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Investigating the dynamics of interchromosomal interactions and CTCF site methylation at the IGF2 locus in mammalian evolution and human disease

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Abbreviations

^{o}C	degree Celcius
µg	microgram
µl	microlitre
µm	micrometre
3C	Chromosome conformation capture
5aza	5-aza-2-deoxycytidine
ACTB	Actin, beta
ACRF	Australian Cancer Research Foundation
APBβ	Amyloid precursor protein
BAC	Bacterial artificial chromosome
BWS	Beckwith-Wiedemann syndrome
cDNA	Complementary DNA
CHORI	Children's Hospital Oakland Research Institute
CpG	5'-C-phosphate-G-3'
CSC	Cancer stem-like cell
CTCF	CCCTC-binding factor protein
ChIP	Chromatin immunoprecipitation
DAPI	4',6-diamidino-2-phenylindole
DMR	Differentially methylated region
DMSO	Dimethyl sulphoxide
ESCs	Embryonic stem cells
FBS	Foetal bovine serum
FISH	Fluorescence *in situ* hybridisation
gDNA	Genomic DNA
IAS1	ICR associated site
IC	Imprinting centre
ICD	Interchromatin domain model
ICN	Interchromosomal network model
ICR	Imprinting control region
IFNγR1	Interferon gamma receptor
IFN-γ	Interferon gamma
IG	Immunoglobin
IGF2	Insulin-like growth factor II
IGF2R	Insulin-like growth factor II receptor
INS	Insulin
iPSC	Induced pluripotent stem cell
LAD	Laminar-associated domain
LCR	Locus control region
LOI	Loss of imprinting
MEF	Mouse embryonic fibroblasts
NCBI	National Centre for Biotechnology Information
ncRNA	Non-coding RNA
NF1	Neurofibromatosis 1
ORc	Olfactory receptor
PBL	Peripheral blood lymphocyte
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RAH	Royal Adelaide Hospital
ROS	Reactive oxygen species
SINE	Short interspersed nuclear element
SNPs	Single nucleotide polymorphisms

SNRPN	Small nuclear ribonucleoprotein polypeptide N
TCR	T cell receptor
tRNA	Transfer RNA
UBE3A	Ubiquitin-protein ligase 3A
UCSC	University of California, Santa Cruz
WSB1	WD repeat and SOCS box-containing 1

Nomenclature

Throughout this thesis, various forms of conventional notation are used in relation to species-specific nomenclature, particularly for mouse, human, bovine and platypus.

Abstract

Long-range physical interactions between distant sections of DNA have been shown to form complex networks of loops controlling gene regulation and other nuclear functions, which are essential throughout development and disease. These chromatin interactions are remarkably frequent, with interaction patterns varying between cell types, developmental stage and in disease. The chromatin insulator CTCF mediates many of these interactions, and is also thought play a role in the definition of topological domains and preventing the spread of heterochromatin. Binding of the CTCF protein can be methylation sensitive, and few studies have investigated the impact of specific methylation changes at CTCF binding sites on long-range interactions at a particular locus. This form of regulation is particularly important to many imprinted genes, which are important for foetal growth and development, such as the growth factor *IGF2*. Altering the regulation at this locus can affect foetal development and has also been shown to be linked to poor prognosis in several cancers.

The aim of this project was to investigate the important *IGF2/H19* locus in relation to long-range interaction and CTCF binding site methylation, in both developmental and disease contexts. We investigated expression of *IGF2* and *H19* as well as the frequency of long range chromatin interactions at the locus in cattle embryos, comparing purebred and hybrid crosses with known differences in birthweight. This work identified different levels of *H19* expression between the different crosses, although no significant difference was observed in the frequency of the *IGF2/H19-WSB1* long-range chromatin interaction. We have suggested that a different mechanism of regulation at the *IGF2/H19* locus may occurring at this early developmental stage. We also investigated the methylation status of seven CTCF binding sites in the *Igf2/H19* imprinting control region in several ovarian cancer tumours and cell lines, as well as looking at expression of key genes and interaction frequency using DNA Fluorescence *in situ* hybridisation. We identified highly variable DNA methylation patterns at CTCF binding sites in serous ovarian cancer tumours at different disease stages and noted that methylation at each site responded with variable sensitivity to treatment with a common demethylating drug in ovarian cancer cell lines.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree. I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968. The author acknowledges that copyright of published works contained within this thesis resides with the copyright holder(s) of those works. I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

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Finally, to my friends and family. Your unwavering support of me throughout this entire process has made this thesis possible. I cannot express how deeply grateful I am to all of you.

Chapter 1 - Introduction

This chapter consists of a conventional thesis introduction.

