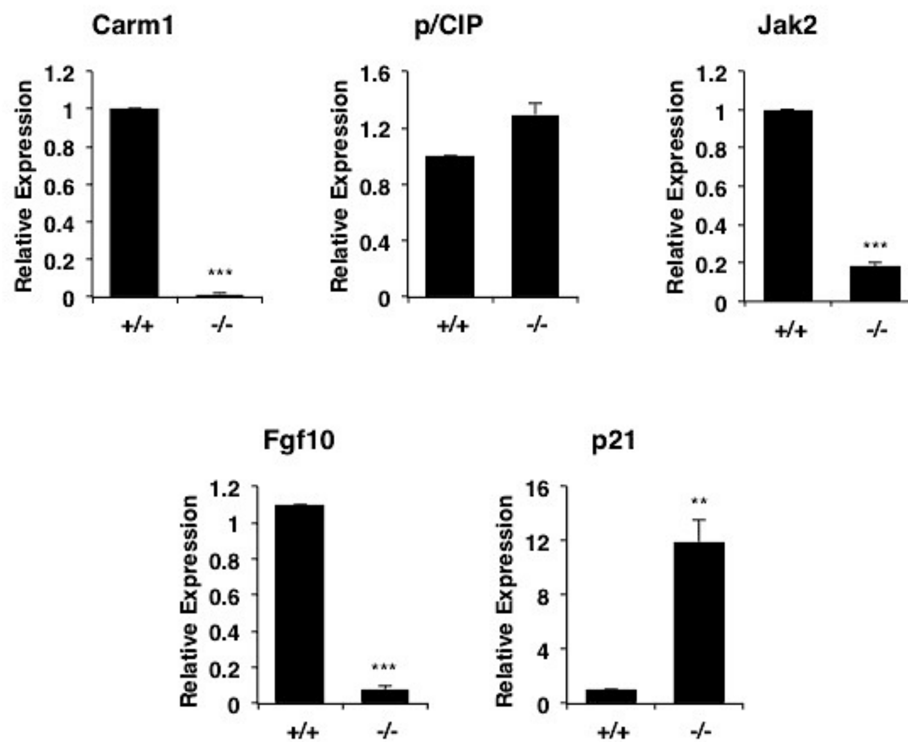

Figure 3-5 Microarray expression analysis of global expression changes in the absence of CARM1

(A) Proportion of differentially expressed genes in CARM1$^{-/-}$ as compared to CARM1$^{+/+}$ MEFs. (B) Realtime RT-PCR analysis of selected differentially expressed genes (p/CIP, Jak2, Fgf10, p21, CARM1) in CARM1$^{-/-}$ and CARM1$^{+/+}$ MEFs. Data is expressed as means and standard error of the mean from repeated experiments, performed independently (n=2 or 3).

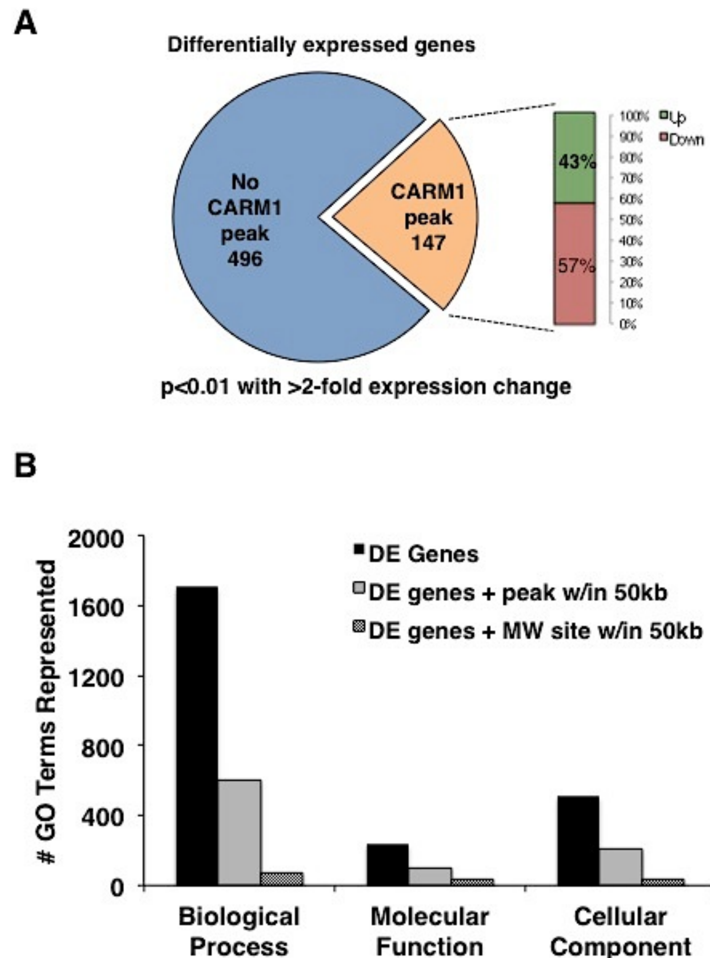

Figure 3-6 Correlating direct genomic recruitment of CARM1 with changes in expression

(A) Pie chart indicating the proportion of genes differentially expressed in CARM1 null MEFs as compared to wildtype, which have a CARM1 peak within 50kb of the TSS as identified by ChIP-Seq. The percentage of these directly targeted, differentially expressed genes that are up- and down-regulated is also indicated. (B) Bar chart comparing the number of GO terms represented among genes differentially expressed (black bars) in the absence of CARM1, with those differentially expressed genes that have a CARM1 peak (grey bars) or MW enriched region (hatched bars) associated within 50kb. GO terms are subcategorized into biological processes, molecular functions, and cellular component.

shown to have reduced diameter and cell volume (Figure 3-7D).

Expression microarray results implicated CARM1 in the regulation of many proteins important for the G1 to S phase transition of the cell cycle (Figure 3-8A). To confirm the critical nature of CARM1 in cell cycle control, the expression of a panel of cell cycle regulators was assessed (Figure 3-8B). We found that p21 mRNA (Figure 3-5B) and protein levels (Figure 3-8B) were increased in the absence of CARM1, with p27 similarly upregulated (Figure 3-8B). Downstream of these proteins, CDK-2 protein levels were undetectable in CARM1 null MEFs. In addition to the ability of p21 and p27 to inhibit its activity, this CARM1-dependent depletion of downstream factors may suggest redundancy in regulation. Also consistent with microarray results, we found that RB was slightly upregulated in the absence of CARM1 (Figure 3-8B). CDK-4 and CDK-6 levels were not significantly altered (not shown), and while the upstream cyclin dependent kinase inhibitors p15 and p16 were both shown by microarray to be significantly downregulated in the absence of CARM1, protein levels were minimal, and we were unable to confirm this change in expression. To determine if the extensive involvement of CARM1 in affecting the expression of cell cycle regulators had functional impact, we subjected wildtype and knockout MEFs to the additional stress of a cell replication inhibitor, hydroxyurea (HU), and assessed cellular proliferation. When treated with HU, the proliferation of CARM1$^{+/+}$ MEFs arrests (Figure 8C). By comparison, the effect of HU on CARM1$^{-/-}$ MEFs was much more severe. Not only was proliferation inhibited in the knockout MEFs; cell number was notably diminished with increasing time post-treatment (Figure 8B). The observations that in the absence of CARM1 the expression of many cell cycle regulators is dramatically altered, and that cells become sensitized to

additional stressors, suggests an important role for CARM1 in maintaining cellular growth and proliferation.

3.3 Discussion

In the present study we assessed the role of CARM1 in transcriptional regulation using a global genomic approach. The importance of CARM1 in transcriptional regulation is becoming increasingly appreciated, and several studies have assessed CARM1 activity on a genome-wide scale, as indicated by the presence of asymmetrically dimethylated histone H3R17 [23], or the presence of coregulators specifically methylated by CARM1 [24]. Furthermore, our previous work examined the recruitment of CARM1 as part of a p/CIP/CARM1 coregulatory complex to a subset of E2-dependent promoters in MCF-7 breast cancer cells [25]. However, these studies have not considered CARM1 recruitment in the absence of an extrinsic stimulus or interacting partners mediating its recruitment.

Herein we present the first direct assessment of genome-wide CARM1 recruitment. Using ChIP-Seq, we identified 7022 genome-wide CARM1 binding events, and highlighted a subset of 432 statistically enriched, high confidence regions. In addition, microarray analysis of wildtype and CARM1-null MEFs revealed 643 differentially expressed genes; 147 of these genes were shown to have a CARM1 binding event occur within 50 kb, and 58 differentially expressed genes have binding that occurs within 5kb (Table 3-2). Therefore, CARM1 recruitment does not appear to be predictive of gene expression, at least not in the absence of an extrinsic signal.