Chapter Overview

This chapter takes the form of a literature review undertaken at the start of candidature and revised as necessary. In this chapter I give an overview of nuclear organisation and the epigenetic regulation involved in maintaining correct nuclear function, with a focus on long-range chromatin interactions. Key factors in these interactions are highlighted, including CTCF and the imprinted *IGF2/H19* region. The evolution of this form of regulation is discussed as well as known issues in disease contexts, with current gaps in the literature considered. Finally, we outline our plans to address these gaps. This chapter provides key information relevant to the manuscripts presented in Chapters 2, 3 and 4 of this thesis.

1.1 - Dynamic nuclear organisation and function

With an estimated 19,000 genes (Ezkurdia *et al.* 2014) and large numbers of regulatory sequences packed into the nucleus, it is unsurprising that the function of this vast amount of genetic information depends heavily on the correct organisation of the chromatin within the nucleus. There have been great advances in understanding genome organisation on the whole chromosome and gene specific level. We now know that chromosomes occupy specific territories during interphase (Figure 1) (Cremer & Cremer 2010). These territories are dynamic hubs and it has been found that gene dense chromosomes tend to localise towards the nuclear interior while gene poor chromosomes tend to cluster at the nuclear periphery (reviewed by (Bickmore 2013; Kalhor *et al.* 2012)).

At a smaller scale bands of the chromosomes undergo further regulation. Sections of the chromosome are epigenetically silenced by DNA methylation and histone modifications, while neighbouring regions may be relaxed, allowing transcription to take place. These open sections of chromatin are then more accessible to transcription factors and other DNA binding proteins. Active regions are thought to localise to the outside of the territory, while inactive regions are usually found in the interior. Interactions with the various components of transcription, replication and other regulatory factors occur throughout the nucleus, but can be concentrated in specific regions or hubs. These interactions are not restricted to transcriptionally active genes. Laminar-associated domains (LADs) are clustered at the nuclear periphery and are enriched for repressive histone marks, CCCTC-binding factor (CTCF) protein binding sites, CpG islands (Guelen *et al.* 2008). Unsurprisingly, genes localised to the area are transcriptionally inactive (Reddy *et al.* 2008). However, it is not only large scale chromosome localisation that influences genome function; physical interactions between chromatin play an important role in regulation of single loci as well. Loops of chromatin extending out from within the chromosome territories occur frequently. One study observed regions from up to four different chromosomes colocalising simultaneously (Zhao *et al.* 2006). It has also been found that there are characteristic interaction profiles associated with maintaining pluripotency (Apostolou *et al.* 2013).

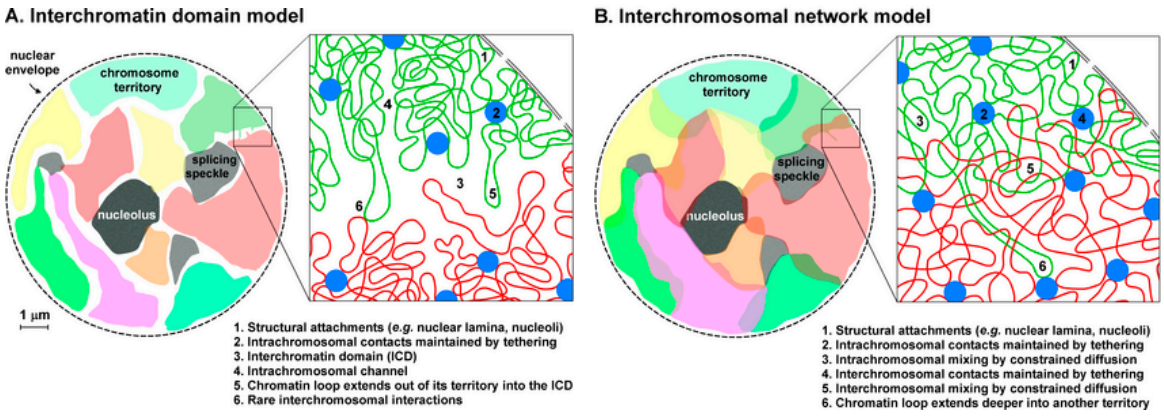

Figure 1: Two models of chromosome territory arrangement. In both models, extensive interactions occur between neighbouring territories. Active genes are thought to loop out from the territory to be transcribed, such as the MHC locus (Mahy et al. 2002), or to interact with a target in another territory while inactive genes remain within the territory. Figure from (Branco & Pombo 2006).

There are two types of interactions that can occur, intrachromosomal interactions, where both regions are located on the same chromosome, and interchromosomal interactions, where the two (or more) interacting regions are located on different chromosomes. It has been shown that both types of interaction can occur at the one locus, such as the TH_2 locus control region (LCR). Intrachromosomal interactions with the promoters of several nearby cytokine genes have been observed, as well as interchromosomal interactions with the promoter of the *interferon gamma (IFN-γ)* gene (Spilianakis *et al.* 2005). It was later shown that these interactions were required to maintain the monoallelic expression of the *interferon gamma receptor* (*IfnγR1)* (Deligianni & Spilianakis 2012). The extent of the interaction networks in various cell types is astounding, with over one million interactions observed in human lung fibroblasts in a recent study using powerful genome wide technologies to establish interaction maps (Jin *et al.* 2013). However, it is only relatively recently that the long-range interactions between the territories has been investigated at both the single locus and genome wide levels.

1.2 – CTCF as a major factor in chromosome interactions

One of the key factors in mediating these interaction networks is the 'master weaver' CTCF (Phillips & Corces 2009). A highly conserved 11-zinc finger protein, CTCF has potential roles in a number of systems, including as an insulator (Bell *et al.* 1999), mediator

of chromatin interactions (Handoko *et al.* 2011; Ling *et al.* 2006; Murrell *et al.* 2004), as well as in regulation of the V(D)J recombination process of the *Immunoglobin (Ig)* and T cell receptor (*Tcr)* loci (Chaumeil & Skok 2012) and in X chromosome inactivation (Filippova *et al.* 2005). It has been described as playing a fundamental role in controlling the networks of intra- and interchromosomal interactions and therefore the higher-order structure of the chromosome territories (Botta *et al.* 2010). It has also been suggested that CTCF and cohesin have a genome-wide role in coordinating cell specific chromatin organisation and transcriptional activity (Hou *et al.* 2010). Cohesin is known to localise with CTCF to binding sites associated with enhancer-promoter interactions (Parelho *et al.* 2008), however it is still unknown what drives the cell specificity of these interactions.

The binding of the CTCF protein occurs through the 11 zinc finger structures (Figure 2), with its versatility in binding coming from alternative uses for each cluster of zinc fingers (Nakahashi *et al.* 2013). In a study investigating the binding of CTCF to the *amyloid precursor protein* (*APBβ*) promoter, it was found that deletion of the core zinc fingers 5-7 completely disrupted binding, while deletion of the peripheral zinc fingers destabilised the bound protein-DNA complex (Quitschke *et al.* 2000). The C terminal end of the CTCF protein is known to bind factors such as SA2, a cohesion subunit, which is involved in the insulator function of CTCF. Deletion or mutation of the C terminus in CTCF resulted in disrupted expression of imprinted genes due to loss of insulator function (Xiao *et al.* 2011). The N terminal is known to harbour a site that recruits Suz12, part of the polycomb repressive complex 2 (Zhang *et al.* 2011).

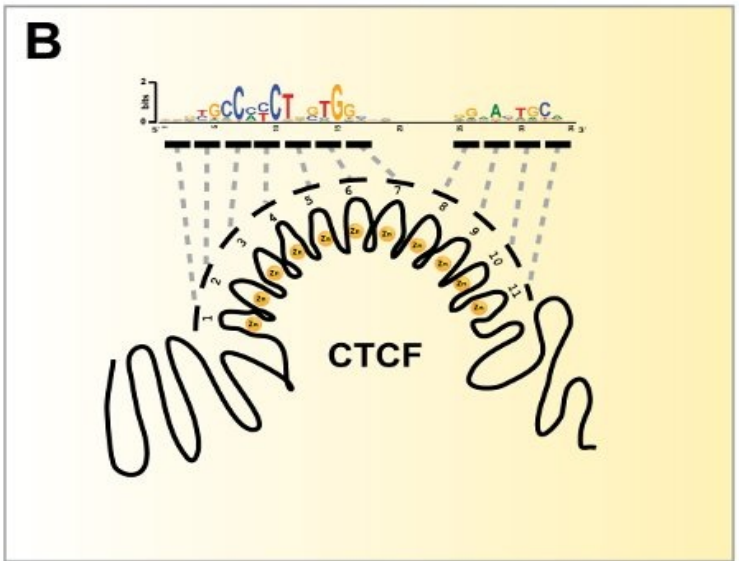

Figure 2: CTCF binds to a DNA motif sequence through the 11 zinc fingers. The core zinc fingers 5-7 are thought to be critical in DNA binding, while the other fingers help stabilise the complex. Figure from (Schmidt *et al* 2012).