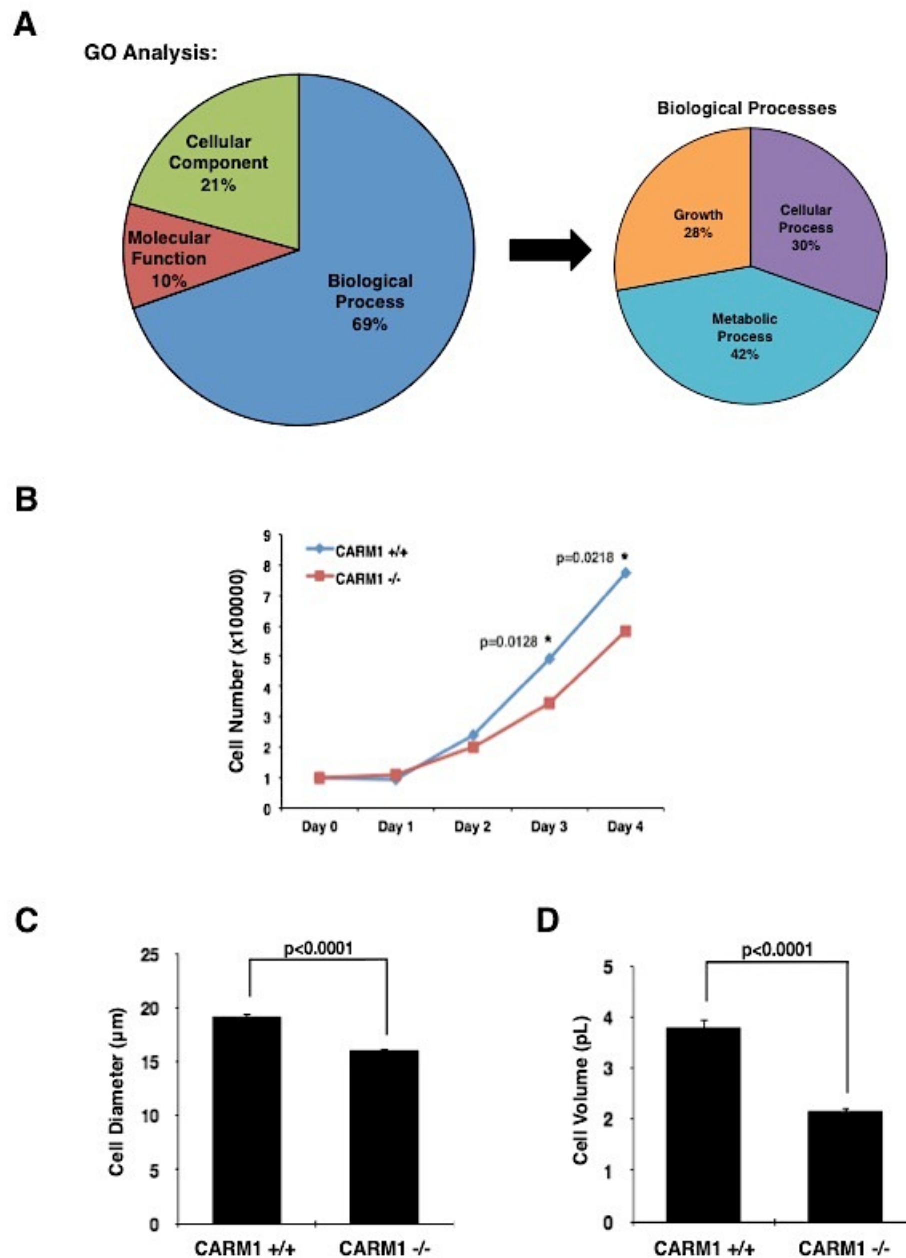

Figure 3-7 Characterizing some of the functional consequences of CARM1 loss

(A) Pie chart representing enrichment of GO terms implicated among genes differentially expressed in CARM1$^{-/-}$ vs. CARM1$^{+/+}$ MEFs. Analysis indicates a prevailing role for CARM1 in biological processes, specifically metabolic processes, and growth, and cellular processes. (B) The effects of CARM1 on cellular proliferation. The Moxi Z automated cell counter was used to assess cellular proliferation in wildtype and CARM1-null MEFs as well as (C) cell diameter, and (D) cell volume.

Characterization of genomic CARM1 activity in MCF7 cells by Lupien *et al.* identified 4088 sites of H3R17 dimethylation in cells that were not treated with E2 [23]. Despite the difference in cell lines between their study and ours, the distribution of H3R17me2 bears striking similarity to the distribution of CARM1 binding events we observed. Corresponding to the observation that 55% of dimethylated H3R17 exists in intergenic regions, we noted that CARM1 also predominates intergenically (66-69%), where binding events occur some distance from transcriptional start sites.

Another consistent feature of both studies is the prevalence of CARM1 binding events (19-20%), and H3R17me2 enrichment (~40%) within the coding sequence of genes [23]. Due to the frequency of CARM1 binding within the coding sequence (CDS), coupled with previous evidence suggesting that the transcriptional elongation-associated Paf1c complex interacts with H3R17me2 [17], we assessed whether these two factors directly interact, and show by co-immunoprecipitation that Paf1c interacts with CARM1 (Figure 3-1D). Wu *et al*. demonstrated that in the absence of CARM1 and a corresponding loss of H3R17me2 genome-wide, Paf1c occupancy at E2-dependent proximal promoters was decreased [17]. We would suggest that this observed loss of occupancy might, at least in part, be a direct consequence of the absence of CARM1.

Investigation of enriched transcription factor binding motifs within CARM1 binding regions was performed to determine whether a previously unidentified protein facilitates CARM1 genomic interaction in an un-induced model. Interestingly, of the five most enriched TF motifs identified in our study, three of them have previously documented roles in transcriptional repression [26-28]. We found that the most enriched transcription factor motif among CARM1-binding regions recognized PAX1, and that

PAX1 motifs correspond with the most statistically enriched, high-confidence binding regions for CARM1 in 45% of cases (Figure 3-3C). We evaluated and confirmed CARM1 recruitment at several regions containing PAX1 recognition sites, and subsequently demonstrated that CARM1 binding at these regions is diminished when PAX1 was depleted (Figure 3-4). In 2012, CARM1 was reported to interact with PAX7 [29]. Deletion construct analysis conducted by that group revealed the CARM1-binding region to be found within the paired domain, and suggested that this ability to interact with CARM1 may be conserved among the paired box domain (Pax) family, all members of which share this domain [30]. Collectively, this evidence suggests that Pax family members, may mediate a subset of genome-wide CARM1 recruitment, and herein we present supporting examples of PAX1-dependent CARM1 recruitment. PAX proteins function as transcription factors, and are important for many cellular processes during embryonic development [31]. During development, PAX1 is expressed in the skeleton, thymus, as well as the 3rd and 4th pharyngeal pouch [31]. Similar to the CARM1 knockout mouse [14], PAX1 null mice have a reduced thymus [32]. In addition, the PAX1 knockout has abnormalities in development of the tail, vertebral column, sternum, and scapula [33]. CARM1 has been shown to be important for endochondral bone formation [34], and CARM1 null mice are much smaller than wildtype counterparts [14]. The similarities between PAX1 and CARM1 knockout mice suggest a potential overlap in developmental regulatory roles. It is likely that the role of PAX1 in recruiting CARM1 to the genome is relevant only during developmental stages, since its expression is generally limited to this stage [30,35].

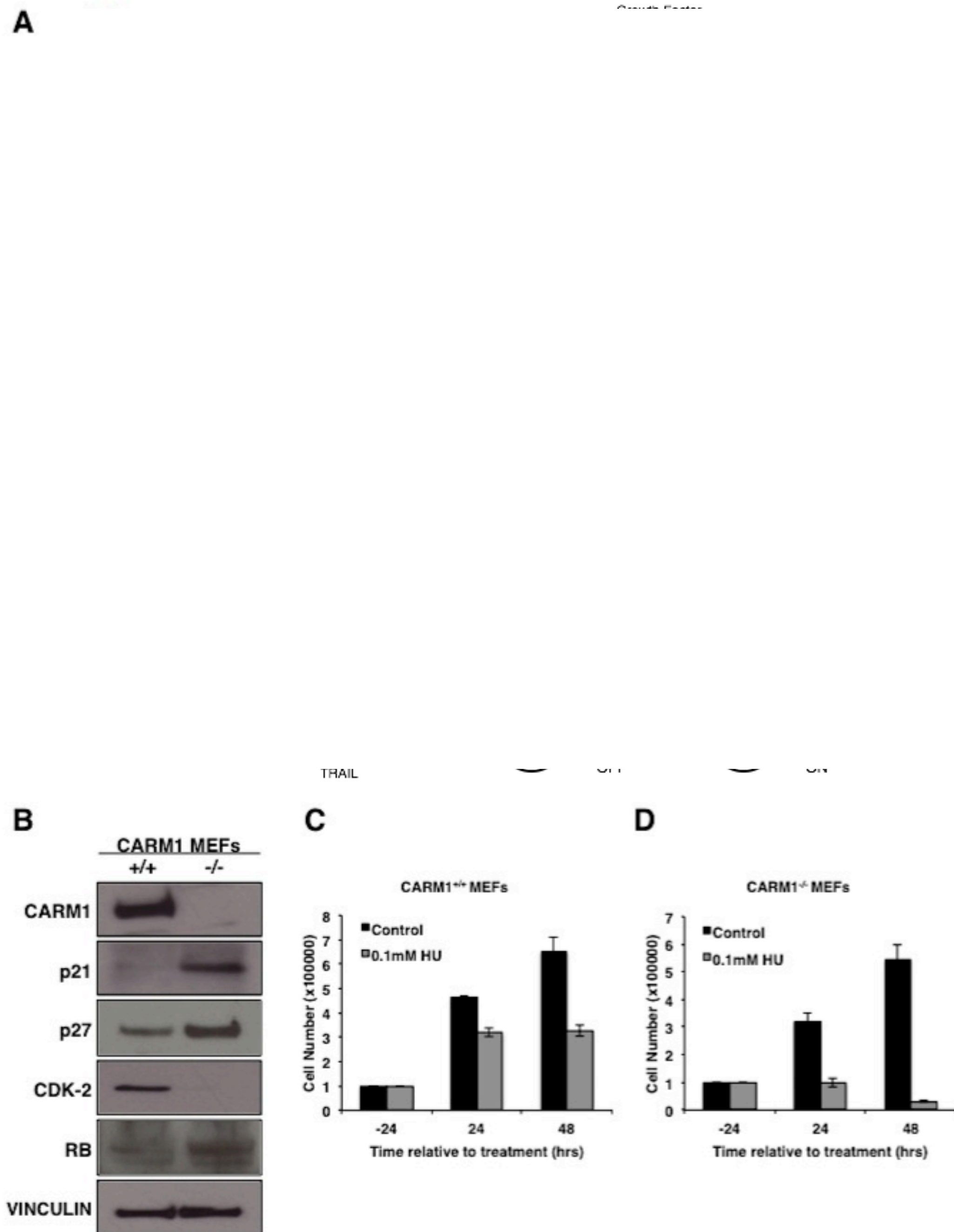

Figure 3-8 CARM1-dependent regulation of the cell cycle proteins.

(A) Overview of proteins involved in the G1-S phase transition of cell cycle, adapted from Cell Signalling Technology. (B) Western Blot analysis analyzing the expression of cell cycle regulatory proteins (p21, p27, CDK-2, RB) in CARM1$^{-/-}$ and CARM1$^{+/+}$ MEFs. Vinculin was used as a loading control, and representative images are shown from at least two independent replicates. (C & D) The Moxi Z automated cell counter was used to assess cellular proliferation in (C) wildtype and (D) CARM1-null MEFs following treatment with 0.1 mM hydroxyurea (HU). MEFs were seeded 24hrs prior to exposure to HU, and proliferation was assayed following 24 and 48hrs of HU exposure. Data is expressed as a bar chart, depicting the mean and standard error of the mean from two independent experiments.

Functional analysis revealed cellular growth to be one of the primary biological processes affected by CARM1 loss (Figure 3-7A). Consistent with this result, we showed that CARM1$^{-/-}$ MEFs proliferate at a reduced rate when compared with CARM1$^{+/+}$ MEFs (Figure 3-7B). Furthermore, the CARM1-null cells were smaller in diameter and had a reduced cell volume (Figure 3-7 C & D). These observations are consistent with our findings that a substantial number of genes involved in cell cycle control were differentially expressed in the absence of CARM1 based on microarray analysis (Figure 3-8A). Cell growth in MCF-7 cells that are depleted of CARM1 has been shown to be reduced [13]. These cells have a corresponding increase in E2F1, and recruitment of CARM1 to the E2F1 promoter is evident [13]. Previous studies also reveal that CARM1 is recruited to the promoter of the cell cycle regulator cyclin E1, which it positively regulates in response to E2 [36]. While cyclin E1 and E2F1 are relatively downstream proteins in the signalling cascade that controls G1-S phase transition in the cell cycle, we notably observed CARM1-dependent differential expression in several of the more upstream regulators (Figure 3-8A). The cyclin-dependent kinase inhibitors p21 and p27 were both upregulated in the absence of CARM1, and CDK-2 expression is also diminished (Figure 3-8B). While our ChIP-Seq did not reveal CARM1 binding events in the promoters of p21 or p27, this does not exclude the possibility that these genes could be directly targeted by CARM1 in an induced system. Alternatively, these cell cycle regulators may be modulated by CARM1-dependent changes in TF and/or coregulator activity. For example, a mechanism for the regulation of p21 involving CARM1 has been previously characterized, wherein p53 and BRCA1 cooperate with p300 and CARM1 in response to DNA damage to induce expression of p21 [3]. Further demonstrating the

critical nature of CARM1-dependent changes in the expression of cell cycle proteins, the addition of a stressor in the form of hydroxyurea (HU) to the compromised cell cycle of the CARM1$^{-/-}$ MEFs resulted in substantial loss of cell viability (Figure 3-8D) as compared to the proliferative pausing seen in the uncompromised wildtype MEFs (Figure 3-8C).

This study represents the first direct assessment of CARM1 recruitment on a genome-wide scale. We describe a pattern of CARM1 binding consistent with other transcriptional regulators, and suggestive of involvement in transcriptional elongation. Furthermore, we identify PAX1 as a novel interacting protein for CARM1 and a likely mechanism for CARM1 recruitment during development. Finally, while direct recruitment of CARM1 does not appear to be essential for the transcriptional change(s) observed, regulation of cellular growth and proliferation appear to be critically regulated by CARM1.

3.4 Materials and Methods

3.4.1 Cell Culture and Reagents

The generation of CARM1$^{-/-}$ mice, and extraction of MEFs has been previously described [14]. CARM1$^{-/-}$ and age-matched wildtype (CARM1$^{+/+}$) MEFs were cultured in Dulbecco's Modified Eagle Medium (Wisent), supplemented with 10% Fetal Bovine Serum (Wisent), and penicillin/streptomycin (Wisent), and maintained at 37°C and 5% CO_2. All siRNA used in this study was purchased from Dharmacon, and transfection protocol was followed according to the manufacturer's recommendations. Cellular

growth characteristics were assessed using the Moxi Z automated cell counter (ORFLO Technologies). Hydroxyurea (HU) was purchased from Sigma (catalogue No.127-07-1). For cell proliferation assays, 10^6 cells were seeded in 6-well plates, allowed to attach for 24hrs and then trypsinized and counted every 24hrs over 4 days. HU was added 24hrs post-seeding, and trypsinizing-cell counting cycles were started 24hrs later.

3.4.2 Protein Extraction and Western Blotting

Cells were harvested in RIPA lysis buffer, consisting of 50mM Tris (pH 8.0), 150mM NaCl, 1% NP-40, 0.1% SDS, and protease inhibitor cocktail. Extracts were incubated on ice for 10 min and cleared by centrifugation for 10 min at 15,000 rpm at 4°C. Protein concentrations were determined, normalized, and proteins separated by SDS-PAGE, transferred to PVDF membrane, and blocked overnight in PBS containing 0.1% Tween-20 and 5% nonfat dried milk, or with TBS containing 0.1% Tween-20 and 5% BSA. Membranes were probed with specific primary antibodies for 2 hrs at room temperature, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 hour. Signals were detected using ECL according to the manufacturer's recommendations (Amersham). Affinity purified anti-p/CIP antibody was generated as previously described [37,38]. All other antibodies used were commercially purchased: CARM1 (Bethyl A300-421A and Epicypher 13-0006), SMAD2/3 (Santa Cruz sc-6202), p21 (Santa Cruz sc-6246), p27 (Santa Cruz sc-528), CDK-2 (Santa Cruz sc-6248), PAF1c (Bethyl A300-173A). An antibody recognizing RB (Santa Cruz sc-7905) was generously provided for our use by Dr. Fred Dick (UWO).

3.4.3 RNA Isolation and real-time PCR

Total cellular RNA was isolated using RNeasy kit (Qiagen) in accordance with the manufacturer's directions. The quality and quantity of RNA was evaluated based on relative absorbance at OD 260/280. For quantitative real-time PCR analysis, 2µg of RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Amplification was detected using predesigned and quality tested 5' nuclease Taqman probes (Applied Biosystems). Reactions were performed in accordance with manufacturer's recommendations and technical duplicates were run in a 96-well format using StepOne Plus Real-time PCR System (Applied Biosystems). Gene expression levels were determined based on the cycle threshold (Ct) and 18S ribosomal RNA was used for normalization. Reactions in the absence of cDNA served as negative controls.

3.4.4 Expression Microarray

Total RNA was extracted from CARM1$^{+/+}$ and CARM1$^{-/-}$ MEFs, as described above. Duplicate experiments were performed. cDNA was prepared from each sample, labeled, and hybridized to Mouse Gene 1.0 ST arrays. Hybridization, washing, scanning, and analysis of gene chips were performed at the University of Western Ontario, London Regional Genomics Centre (London, Ontario, Canada). Probe level data was generated and summarized to gene level data in Partek Genomics Suite and adjusted for background. A 1-way ANOVA was performed contrasting expression in CARM1$^{-/-}$ and CARM1$^{+/+}$ MEFs and a list of genes that showed a 2-fold or greater change in expression with an unadjusted p-value of 0.01 was generated.

3.4.5 Chromatin Immunoprecipitation and ChIP-Seq

CARM1$^{+/+}$ and CARM1$^{-/-}$ MEFs were cross-linked with 1% paraformaldehyde at room temperature for 10 min. Cross-linking was quenched by washing cells twice with ice cold PBS containing PMSF. Cell pellets were lysed in lysis buffer (50mM Tris-HCl [pH 8.1], 10mM EDTA, 1% SDS, and protease inhibitors) and incubated on ice for 10 min. Lysates were sonicated to yield chromatin fragments approximately 300bp – 1000bp in length, and ChIP experiments were performed as previously described [25,39], using antibodies against CARM1 and IgG (2-8μg).

Immunoprecipitated DNA was purified using QIAquick PCR Purification Kit (Qiagen), and was analyzed either by conventional end-point PCR, or by quantitative PCR following ChIP (qChIP), as previously described [25,39]. Quantitation was performed on a StepOne Plus Real-time PCR System (Applied Biosystems). The signal from IgG ChIP experiments was subtracted from the signal obtained using the specific antibody.

The chromatin immunoprecipitation assay used prior to deep sequencing analysis was modified such that sonication conditions produced genomic fragments in the 250-350 bp range. The isolated DNA was sequenced at the University of British Columbia (http://www.cmmt.ubc.ca/facilities/services/sequencing) using the Illumina sequencing platform. The obtained reads were then aligned to the mouse genome (MM9) in Partek Flow using Bowtie (version 0.12.7). Peaks were called using 125bp window in Partek Genomics Suite. Primers used for ChIP experiments are listed in Appendix C.

3.4.6 Transcription Factor Motif Enrichment Analysis

Partek Genomics Suite was used to identify enriched TF binding motifs within CARM1 binding regions. Briefly, peak sequences were scored against motif models from the JASPAR database (http://jaspar.cgb.ki.se/). Motif instances within CARM1 peaks were called if their score exceeded 0.7 sequence quality. CARM1 bound sequences were also interrogated using Partek Genomics Suite to identify a *de novo* binding motif using the Gibbs motif sampling method [40]. A 14bp *de novo* binding motif was identified by this approach CARM1 peak sequences.

3.4.7 Gene Ontology Analysis

GO analysis was performed using Partek Genomics Suite. Enriched functional groups were identified using Fischer's Exact. Functional groups were reported if 2 or more genes were represented. Gene Ontology default background file (Mus musculus-2012-11-19-MoGene-1_0-st-v1) was used for gene background correction.

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Chapter 4

4 General Discussion

4.1 Thesis Summary

The focus of this thesis is the role of CARM1 in global gene regulation. CARM1 is known to methylate a wide array of proteins important for transcription, including transcription factors, coregulators, and components of the core transcriptional machinery [1-3]. However, its characterization to date has been limited by an emphasis on its ability to interact with and regulate the function of specific transcriptional regulators on a gene-by-gene basis and in response to extrinsic signals [4-6]. In Chapter 2, I extend the current understanding of CARM1 in one of its more well-defined roles, as a secondary coregulator in E2-dependent gene expression. Using a global genomic approach, I have characterized its participation as part of a coregulatory E2-dependent p/CIP/CARM1 complex, identifying a subset of gene promoters to which this complex is recruited in response to E2 [7]. I focus on the recruitment of a functional p/CIP/CARM1 complex to the proximal promoter of Jak2, and demonstrate that complex recruitment leads to transcriptionally permissive changes in histone modifications.

In Chapter 3 I identify the genomic sites with which CARM1 interacts in a specific cell system irrespective of binding partner(s) and in the absence of induction. This characterization of global CARM1-binding events suggests a role for CARM1 in transcriptional elongation. Additionally, I have identified the PAX1 transcription factor as a potential binding partner for CARM1 based on the inability of CARM1 to bind regions containing PAX1 consensus motifs following siRNA-mediated depletion of

PAX1. Functional analysis of CARM1-dependent genes implicates a critical role for CARM1 in cellular growth and proliferation. CARM1 has previously been shown to affect cell growth [8], and affect specific cell cycle regulators [8-10]. I demonstrate that hydroxyurea treatment of wildtype MEFs, in which cell cycle proteins are expressed normally, results in proliferative pausing. By comparison, in CARM1 null MEFs there are widespread changes in the expression of cell cycle regulators and hydroxyurea treatment results in loss of viability, suggesting a role for CARM1 in sensitizing cells to cell cycle stress.

4.2 p/CIP/CARM1 Complex Recruitment

p/CIP is a well validated nuclear receptor coactivator that has been shown to associate with numerous ER-dependent promoters including the pS2 (also known as TFF1) in response to E2 treatment in MCF-7 cells [11]. p/CIP is known to act as a scaffold protein, and facilitate the assembly of coregulatory complexes [11,12]. In addition, p/CIP and CARM1 have been shown to synergistically coactivate transcription [13]; and subsequent studies showed by sequential ChIP analysis that a p/CIP/CARM1 complex is recruited to pS2, facilitating its transcriptional activation [4,5].

Using a ChIP-DSL approach, 204 additional proximal promoters to which the p/CIP/CARM1 complex binds in response to E2 were identified (Table B-1). Although ChIP-DSL technology is now outdated, the assay is highly sensitive, and eliminates many of the biases of the ChIP-on-chip whole-genome tiling arrays more commonly used at the time. ChIP-DSL was described in 2007 [14]. This technique uses immunoprecipitated

DNA only as a template to mediate annealing and ligation of aligned oligonucleotide pairs, instead of directly amplified DNA for hybridization. Adjacent oligonucleotide pairs ligate to form complete amplicons, which corresponding to regions from unique gene promoters. The use of unique promoter sequences eliminates potential interference from repetitive or related sequences during amplification. The primary limitation of this approach is that analysis is restricted to the 1 kb of upstream regulatory promoter sequence of genes found on the array. However, a major advantage of the approach is that targeted sequences are directly associated with known genes, thereby eliminating the complicated process of assigning responsive genes to distant binding sites.