CTCF binding is known to be methylation sensitive (Kanduri *et al.* 2000; Wang *et al.* 2012b), however it also has a role in maintaining methylation patterns such as the maintenance of imprinting at the *insulin-like growth factor 2* (*IGF2)* locus (Szabo *et al.* 2004). This impact on methylation can be a component of disease, as seen when CTCF haploinsufficiency in mice resulted in destabilised DNA methylation genome-wide and predisposed them to cancer (Kemp *et al.* 2014). CTCF also has a role in further refining nuclear architecture as a factor in the definition of topological domains (Apostolou *et al.* 2013). The boundaries of these domains are not only enriched for CTCF binding sites, but also tRNAs, housekeeping genes, and SINE retrotransposons (Dixon *et al.* 2012).

1.2.1 - Evolution of CTCF binding sites

Due to the relevance of the CTCF protein in mediating many of these interactions, it is interesting to look at the evolution of the CTCF binding sites. Although the CTCF protein itself is highly conserved from drosophila to humans (Moon *et al.* 2005; Ohlsson *et al.* 2001), little is known about the evolution of the binding sites. Many binding sites are conserved throughout the vertebrates (Martin *et al.* 2011), however there has also been species specific binding sites identified in mammalian species (Schmidt *et al.* 2012). It was suggested that CTCF binding sites have moved around the genome through SINE activity, with ancient retrotransposon activity resulting in the most conserved binding sites (Schmidt *et al.* 2012). It was proposed that by carrying CTCF binding sites through the genome as they jump,

retrotransposons are protected from epigenetic silencing through expanding methylation due to the barrier function of CTCF (Schmidt *et al.* 2012). The movement of CTCF binding sites via SINEs may account for the prevalence of the motif throughout the genome, with some 13,800 sites in the human genome (Kim *et al.* 2007).

Recent analysis has refined the binding motif of CTCF, identifying a second downstream motif (Figure 3). Binding events involving both motifs were found to be more conserved between the mammalian species tested, and showed stronger enrichment in ChIP experiments (Schmidt *et al.* 2012). These binding sites can be further classified into high and low occupancy sites based on several conditions. Low occupancy sites are more cell-type specific than high occupancy sites, and are generally associated with high gene expression and active histone marks (Essien *et al.* 2009). Low occupancy sites also tend to be conserved as low occupancy sites between species (Essien *et al.* 2009). More recently, slight differences in CTCF binding motifs appear to alter the regulatory function of CTCF at certain genes during stem cell differentiation (Plasschaert *et al.* 2014).