In 2010, global E2-dependent binding sites for p/CIP were identified [15]. This study suggested that only a minority (~3%) of E2-dependent p/CIP binding occurs within 500 bp of the TSS, a finding that is consistent with accumulating genomic analysis indicating that ER primarily interacts with regions some distance (>10kb) from the TSS [16,17]. In addition, the level of CARM1 activity (as assayed by its methylation of H3R17 and/or indirectly p/CIP binding [based on recognition of arginine-methylated p/CIP], across the genome also appears to cluster predominantly upstream of the 1kb promoter region [18]. Despite the fact that genomic binding of these factors is becoming increasingly understood to be more prevalent outside of the promoter context, promoter proximal interactions do occur in response to E2 and these binding events can have transcriptional consequences. Our discovery of 204 E2-dependent target promoters for the p/CIP/CARM1 complex corresponds with the observations of a limited set of proximal promoter binding sites for p/CIP, and with the evidence of minimal CARM1 methylation activity at promoter elements.

Both p/CIP and CARM1 have been implicated in aggressive breast cancer [19-21], with demonstrated roles in E2-induced proliferation of the MCF-7 breast cancer cell line [22-24]. Ingenuity Pathway Analysis conducted on the identified p/CIP/CARM1 targets implicated a notable proportion of these genes as having an involvement in cancer, with several cancer initiation- and progression-related molecular and cellular processes ranking highly (Figure 2-5). Collectively, these data suggests that while promoter-proximal recruitment of E2-dependent transcriptional regulators is rare, recruitment of the p/CIP/CARM1 complex to a subset of promoters is likely functionally relevant.

4.3 Transcriptional effect of the E2-dependent p/CIP/CARM1 complex recruitment

Past studies featuring microarray analysis following E2-stimulation of MCF-7 cells can be broadly categorized based on the length of hormone treatment. There is an observable difference in the pattern of expression change, such that at early time points (<6 h) more genes are upregulated and more variation is evident between time points, while at later time points (>12 h) there is a more stable pattern of expression change and the majority of genes are downregulated [16,25]. We examined changes in mRNA expression following 12 h of stimulation with E2, and showed that approximately 20% of p/CIP/CARM1 complex target genes identified by ChIP-DSL were transcriptionally altered (16.2% upregulated and 3.9% downregulated). The relatively small number of differentially expressed target genes after 12 h suggests that proximal recruitment of the p/CIP/CARM1 complex is not predictive of E2-dependent gene expression at this late

time point. However, that does not necessarily mean that the p/CIP/CARM1 complex is not active in regulating transcription. Transcription of protein coding genes is primarily performed by RNAPII [26]. As an alternate method of determining immediate transcriptional response assessment of RNAPII recruitment to proximal promoters in response to extrinsic signal, and corresponding to coregulator or transcription factor recruitment, is suggestive of active gene expression [16,17]. When the list of p/CIP/CARM1 complex targets is compared with RNAPII-bound promoters [16], 86/204 (42%) genes were bound by both p/CIP/CARM1 and RNAPII in response to E2. This observation supports the suggestion that proximal promoter recruitment may be more relevant for more immediate transcriptional response. A recently developed technique, 'Global Run-On Sequencing' (GRO-seq), would allow for further confirmation that p/CIP/CARM1 plays a role in rapidly inducing gene expression. This assay maps the position, amount, and orientation of transcriptionally engaged RNA polymerases genome-wide [27]. GRO-seq analysis following E2 treatment was recently published and showed that E2-signaling regulates a large proportion of the transcriptome, and demonstrated that intergenic ER binding is transcriptionally relevant [28]. Unexpectedly, the observed transcriptional effects were revealed to be quite transient, suggesting that longer E2 treatment times previously utilized to allow for mature mRNA accumulation likely have not provided a comprehensive understanding of E2-dependent transcriptional regulation.

4.4 CARM1 is the functional component of p/CIP/CARM1 complex

Several studies have demonstrated a correlation between recruitment of CARM1, methylation of histone H3, and activation of several steroid responsive genes [29-31]. The methyltransferase activity of CARM1 is critical for this ability to enhance transcriptional activation by NRs and p160 coactivators [32]. Interestingly, methylation of H3R17 and/or p/CIP has been detected at 70% of the ER cistrome in MCF-7 cells, and CARM1 activity genome-wide was found to be predictive of 'active' ER sites [18].

Importantly, we noted a statistically significant E2-dependent enrichment in H3R17me2 on the Jak2 promoter, corresponding with p/CIP/CARM1 recruitment, suggesting the complex [and specifically CARM1] is functional (Figure 2-6). Reinforcing this suggestion, we observed a decrease in E2-dependent H3R17me2 of the Jak2 promoter following depletion of p/CIP, as well as a reduction in E2-induced transcription of Jak2 (Figure 2-7). Depletion of CARM1 caused a more dramatic loss in E2-dependent Jak2 transcription, suggesting that its enzymatic activity is in fact important for the regulation of Jak2 by the p/CIP/CARM1 complex. JAK2 is a central component of the Jak/Stat signalling pathway and is responsible for phosphorylation and activation of the STAT family of proteins, which normally reside in the cytoplasm and, upon activation, translocate to the nucleus and bind to specific target genes involved in cell proliferation and survival [33]. JAK2 null mice display phenotypes similar to the ERα and p/CIP knockout animals including defects in mammary gland cell proliferation and apoptosis [34,35]. Furthermore, E2 is known to stimulate phosphorylation and activation of STAT3 and 5, although the mechanism has not been fully elucidated [36-38]. We demonstrate a

reduction of E2-indced STAT3 phospohorylation following siRNA-mediated depletion of p/CIP and/or CARM1 (Figure 2-7), suggesting that targeting of JAK2 by the p/CIP/CARM1 complex may contribute to E2-dependent modulation of this signalling pathway.

4.5 Characterizing genome-wide CARM1 binding

In Chapter 3 I describe our analysis of global CARM1 recruitment in MEFs. The use of CARM1 wildtype and knockout MEFs provides a physiologically normal cell system in which the relevance of direct CARM1-dependent gene regulation can be studied. Conducting such an examination in the absence of any extrinsic signal is novel and allows for a broader understanding of CARM1 action, without limiting its functionality to specialized cellular programs or particular disease states. This study represents the first time that CARM1 binding has been directly considered on a genome-wide scale. Using ChIP-Seq, 7022 CARM1-dependent peaks were identified, and using statistical filtering methods 432 high-confidence binding regions were delineated. Analysis of the distribution of binding events revealed a predominance of binding events occurring at regions distal from the TSS of genes (Figure 3-1). Approximately 6% of CARM1 peaks correspond to promoter regions (defined as 5kb upstream and 1kb downstream of TSS), representing 421/7022 peaks. The observation that only a small proportion of recruitment occurs within the proximal promoter has become a consistent feature of the genome-wide characterization of transcriptional regulators. The existence of primarily distal binding sites raises the question of how such binding events can influence transcription. Technologies have evolved to address these questions, and the

use of ‘chromosome conformation capture’ (3C) and/or ‘chromatin-interaction analysis by paired end tag sequencing’ (ChIA-PET) have established a paradigm of chromosomal looping that brings distal ER binding sites into contact with TSS [39,40]. Importantly, RNAPII occupancy is enriched at genes where looping occurs [40], suggesting that this mechanism of bringing distal regulatory elements into contact with the TSS is functionally relevant for transcriptional control. Hah *et al*. later demonstrated that, in addition to this looping, a class of primary transcripts (eRNA) is expressed from ER-associated distal enhancer regions [41]. Future work to assess whether looping events bring CARM1 into contact with TSS would supplement our understanding of its role in regulating transcriptional initiation. An additional avenue to be pursued, which will more fully clarify CARM1’s transcriptional relevance, will be to characterize the global deposition of the CARM1-dependent histone modification, H3R17me2, by ChIP-Seq. The presence of H3R17me2 is reflective of CARM1 activity, and will provide insight into the mechanism of CARM1 action at different genomic locations.

We have previously demonstrated that E2-dependent interaction of CARM1 with the genome can be mediated through p/CIP [and the ER] [7]. Additionally, CARM1 is known to be recruited in a complex with transcription factors such as E2F1 [8], β-catenin [42], and NF-kB [43]. In an attempt to identify other transcription factors that may facilitate CARM1 binding, we used a bioinformatic approach and assessed the enrichment of TF binding motifs present within identified CARM1 peaks. The five most enriched TF motifs were found to correspond with Hunchback (PAX1), ZNF354C, YY1, Gfi, and MZF1 (Figure 3-3A). Notably, YY1 has been shown to recruit PRMT1, a CARM family member, to activated promoters [44]. This does not necessarily suggest

that a CARM1-YY1 interaction exists. However, since the central (methyltransferase region) of CARM1 shares a high degree of homology with PRMT1 [13,32], it seems possible that a similar, albeit context-dependent, association with YY1 may occur. Our experimental focus, however, was on the most enriched motif, PAX1. *De novo* motif discovery produced a strikingly similar motif to the PAX1 consensus site (Figure 3-3B). Next, we validated using quantitative ChIP analysis that CARM1 was binding at regions containing PAX1 motifs (Figure 3-4A). Ultimately, the strongest evidence suggesting that PAX1 may facilitate CARM1 genomic recruitment came from the observation that when PAX1 was depleted using siRNA, there was a reduction in CARM1 enrichment at analyzed regions (Figure 3-4B). While this is the first evidence that PAX1 may facilitate genomic recruitment of CARM1, it is not the first time CARM1 has been shown to interact with the paired box (Pax) protein family. CARM1 has been shown to interact with PAX7 [45], with the CARM1-binding region present within the paired domain. Taken together, these findings suggest that the ability to interact with CARM1 may be conserved among the paired box domain (Pax) family. It is likely that the role of PAX proteins in recruiting CARM1 to the genome is relevant only during developmental stages, since their expression is generally limited to this stage [46,47].

4.6 CARM1 in Transcriptional Elongation

Arginine methylation of transcriptional elongation factors by PRMT family members has been previously demonstrated, with PRMT1 and PRMT5 affecting association between SPT5 and RNAPII, resulting in transcriptional pausing [48]. In

addition, PRMT7 can symmetrically dimethylate eukaryotic elongation factor 2 (eEF2) [49]. The evidence linking CARM1 with transcriptional elongation has been more indirect. Several components of the transcriptional elongation-associated Paf1c complex have been shown to interact with the CARM1-specific histone modification H3R17me2 [50]. CARM1 can also methylate the C-terminal domain of RNAPII, and contribute to transcriptional activation by creating docking sites for effector protein(s) [3].

Our assessment of the genomic distribution of CARM1 peaks revealed that 20% (1404/7022) of binding events occurred within gene bodies (Figure 3-1). These binding events could reflect a role for CARM1 in alternative splicing and mRNA processing, a function in which CARM1 has already been implicated through its methylation of several splicing factors [51]. Alternately, the predominance of binding sites within the CDS of genes could also be indicative of a direct role for CARM1 in transcriptional elongation. To address this possibility, we performed co-immunoprecipitation experiments in CARM1$^{+/+}$ MEFs and demonstrated an interaction between CARM1 and Paf1c (Figure 3-1D). Furthermore, we attempted to discern an association between CARM1 and activated RNAPII, however these experiments are currently ongoing. Taken together, the predominance of CDS binding sites and the demonstration that CARM1 directly interacts with the elongation-associated Paf1 compex presented within this thesis provides evidence to support the involvement of CARM1 in transcriptional elongation. Future work will need to be conducted to fully elucidate its regulatory role in this stage of gene expression.

4.7 Functional Impact of CARM1

Functional analysis of the genes targeted by the p/CIP/CARM1 coregulatory complex implicated cancer as one of the primary diseases affected by their misregulation. Furthermore, processes including cellular growth and proliferation, development, cell-to-cell signaling, and morphology were associated with this gene set (Figure 2-5). It is interesting to note that when functional analysis is conducted on genes differentially expressed in the absence of CARM1, similar cellular processes were implicated (Figure 3-7). Since sustained proliferation is one of the most fundamental characteristics of cancer cells [52], and functional analysis implicated growth characteristics as being critically regulated by both CARM1- and p/CIP/CARM1-regulated genes, we further analyzed the impact of CARM1 in this process.

CARM1 has been previously implicated in growth as well as cell cycle control. CARM1 knockout mice are small in size, and NF-kB dependent gene expression, important for cellular events such as apoptosis, cell proliferation, and differentiation, is impaired in CARM1-null MEFs [53]. CARM1 also plays a role in ERα-dependent breast cancer cell differentiation and proliferation [24,54]. In response to DNA damage CARM1 methylates the p300 KIX domain, promoting recruitment of BRCA1 by p300 to the p53-responsive promoter of p21 and inducing increased p21 expression [10]. CARM1 is also recruited to the promoter of cyclin E1 (CCNE1) in an E2F-dependent manner, and functions as a positive regulator of transcription [8]. Our analysis of growth characteristics revealed that CARM1 null MEFs proliferated at a reduced rate when compared to wildtype (Figure 3-7B) and had a smaller cell diameter and cell volume (Figure 3-7D). Furthermore the expression of a large number of proteins known to

regulate cell cycle was altered in the absence of CARM1 (Figure 3-8). To determine if involvement of CARM1 in regulating the expression of cell cycle regulators has functional impact, we subjected wildtype and knockout MEFs to the additional stress of a cell replication inhibitor, hydroxyurea (HU), and found a substantial loss of cell viability when CARM1 was absent. The suggestion that loss of CARM1 may sensitize cells to replicative stress needs to be further evaluated in the future, and the model tested in cancer cell lines known to have elevated CARM1. Alternately, and arguably of more translational relevance, CARM1 expression could be assessed, along with the expression of some of its downstream targets (ex. JAK2, p21) in breast tumor samples. Stratifying tumors based on the presence or absence of CARM1 may allow for diagnostic and/or prognostic inferences to be made with respect to the delineation of breast cancer subtypes, and could ultimately suggest different therapeutic strategies. For example, reduction of CARM1, or of its methyltransferase activity through the use of CARM1 inhibitors [55,56], may prove to be a successful treatment approach when used in combination with cell cycle inhibitors for cancers expressing high levels of CARM1.

4.8 Conclusions

The use of whole-genome approaches to characterize the role of the arginine methyltransferase CARM1 in transcriptional regulation has advanced our understanding of the myriad roles it plays. The work presented within this thesis describes the recruitment of a functional p/CIP/CARM1 coregulatory complex to gene promoters in an E2-dependent manner. Bioinformatic analysis suggests that direct binding via a classical ERE is not the primary mechanism by which the complex is recruited, reinforcing

accumulating evidence that transcription factor crosstalk commonly occurs in ER-mediated transcription. I focus on the recruitment of a functional p/CIP/CARM1 complex to the proximal promoter of Jak2, and demonstrate that regulation of Jak2 expression is contingent on the presence of CARM1.

CARM1 recruitment was also characterized independent of p/CIP, and found to bind predominantly at intergenic regions, consistent with the profiles of many other transcriptional regulatory proteins. Enrichment of CARM1 binding events was also found within the coding sequence of genes, suggesting an involvement in transcriptional elongation; in support of this, interaction between CARM1 and the elongation-associated Paf1c was confirmed. In addition, we identified the transcription factor PAX1 as a novel mechanism through which CARM1 can interact with the genome. Overall, this study suggested that direct genomic recruitment of CARM1 is not critical for its transcriptional and functional effect(s) in an un-induced system; however, we observed an important regulatory role for CARM1 in cellular growth and proliferation.

Moving forward, there are many more avenues to be pursued before we gain a complete understanding of the many ways in which CARM1 contributes to the regulation of gene expression, and affects diverse cellular processes. As technology advances, we will be able to more fully characterize the coordinated action of transcriptional regulators such as CARM1.

4.9 References

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Appendices

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Appendix B: Supplementary Material for Chapter 2: β-Estradiol-dependent activation of the JAK/STAT pathway requires p/CIP and CARM.

A

Overlapping Motif (columns); Query Motif (rows)

	ERE	Sp1	AP-1	FoxA1	C/EBPα	Oct
ERE (24)		29.17%	4.17%	12.50%	41.67%	33.33%
Sp1 (49)	14.29%		10.20%	10.20%	36.73%	24.49%
AP-1 (18)	5.56%	27.78%		11.11%	27.78%	16.67%
FoxA1 (21)	14.29%	23.81%	9.52%		28.57%	19.05%
C/EBPα (63)	15.87%	28.57%	7.94%	9.52%		28.57%
Oct (42)	19.05%	28.57%	7.14%	9.52%	42.86%	

B

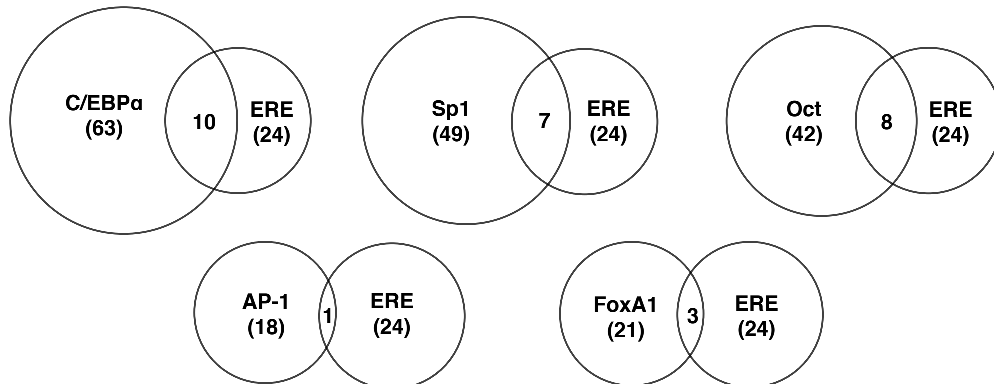

Figure B-1 Co-occurrence of TF binding motifs in p/CIP/CARM1 target promoters

(A) Chart showing the overlap between ERE, Sp1, AP-1, FoxA1, C/EBPα, and Oct consensus sites. The left column indicates the query motif(s), and the percentage of those promoters containing overlapping motifs is listed left to right. Cells are color coded according to the proportion overlap: red: >25% overlap, orange: 14-24.9% overlap, yellow <13.9% overlap. (B) Venn diagrams showing overlap between p/CIP/CARM1 target promoters containing EREs and those containing C/EBPα, Sp1, Oct, AP-1, or FoxA1 consensus sites.

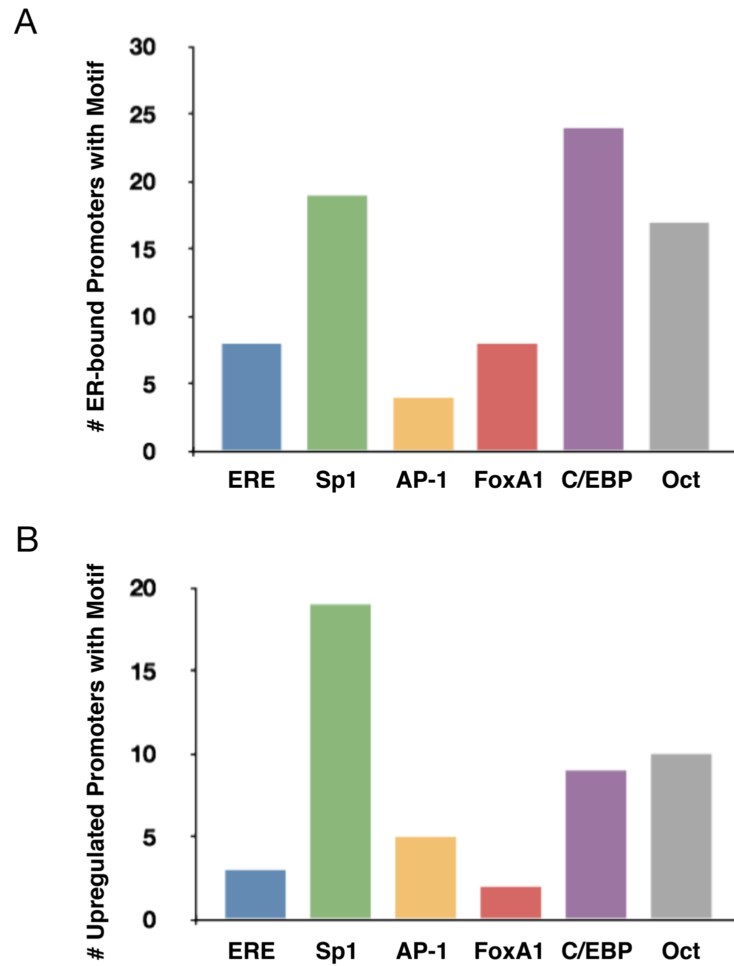

Figure B-2 Additional binding site analysis of p/CIP/CARM1 target promoter sequences

Bar graphs showing the number of (A) ER-bound and (B) transcriptionally upregulated p/CIP/CARM1 target promoters that contain ERE, Sp1, AP-1, FoxA1, C/EBP, and Oct binding motifs.

Table B-1 Genes directly targeted by the p/CIP/CARM1 complex.