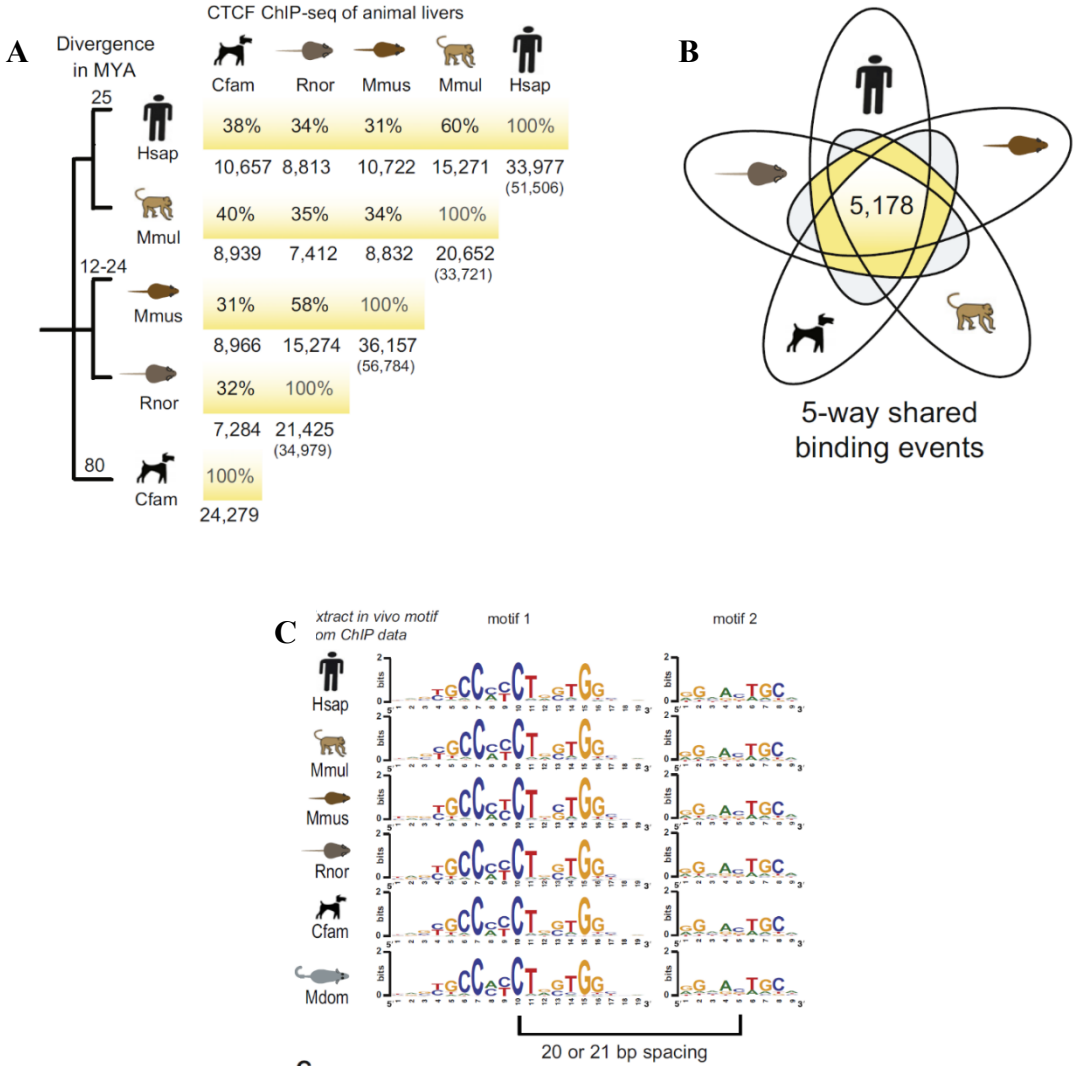

Figure 3A: Pairwise analysis of orthologous regions between species analysed for CTCF binding events. **B:** CTCF binding events shared by all five placental mammals. **C:** De novo identified CTCF binding motifs in each species. Figure from (Schmidt *et al.* 2012).

1.3 - Conservation of chromatin interactions across mammalian evolution and in different cell types

A recent study investigating lamina association, replication timing, and interactions has found that around 10% of the higher order structure appears to be diverged between humans and mice (Chambers *et al.* 2013). Interestingly, it was observed that much of the difference in structural features appears to occur in regions containing developmental genes (Chambers, Bickmore & Semple 2013). Although imprinting is not always conserved between species, other epigenetic features such as monoallelic expression and replication timing are often seen at the same loci that are known to undergo interactions (Wright 2014).

Some of these interactions are conserved across species, as with the interaction between the imprinted *Igf2/H19* region and the *WD repeat and SOCS box-containing 1* (*Wsb1)* region originally identified in mice (Ling *et al.* 2006). This interaction only occurs between the maternal *Igf2* and paternal *Wsb1* alleles, despite biallelic expression of *Wsb1* (Ling *et al.* 2006). This interaction has also been observed in chicken and platypus, as well as mammalian species (Figure 4) although sequence analysis was unable to identify CTCF binding sites in the platypus *H19* imprinting control region (ICR) (Wright 2014). In all species, the interaction occurred at a non-random frequency, even in species where the *Igf2* gene is not imprinted. Interestingly, random monoallelic expression was also observed at the *Igf2* locus in the platypus suggesting elements of epigenetic regulation predating genomic imprinting (Wright 2014).

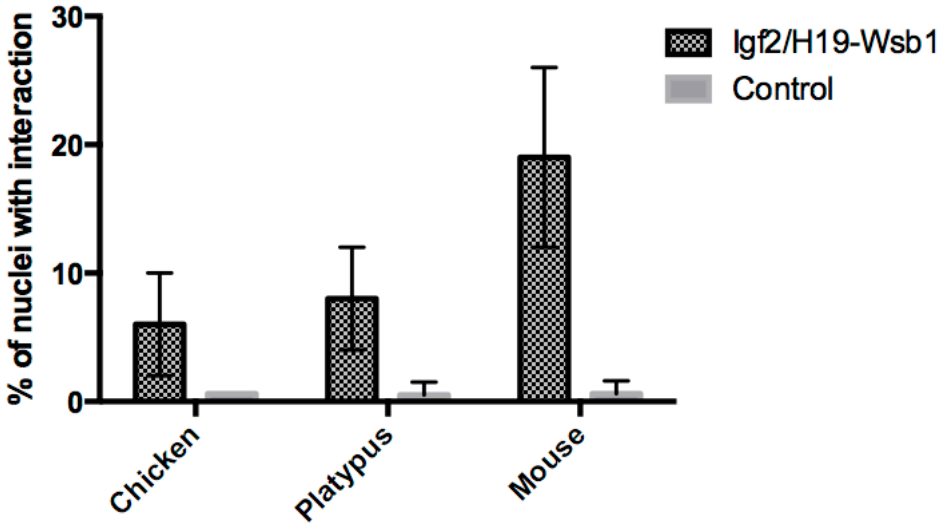

Figure 4: Conservation of the *Igf2/Wsb1* interaction in amniotes. Interactions were identified using DNA FISH dot assays. Overlapping signals were counted as an interaction; the non-interaction control was between *mesoderm-specific transcript* (*Mest)* and *Wsb1*. Error bars show the standard deviation between samples for each of the species. Figure from Wright *et. al.* unpublished.

Recent studies have also suggested that interactions appear to be conserved between different species with regard to the differentiation state of the cells (Ryba *et al.* 2010). These interactions play a significant role in stem cell pluripotency. A study investigating the key pluripotency homeobox gene *Nanog* identified differentiation state specific interaction patterns (Figure 5) and distinctly different interaction network patterns when the mediator and cohesion complexes responsible for the interactions were knocked down (Apostolou *et*

al. 2013). The two cell types used were mouse embryonic stem cells (ESCs) and mouse embryonic fibroblasts (MEFs) to represent an undifferentiated state and a differentiated cell type. The changes to the interactome profile occurred before obvious changes were observed in transcriptional or phenotypic measures (Apostolou *et al.* 2013).

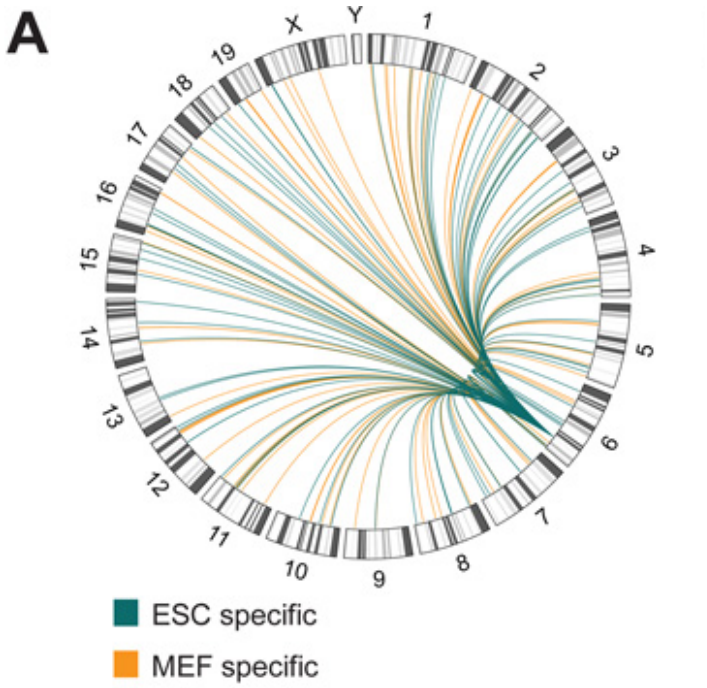

Figure 5: Differentiation state specific interactions with the *Nanog* locus in mouse embryonic stem cells (ESCs) and embryonic fibroblasts (MEFs). Figure from (Apostolou *et al.* 2013).

There can still be great variability, observed both between cell types and even cell-to-cell in a single population, due to the stochastic nature of the chromosome arrangement. It also shows that interaction profiles are heavily influenced by the epigenetic characteristics of a given cell type.

1.4 – Architecture of the *IGF2/H19* region and relevance for disease, development and hybridisation

The *IGF2/H19* locus (on chromosome 11p15.5 in humans) is a reciprocally imprinted locus whereby only the paternal *IGF2* and maternal *H19* alleles are expressed. *H19* is a non-coding RNA (ncRNA) gene that is thought to have tumour suppressor activity (Hao *et al.* 1993), while *IGF2* is a potent mitogenic growth factor involved in foetal growth (Constancia *et al.* 2002). It is generally expressed at varying levels in different tissues (Vandijk *et al.* 1991; Vu & Hoffman 1994), and expression is modulated through developmental stages (Issa *et al.* 1996).

Parental specific methylation patterns are observed at the Differentially Methylated Region (DMR) and Imprinting Control Region (ICR), and are heavily involved in the regulation of the *IGF2/H19* locus (Figure 6). DNA methylation is a form of epigenetic regulation that is important in chromatin interactions. A well characterised mechanism of epigenetic silencing, DNA methylation is commonly seen in regions of transcriptionally inactive heterochromatin along with repressive histone modifications. It is a significant mechanism in the process of monoallelic expression (Wang *et al.* 2007) as observed in cases of allelic exclusion, X-inactivation and in imprinted gene clusters.