Gene ID	Accession Number	Description
TLR3	NM_003265	Transmembrane receptor
DBC1	NM_014618	Peptidase
LAP3	NM_015907	Peptidase
CLDN12	NM_012129	Calcium-independent cell-cell adhesion
CRHR1	NM_004382	G-protein coupled receptor
P181_E7	XM_372688	Transporter
KRAS2	NM_033360	GTPase enzyme
M6PR	NM_002355	Mannose-6-phosphate receptor (cation dependent)
PDCD8	NM_004208	Enzyme/cell death
CIRBP	NM_001280	Cold inducible RNA binding protein
DLGAP3	XM_035601	Discs, large (Drosophila) homolog-associated protein 3
AES	NM_198969	Amino-terminal enhancer of split/transcriptional regualtor
BTN2A2	NM_006995	Butyrophilin, subfamily 2, member A2
C10orf111	NM_153244	Unknown
STARD6	NM_139171	StAR-related lipid transfer (START) domain containing 6
GNG5	NM_005274	Guanine nucleotide binding protein (G protein), gamma 5
UNC13A	XM_038604	Unc-13 homolog A (C. elegans)
SLC4A5	NM_021196	Solute carrier family 4, sodium bicarbonate cotransporter, member 5
PTER	NM_030664	Phosphotriesterase related
TRAF3	NM_003300	TNF receptor-associated factor 3/apoptosis
C14orf29	NM_181533	Peptidase
ZNF800	NM_176814	Unkown
ARMC3	NM_173081	Armadillo repeat containing 3
LETM1	NM_012318	Leucine zipper-EF-hand containing transmembrane protein 1
IL15RA	NM_002189	Interleukin 15 receptor, alpha
MAPK4	NM_002747	Mitogen-activated protein kinase 4
GTF2E2	NM_002095	General transcription factor IIE, polypeptide 2, beta 34kDa
SEC31L1	NM_016211	SEC31 homolog A (S. cerevisiae)/vesicle transport
KRTHA4	NM_021013	Keratin 34
IGSF4/CADM1	NM_014333	Cell adhesion molecule 1/tumour suppressor
CASP6	NM_001226	Caspase 6, apoptosis-related cysteine peptidase
KIAA1340	XM_044836	KLHDC5 kelch domain containing 5
DRF1	NM_025104	DBF4 homolog B (S. cerevisiae)/cell cycle regulator
OCA2	NM_000275	Oculocutaneous albinism II
LOC343066	XM_291392	Arylacetamide deacetylase-like 4
SLC26A1	NM_022042	Solute carrier family 26 (sulfate transporter), member 1
FRMD1	NM_024919	FERM domain containing 1
AIP1	NM_012301	MAGI2 membrane associated guanylate kinase
APRG1	NM_178338	Unknown/tumour suppressor
CBR3	NM_001236	Carbonyl reductase 3
FCGRT	NM_004107	Fc fragment of IgG, receptor, transporter, alpha
N4BP1	NM_153029	NEDD4 binding protein 1
WDFY2	NM_052950	WD repeat and FYVE domain containing 2
ZNF567	NM_152603	Transcription factor
RAP140	NM_015224	Chromosome 3 open reading frame 63
PELO	NM_015946	Pelota homolog (Drosophila)
OR51B4	NM_033179	Olfactory receptor, family 51, subfamily B, member 4
SERPINB2	NM_002575	Serpin peptidase inhibitor, clade B (ovalbumin), member 2

DKFZP564I122	XM_032397	Methylmalonic aciduria protein
SEC31L1	NM_014933	SEC31 homolog A (S. cerevisiae)
TM4SF8	NM_005724	Tetraspanin 3
NJMU-R1	NM_022344	Open reading frame 75
CTNND1	NM_001331	Catenin (cadherin-associated protein), delta 1
CDH7	NM_033646	Cadherin 7, type 2
FLJ14054	NM_024563	Open reading frame 23
OR5T3	XM_372393	Olfactory receptor, family 5, subfamily T, member 3
TPARL	NM_018475	Transmembrane protein 165
CR2	NM_001877	Complement component (3d/Epstein Barr virus) receptor 2
ZIM3	NM_052882	Zinc finger, imprinted 3/transcription factor
VPS26A	NM_004896	Vacuolar protein sorting 26
MOCOS	NM_017947	Enzyme
POLR3F	NM_006466	Enzyme
MBD3	NM_003926	Methyl-CpG binding domain protein 3
FLJ25467	NM_144719	Coiled-coil domain containing 13
A2LP	NM_007245	Homo sapiens ataxin 2-like (ATXN2L), transcript variant A
OR6C1	XM_372459	Homo sapiens olfactory receptor, family 6, subfamily C, member 1
RAD9B	NM_152442	RAD9 homolog B (S. cerevisiae)/DNA replication
ECT2	NM_018098	Epithelial cell transforming sequence 2 oncogene
PCSK5	NM_006200	Proprotein convertase subtilisin/kexin type 5
GATM	NM_001482	Glycine amidinotransferase (L-arginine:glycine amidinotransferase)
TUBGCP6	NM_020461	Tubulin, gamma complex associated protein 6
KCTD16	XM_098368	Potassium channel tetramerisation domain containing 16
CHRNA6	NM_004198	Cholinergic receptor, nicotinic, alpha 6
TGFB1	NM_000660	Growth factor
NEK4	NM_003157	Enzyme
CPT1C	NM_152359	Carnitine palmitoyltransferase 1C
OR7A10	XM_372712	Olfactory receptor, family 7, subfamily A, member 10
FLJ23584	NM_024588	Hypothetical protein
RPS4X	NM_001007	Ribosomal protein S4, X-linked
FLJ34969	NM_152678	FAM116A family with sequence similarity 116, member A
MGC11386	NM_032933	C18orf45
ELAC1	NM_018696	ElaC homolog 1 (E. coli)/trna processing
STARD4	NM_139164	StAR-related lipid transfer (START) domain containing 4
HRASLS	NM_020386	HRAS-like suppressor
P188_B6	XM_374513	Similar to KIAA1218 protein
ENO2	NM_001975	Enolase 2 (gamma, neuronal)
P176_D4	XM_371127	Homo sapiens similar to putative protein
PKP4	NM_003628	Plakophilin 4/cell adhesion
KIF24	NM_018278	Homo sapiens kinesin family member 24 (KIF24)
OR2AP1	XM_062467	Olfactory receptor, family 2, subfamily AP, member 1
P155_A2	XM_036408	Unknown orf (KIAA1228)
IFI6	NM_022873	Interferon, alpha-inducible protein 6/apoptosis
DPH2L2	NM_001384	DPH2 homolog (S. cerevisiae)
FUT7	NM_004479	Fucosyltransferase 7 (alpha (1,3) fucosyltransferase)
SMARCAL1	NM_014140	SWI/SNF related, matrix associated, regulator of chromatin, a-like1
CD9	NM_001769	CD9 molecule/cell adhesion
LOC133609	XM_068430	Homo sapiens similar to 60S acidic ribosomal protein P1
SRISNF2L	NM_015106	RAD54-like 2 (S. cerevisiae)
POLR2E	NM_002695	Polymerase (RNA) II (DNA directed) polypeptide E, 25kDa
NFKB1	NM_003998	Nuclear factor of kappa light polypeptide gene enhancer
C9orf95	NM_017881	Orf

GNL1	NM_005275	Guanine nucleotide binding protein-like 1
KRT20	NM_019010	Keratin 20/apoptosis
TMEM16F	XM_113743	Transmembrane protein 16F
MGC15882	NM_032884	C1orf94
IVNS1ABP	NM_006469	Influenza virus NS1A binding protein
DDX54	NM_024072	DEAD (Asp-Glu-Ala-Asp) box polypeptide 54/tranascriptional regulator
WBP11	NM_016312	WW domain binding protein 11
OR10A5	NM_178168	OR10A5 olfactory receptor, family 10, subfamily A, member 5
SGK2	NM_170693	Serum/glucocorticoid regulated kinase 2
BMI1	NM_005180	BMI1 polycomb ring finger oncogene
LOC284275	XM_211413	Hypothetical protein
K5B	NM_173352	Keratin 5b
MT1E	NM_175617	Metallothionein 1E
MT4	NM_032935	Metallothionein 4
OTUB2	NM_023112	OTU domain, ubiquitin aldehyde binding 2
THEG	NM_199202	Theg homolog (mouse)
CARD9	NM_022352	P111_E11
ARHGAP8	NM_181335	Rho GTPase activating protein 8
ITPKB	NM_002221	Inositol 1,4,5-trisphosphate 3-kinase B
LOC389253	XM_374104	Hypothetical protein
C18orf26	NM_173629	Orf
POLR2F	NM_021974	Polymerase (RNA) II (DNA directed) polypeptide F
LAMA1	NM_005559	Laminin, alpha 1/cell adhesion protein
P139_A6	NM_152567	Arabidopsis thaliana chromosome 1
SOD1	NM_000454	Superoxide dismutase 1, soluble
P163_B12	XM_114325	Homo sapiens ubiquitin specific protease 19 (USP19)
TSSC4	NM_005706	Tumor suppressing subtransferable candidate 4
P181_D9	XM_372649	Hypothetical protein CBG22662
MAP3K7IP1	NM_006116	Mitogen-activated protein kinase kinase kinase 7 interacting protein
P2RY1	NM_002563	Purinergic receptor P2Y, G-protein coupled, 1
FLJ46299	XM_093813	Homo sapiens similar to hypothetical protein (LOC166348)
TSHB	NM_000549	Thyroid stimulating hormone, beta
RDH10	NM_172037	Retinol dehydrogenase 10 (all-trans)
KCNQ1	NM_000218	Potassium voltage-gated channel, KQT-like subfamily, member 1
RPS15	NM_001018	Ribosomal protein S15/translation
LOC92691	NM_138390	Unknown
KCTD3	NM_016121	Potassium channel tetramerisation domain containing 3
HBD	NM_000519	Hemoglobin, beta /// hemoglobin, delta
LOC387635	XM_370532	Similar to ubiquitin-conjugating enzyme E2 variant 1
PRKRIR	NM_004705	Protein-kinase, interferon-inducible RNA dependent inhibitor,
RAB6IP1	XM_290550	RAB6 interacting protein 1
RFXAP	NM_000538	Regulatory factor X-associated protein
HDC	NM_002112	Histidine decarboxylase
ZNF197	NM_006991	Zinc finger protein 197/transcription factor
PCP4	NM_006198	Purkinje cell protein 4
LRFN4	NM_024036	Leucine rich repeat and fibronectin type III domain containing 4
ABAT	NM_000663	4-aminobutyrate aminotransferase
P186_C2	XM_373988	Homo sapiens similar to protein 40kD (LOC388290), mRNA.
COL17A1	NM_000494	Collagen, type XVII, alpha 1
PTPRJ	NM_002843	Protein tyrosine phosphatase, receptor type, J
PXMP4	NM_007238	Peroxisomal membrane protein 4, 24kDa
JAK2	NM_004972	Janus kinase 2 (a protein tyrosine kinase)

PRSS15	NM_004793	lon peptidase 1, serine protease
C6orf146	NM_173563	Orf
LOC389439	XM_374187	Homo sapiens LOC389439 (LOC389439), mRNA
TRO	NM_177557	Trophinin/cell adhesion
GPR132	NM_013345	G protein-coupled receptor 132
HARS	NM_002109	Histidyl-tRNA synthetase
MDS009	NM_020234	DTWD1 DTW domain containing 1
VN1R5	NM_173858	Vomeronasal 1 receptor 5
CCNA2	NM_001237	Cyclin A2/cell cycle
DMP1	NM_004407	Dentin matrix acidic phosphoprotein
PCDHGC5	NM_018929	Protocadherin gamma subfamily C, 5
POLG	NM_002693	Polymerase (DNA directed), gamma
ZBED2	NM_024508	Zinc finger, BED-type containing 2
HSD17B12	NM_016142	Hydroxysteroid (17-beta) dehydrogenase 12
LOC126298	XM_065026	Homo sapiens immunity-related GTPase family, Q (IRGQ), mRNA
CSNK1A1L	NM_145203	Casein kinase 1, alpha 1-like
CYC1	NM_001916	Cytochrome c-1
AKAP13	NM_006738	A kinase (PRKA) anchor protein 13
LMOD1	NM_012134	Leiomodin 1 (smooth muscle)
RBM29	NM_032213	RNA binding motif and ELMO/CED-12 domain 1
P097_A6	NM_017597	Homo sapiens hypothetical protein
LOC338731	XM_294688	Hypthetical protein
LOC131873	XM_067585	Hypothetical protein
MAP6	XM_166256	Microtubule-associated protein 6
SS18L1	NM_015558	Synovial sarcoma translocation gene on chromosome 18-like 1
PIAS2	NM_004671	Protein inhibitor of activated STAT, 2
DPYS	NM_001385	Dihydropyrimidinase
RPS27L	NM_015920	Ribosomal protein S27-like
FXR1	NM_005087	Fragile X mental retardation, autosomal homolog 1
NAGA	NM_000262	N-acetylgalactosaminidase, alpha-
IDH3G	NM_004135	Isocitrate dehydrogenase 3 (NAD+) gamma
PROS1	NM_000313	Protein S (alpha)
DYRK1A	NM_101395	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A
EDC3	NM_025083	Homo sapiens enhancer of mRNA decapping 3 homolog (S. cerevisiae)
TNFRSF19L	NM_152222	RELT tumor necrosis factor receptor
SLC39A10	XM_047707	Solute carrier family 39 (zinc transporter), member 10
SAMD3	NM_152552	Sterile alpha motif domain containing 3
FLRT2	NM_013231	Fibronectin leucine rich transmembrane protein 2/cell adhesion
MGC14156	NM_032906	PIGY phosphatidylinositol glycan anchor biosynthesis, class Y
BPI	NM_001725	NEDD4 binding protein 1
LOC341511	XM_292109	Homo sapiens similar to 60S ribosomal protein L23a
C6orf188	NM_153711	Hypothetical protein
MXD3	NM_031300	MAX dimerization protein 3
LGR6	NM_021636	Leucine-rich repeat-containing G protein-coupled receptor 6
PCNA	NM_002592	Proliferating cell nuclear antigen
P162_C9	XM_096669	Homo sapiens hypothetical LOC144705
GAD1	NM_013445	Glutamate decarboxylase 1 (brain, 67kDa)
FLJ11331	NM_018392	C4orf21
CMYA4	NM_173167	Unc-45 homolog B
P181_E10	XM_372694	imilar to RIKEN cDNA C230094B15
P188_D7	XM_374566	Homo sapiens similar to T-cell receptor beta

[a] Genes show 2-fold enrichment or greater and p value <0.05.

Table B-2 Enriched motifs within p/CIP/CARM1 target promoters.

Motif	p-value	Yes	No	Enrichment (Yes/No)
V$KID3_01	0.000	0.5028	0.0000	inf
V$ZF5_B	3.99E-41	0.2712	0.0000	inf
V$ETF_Q6	2.77E-40	0.2655	0.0000	inf
V$SP1_Q2_01	1.08E-24	0.1977	0.0089	22.1458
V$PAX4_01	1.31E-22	0.1469	0.0000	inf
V$POU3F2_02	1.96E-22	0.1695	0.0054	31.6394
V$POU3F2_01	4.34E-22	0.2203	0.0250	8.8136
V$OG2_01	1.44E-21	0.1751	0.0089	19.6149
V$CDXA_02	3.52E-21	0.1921	0.0161	11.9526
V$PAX4_02	1.91E-18	0.1412	0.0054	26.3661
V$CETS1P54_03	2.12E-18	0.1186	0.0000	inf
V$FOXJ2_02	8.53E-17	0.1525	0.0107	14.2376
V$PAX4_03	1.47E-17	0.1130	0.0000	inf
V$PLF_02	4.25E-16	0.1299	0.0071	18.1918
V$SREBP1_01	4.49E-16	0.1243	0.0054	23.2022
V$KROX_Q6	4.49E-16	0.1243	0.0054	23.2022
V$TST_01	4.63E-16	0.1525	0.0161	9.4918
V$PAX5_02	7.09E-16	0.1017	0.0000	inf
V$WT1_Q6	7.09E-16	0.1017	0.0000	inf
V$HOX13_01	1.25E-14	0.1412	0.0161	8.7887
V$CART1_01	8.66E-14	0.1130	0.0071	15.8188
V$OCT4_02	1.01E-13	0.1073	0.0054	20.0383
V$NKX25_02	1.69E-13	0.1243	0.0125	9.9435
V$AP4_01	1.69E-13	0.1243	0.0125	9.9435
V$AP2_Q6	2.58E-13	0.1186	0.0107	11.0737
V$IPF1_Q4	5.14E-13	0.1356	0.0196	6.9029
V$GABP_B	6.07E-13	0.1017	0.0054	18.9836
V$SRY_02	6.07E-13	0.1017	0.0054	18.9836
V$CEBPGAMMA_Q6	8.91E-13	0.1186	0.0125	9.4915
V$CHOP_01	7.48E-12	0.1073	0.0107	10.0191
V$CDPCR3_01	7.48E-12	0.1073	0.0107	10.0191
V$AP2_Q6_01	1.41E-11	0.1130	0.0143	7.9094
V$NCX_01	1.45E-11	0.1864	0.0607	3.0708
V$TEL2_Q6	1.22E-10	0.1017	0.0125	8.1356
V$XVENT1_01	1.28E-10	0.1186	0.0214	5.5366
V$MAZ_Q6	1.55E-10	0.1356	0.0321	4.2184
V$OCT1_02	1.88E-10	0.1073	0.0161	6.6794
V$PAX4_04	8.90E-10	0.1017	0.0161	6.3279

V$BRCA_01	1.11E-09	0.1073	0.0196	5.4648
V$FAC1_01	2.42E-09	0.1243	0.0321	3.8669
V$OCT1_03	4.90E-09	0.1017	0.0196	5.1772
V$HNF3ALPHA_Q6	4.90E-09	0.1017	0.0196	5.1772
V$RFX1_02	4.97E-09	0.1186	0.0304	3.9083
V$PAX3_B	5.26E-09	0.1130	0.0268	4.2184
V$TBX5_01	5.26E-09	0.1073	0.0232	4.6241
V$HMGIY_Q6	1.06E-08	0.1017	0.0214	4.7457
V$CP2_02	1.06E-08	0.1017	0.0214	4.7457
V$VDR_Q3	1.06E-08	0.1017	0.0214	4.7457
V$PAX6_01	1.63E-08	0.1356	0.0464	2.9204
V$HAND1E47_01	3.49E-08	0.1638	0.0732	2.2379
V$CEBPDELTA_Q6	8.21E-08	0.1017	0.0268	3.7966
V$STAT1_01	8.21E-08	0.1017	0.0268	3.7966
V$CKROX_Q2	1.19E-07	0.1412	0.0589	2.3968
V$GATA4_Q3	1.51E-07	0.1017	0.0286	3.5594
V$PAX8_01	4.28E-07	0.2429	0.1696	1.4321
V$GRE_C	4.35E-07	0.1243	0.0500	2.4859
V$AP2ALPHA_01	7.82E-07	0.1017	0.0339	2.9973
V$CEBP_Q3	9.97E-07	0.1073	0.0393	2.7324
V$VMYB_02	1.28E-06	0.1017	0.0357	2.8475
V$CREB_Q4_01	1.57E-06	0.1073	0.0411	2.6136
V$TAXCREB_02	2.06E-06	0.1017	0.0375	2.7119
V$PAX6_Q2	3.07E-06	0.1243	0.0589	2.1092
V$AP1_Q2_01	5.01E-06	0.1017	0.0411	2.4761
V$RFX_Q6	5.56E-06	0.1073	0.0464	2.3120
V$BCL6_Q3	5.92E-06	0.1130	0.0518	2.1819
V$LRF_Q2	6.11E-06	0.1186	0.0571	2.0763
V$PPARG_02	7.58E-06	0.1017	0.0429	2.3729
V$PAX5_01	8.55E-06	0.1243	0.0643	1.9335
V$PAX_Q6	1.12E-05	0.1525	0.0946	1.6118
V$USF_Q6_01	1.13E-05	0.1017	0.0446	2.2780
V$LEF1TCF1_Q4	1.18E-05	0.1243	0.0661	1.8812
V$MAF_Q6_01	1.65E-05	0.1017	0.0464	2.1903
V$PAX2_02	1.70E-05	0.1073	0.0518	2.0729
V$MYB_Q3	3.36E-05	0.1073	0.0554	1.9391
V$TTF1_Q6	4.63E-05	0.1073	0.0571	1.8785
V$PPARA_01	4.65E-05	0.1299	0.0804	1.6171
V$ER_Q6	4.75E-05	0.1017	0.0518	1.9638
V$CACD_01	6.31E-05	0.1073	0.0589	1.8216

V$NKX25_Q5	6.58E-05	0.1017	0.0536	1.8983
V$TAL1BETAE47_01	6.58E-05	0.1017	0.0536	1.8983
V$ZIC2_01	9.01E-05	0.1017	0.0554	1.8371
V$SPZ1_01	1.22E-04	0.1017	0.0571	1.7797
V$CDP_02	1.63E-04	0.1017	0.0589	1.7257
V$STAT_Q6	1.63E-04	0.1017	0.0589	1.7257

* Cutoff conditions p < 0.001, Yes/No > 1.2

Table B-3 Primer List.

ChIP Validations:		
Gene Target	**5’**	**3’**
pS2	GGCCATCTCTCACTATGAATCACTTCTGC	GGCAGGCTCTGTTTGCTTAAAGAGCG
CyclinA2	GCTAACTAGACGTCCCAGAGC	GGGGAGAGGTAGGATTTAGG
NFkB1	GCCTGGTACACTATAGCAGTC	ATCGCCCTCTGAACTTCAAC
WBP11	GGGCGAAGGCTAGAGT_AAGT	GATTGCTTATATGGGCGGTG
DYRK1A	CCTCTTCTGCTGATTATCAGT	CTGAGACATTTCTCACAACC
IL15RA	GTCTGCTCTCCGATGACTTTG	CAGGACCTTACCCACGCAAG
MAPK4	GAACTCTGCACCCTGGTTTC	TTGCACTTGGGTTCCTTTTC
TGFB1	GGTCGGGAGAAGAGGAAAAA	CTGAGGGACGCCGTGTAG
NEK4	CTTTGGCTGGAACAAATGGT	CCCTAAAAACTCGCCTGCTA
KRAS2	ATTTCCCCATGACACAATCC	ACCCTGTAGCACACCCTCAC
Assessing Chromatin Modifications on JAK2:		
Assay	**5’**	**3’**
ChIP	AAGGTGGCTGATGGGAGTC	CTTTCGGCTTTTCCTTCCAC
qChIP	GGTGGCTGATGGGAGTCAGG	GCTTTCGGCTTTTCCTTCCACC

Table B-4 Antibody List.

	Antibody Name	**Source**	**Catalogue #**
p/CIP		* In House	
CARM1	CARM1 Antibody	Cedarlane	A300-421A
ER	ERα (HC-20)	Santa Cruz Biotechnology	sc-543
JAK2	JAK2 (C-14)	Santa Cruz Biotechnology	sc-34479
H3R17me2	Anti-dimethyl-Histone H3 (Arg17) (rabbit immunoaffinity purified IgG)	Upstate	07-214
H3K4me3	Mouse monoclonal to Histone H3 (tri methyl K4)	AbCam	ab1012
H3R2me2	Anti-dimethyl-Histone H3 (Arg2) (rabbit antiserum)	Millipore	07-585
H3Ac(K9, K14)	Anti-acetyl-Histone H3 (rabbit polyclonal IgG)	Upstate	06-599
STAT3	Stat3 (79D7) (rabbit mAb)	Cell Signaling Technology	#4904
P-STAT3	Phospho-Stat3 (Tyr705)	Cell Signaling Technology	#9131

Appendix C: Supplementary Information for Chapter 3: Whole Genome analysis of CARM1 in wildtype and CARM1-knockout Mouse Embryonic Fibroblasts.