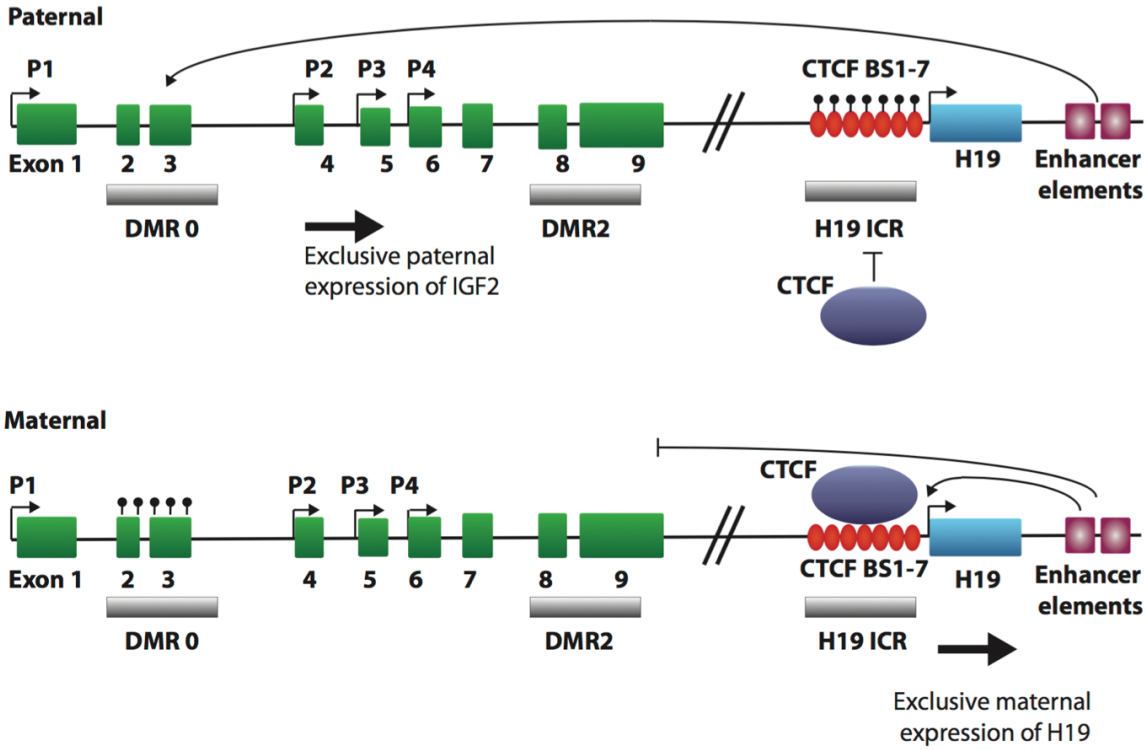

Figure 6: Regulation of the *IGF2/H19* locus. *IGF2* has four promoters (P1-P4) that are used in different tissues and developmental stages. Exclusive monoallelic expression from the paternal allele is observed from P2-P4. Biallelic expression from P1 has been observed in adult liver. The shaded rectangles represent the DMRs and the *H19* ICR. Seven CTCF binding sites are located in the ICR and the CTCF protein binds to the unmethylated maternal allele. The methylated (sticks) paternal allele is not bound by CTCF. Figure adapted from (Murphy *et al.* 2006).

In the context of *IGF2/H19* regulation, there are seven CTCF binding sites located within the ICR just upstream of *H19* that are methylated on the paternal copy, preventing CTCF from binding (Hark *et al.* 2000). Enhancer elements downstream of *H19* are brought to the *IGF2* promoters by an intrachromosomal loop structure, promoting expression of the paternal *Igf2*. The maternal allele however is not expressed, as CTCF binds to the unmethylated ICR and prevents the loop from forming (Kurukuti *et al.* 2006). The enhancers are then used to promote expression of the maternal *H19*. The maternal allele of *IGF2* has also been shown to undergo an interchromosomal interaction with the paternal allele of the non-imprinted *WSB1/NF1* locus (Ling *et al.* 2006). Interestingly, interactions between *Igf2/Wsb1* have been observed in the chicken and platypus, species where imprinting is not observed, however CTCF binding sites were not able to be identified in the *H19* ICR (Wright *et. al.* unpublished). This may be due to sequence divergence in the CTCF binding site in monotremes. Both regulatory interactions rely on the binding of the CTCF protein. It is thought that as well as promoting maternally exclusive expression of *H19*, the binding of CTCF silences the maternal expression of *IGF2* through histone modifications by recruiting a polycomb repressive complex (Li *et al.* 2008). Replacement of CTCF with a decoy protein resulted in loss of imprinting and the intrachromosomal loop in human cell lines, highlighting the importance of CTCFs role in regulation of the *IGF2/H19* locus through interaction with Suz12, a component of the polycomb repressive complex (Zhang *et al.* 2011). The four *IGF2* promoters are differentially expressed, with monoallelic expression from P2-P4 and biallelic expression from P1, found only in the liver (Stringer *et al.* 2012).