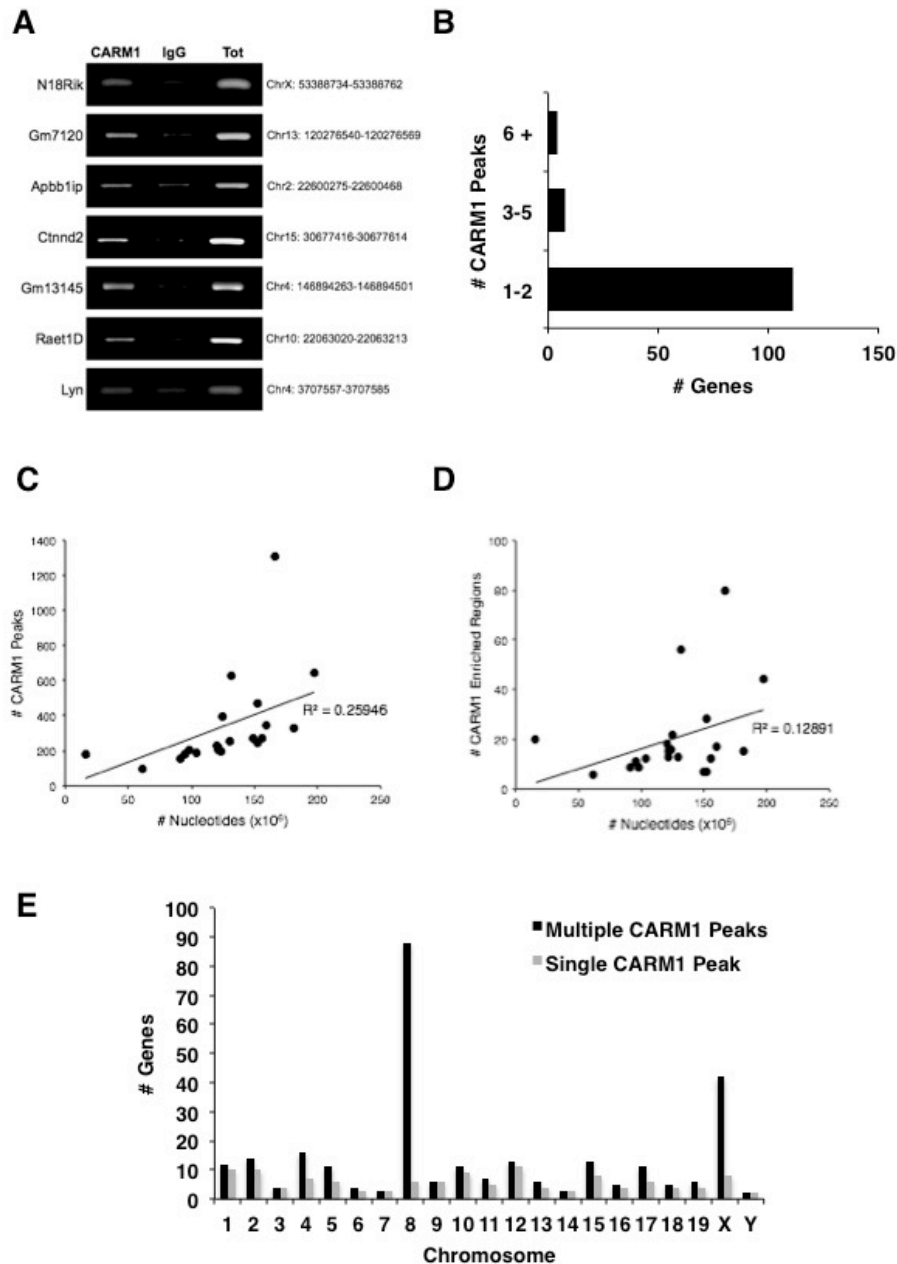

Figure C-1 Additional characterization of CARM1 genome-wide binding

(A) ChIP analysis validating CARM1 recruitment to indicated genomic regions, identified by ChIP-Seq. (B) CARM1 binding frequencies; analysis of the number of genes with 1-2, 3-5, or 6+ CARM1 binding events within 50kb of TSS. Correlation of CARM1 (C) binding sites or (D) MW-enriched regions with chromosome length. (E) Bar chart depicting the prevalence of number of multiple CARM1 binding events within 50kb of the TSS on each chromosome.

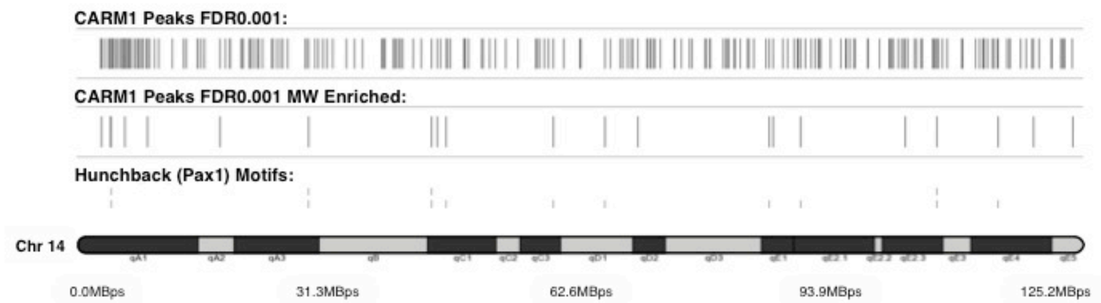

Figure C-2 Chromosomal view depicting co-occurrence of CARM1 enriched regions and PAX1 motifs

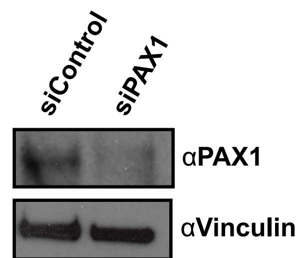

Figure C-3 siRNA-mediated depletion of PAX1 in wildtype MEFs

Primers used for ChIP Validations:

Apbb1ip F: AACGGGGAGTGGGAAAGAT
Apbb1ip R: AAGCGGGTGGTAAACTCCAT
Ctnnd2 F: GCCTTGCCCTAAGAGTAGCTT
Ctnnd2 R: ACGTGTGGCCCTAGAGCTT
Gm7120 F: GGAGCTCCTCTGCTGGAATA
Gm7120 R: ACAGTCACACAGCCACACC
Raet1d F: GGAGCAAGCGAAAATGATTG
Raet1d R: CCTGAACAGGTATCAATGCAAA

Primers used for Pax-1 ChIPs:

Chr 1- Col9a1 F: AACAATTCATGCGCATTCTG
Chr 1 - Col9a1 R: GGCAGAACTCGGACAGTCAT
Chr4 - Mllt3 F: TCAAGCTTGTTCAGATGTGAAATTA
Chr4 - Mllt3 R: CCCACTTTGCCAGTTTAGGA
Chr5 - Mag2 F: GGATAGTGATTCCCCCAGAAG
Chr5 - Mag2 R: ATGTCCATCTTGCCAAAAGC
Chr9 - Mir101c F: GACTGTGCAATTGGGGAGTAA
Chr9 - Mir101c R: CTGCCCAACCTAAAGTCCTG

Curriculum Vitae

Niamh Coughlan

EDUCATION

2007 – 2014 Ph.D. Department of Biochemistry
University of Western Ontario, London, Ontario
Supervisor: Dr. Joe Torchia
Research: Arginine methylation, and the protein arginine methyltransferase CARM1 in transcriptional regulation.

2003 – 2007 B.MSc. Honors Double Major in Biochemistry and Physiology
University of Western Ontario, London, Ontario

AWARDS & DISTINCTIONS

2009 – 2013 Studentship from the Translational Breast Cancer Research Unit
Award held at CRLP, London Regional Cancer Program, UWO.
Award competitively renewed on an annual basis.

2011 USC Teaching Honour Roll Award of Excellence

2011 - 2012 Ontario Graduate Scholarship
Award held at CRLP, London Regional Cancer Program, UWO.

2009 - 2012 Studentship from the CIHR-Strategic Training Program for Cancer Research and Technology Transfer (CaRTT)
Award held at CRLP, London Regional Cancer Program, UWO.
Award competitively renewed for 2011-2012.

2011 Division of Experimental Oncology Graduate Student Travel Award

2011 UWO Society of Graduate Students Travel Subsidy

2008 - 2012 Schulich Graduate Scholarship (SGS)

RELATED WORK EXPERIENCE

2008 – 2012 Teaching Assistant
Biochemistry 3380G (Biochemistry Lab)
University of Western Ontario, London, Ontario

ACADEMIC CONTRIBUTIONS

Peer Reviewed Publications:

Coughlan N, Thillainadesan G, Isovic M, Andrews J, and Torchia J. β-Estradiol dependent activation of the JAK/STAT pathway requires p/CIP and CARM1. Biochim Biophys Acta. 2013 Jun;1833(6):1463-75.

Corkery D, Thillainadesan G, **Coughlan N**, Mohan RD, Isovic M, Tini M, Torchia J. Regulation of the BRCA1 gene by an SRC3/53BP1 complex. BMC Biochem 2011;12:50

Manuscripts in Preparation:

Coughlan N, Isovic M, Kolendowski B, Thillainadesan G, Torchia J. Whole Genome analysis of CARM1 in wildtype and CARM1-knockout Mouse Embryonic Fibroblasts.

Coughlan N, Bedford M, Torchia J. The role of TDRD3 in the regulation of p/CIP.

Invited Oral Presentation(s):

Great Lakes Nuclear Receptor Conference (Ann Arbor, MI), October 22-23, 2010
Title: Identification of 17b-estradiol-dependent targets of the p/CIP/CARM1 complex

Oral Presentations:

2009 - 2013 Cancer Research Program Seminar Series (LRCP, London, Ontario)
Annual presentation of research in progress, various titles

2008 - 2013 Dept. of Biochemistry Graduate Student Seminar Series
Annual presentation of research in progress, various titles

Poster Presentations:

2008 - 2013 Oncology Research and Education Day (London, Ontario)
Annual research poster at regional conference, various titles

March 2010 Margaret Moffat Research Day (London, Ontario)
Coughlan N, Thillainadesan G, Isovic M, Andrews J, Torchia J.
"*The Role of the p/CIP/CARM1 complex in E2-Dependent Gene Regulation*"

2008 UWO Biochemistry Dept. Showcase of 1st Year Graduate Studies
Niamh Coughlan, Mark T. Bedford, and Joseph Torchia
"The Role of Arginine Methylation in Gene Regulation - Biochemical & Functional Studies of Tudor Domain Containing Protein 3"