Despite the complexity of its regulation, the *IGF2/H19* region is a great example of how higher order chromatin structure can influence gene regulation and expression. The combination of parent-of-origin allele specific expression, its role in both development and disease, and its involvement in higher order chromatin structures, makes this locus the perfect candidate for investigating interchromosomal networks and gene expression in both evolutionary and disease contexts.

Aberrant expression of the *IGF2* locus has been observed in many types of cancer including colorectal cancer (Cheng *et al.* 2010; Cui *et al.* 2002), osteosarcoma (Ulaner *et al.* 2003), prostate cancer (Tennant *et al.* 1996), and ovarian cancer (Huang & Murphy 2013; Murphy *et al.* 2009; Murphy *et al.* 2006). It is worth noting that elevated *IGF2* alone was not found to induce tumours, but significantly increased growth and has been associated with poor prognosis in epithelial ovarian cancer (Lu *et al.* 2006). This may be linked to loss of

imprinting (LOI) in cancers (Kim *et al.* 1998), altered DNA methylation (Kulis & Esteller 2010), or changes in interaction networks (Zeitz *et al.* 2013).

This intriguing region is not only relevant in disease. Abnormal regulation of this region has been implicated in overgrowth phenotypes observed in several species hybrid systems. Hybrid animals have been a source of scientific interest for many years, since sterility and other issues were observed to differ between the sexes or reciprocal crosses of the same parent breeds (Haldane 1922). Hybrid overgrowth phenotypes, where one hybrid offspring is significantly larger than the reciprocal cross, were originally observed in the *Peromyscus polionotus* and *Peromyscus maniculatus* species (Dawson 1965; Vrana *et al.* 1998). This overgrowth has been linked to incorrect expression of imprinted genes (Schütt *et al.* 2003; Vrana *et al.* 2000). Likewise, in bovine hybrids, large birthweight discrepancies are observed between the *Bos primigenius taurus* and *Bos primigenius indicus* reciprocal hybrids (Brown *et al.* 1993). Imprinted genes in cattle, such as *insulin-like growth factor 2 receptor (IGF2R), IGF2* and *H19* have all been shown to be deregulated in cloned cattle (Long & Cai 2007) or hybrids (Chen *et al.* 2015; Goodall & Schmutz 2007). Although the likely cause of these phenotypes has been narrowed down to altered imprinting regulation, many of the existing studies have shown this by looking at expression changes of the imprinted genes. However, little has been shown regarding the more complex epigenetic regulation of these genes, including long-range chromatin interactions.

1.5 – Monoallelic expression and genomic imprinting

Monoallelic expression is where only one allele is expressed, with the other often being epigenetically silenced. The three common forms of epigenetic regulation of monoallelic expression are genomic imprinting, X-chromosome inactivation, and allelic exclusion (reviewed by (Zakharova *et al.* 2009)). One of the best studied examples where chromatin interactions are required for monoallelic expression is in the allelic exclusion of the olfactory receptor (ORc) genes. There are 339 human ORc genes arranged in clusters (Glusman *et al.* 2001), with the largest cluster containing 116 genes (Malnic *et al.* 2004). However, despite the apparent abundance of ORc genes, each sensory neuron only expresses one receptor (Chess *et al.* 1994). Furthermore, only one allele of the chosen gene is active, the other allele being epigenetically silenced, resulting in monoallelic expression (Chess *et al.* 1994). Neurons expressing various receptors are used in combination to detect an impressive range of odorants (Malnic *et al.* 1999).

The process of allelic exclusion in the ORc genes occurs through the action of an enhancer known as the *H* element. A long-range interaction occurs between the *H* enhancer element and the promoter of the active ORc allele (Lomvardas *et al.* 2006). Duplication of the *H* element in mice causes a single neuron to express multiple functional receptors (Lomvardas *et al.* 2006). However, deletion of the *H* element was found to influence selection of locally clustered ORc genes, suggesting other factors may also be involved (Fuss *et al.* 2007). Regardless of the specificity of the H element in question, there is no doubt long-range interactions and higher-order chromatin structure play a large role in the regulation of the olfactory receptor genes.

Genomic imprinting is a specific form of methylation silencing that results in monoallelic expression as it occurs in a parent-of-origin specific way. Imprinted genes are typically associated with development (Tycko & Morison 2002), and are a unique feature in the therian mammals and some plants (Nowack *et al.* 2007). Imprinted genes are by definition monoallelically expressed. Genomic imprinting is a fascinating form of epigenetic regulation as imprinted genes are often found in clusters controlled by a *cis*-acting Imprinting Control Region (ICR) (Bartolomei & Ferguson-Smith 2011; Edwards & Ferguson-Smith 2007). Due to the differential expression of the two alleles, imprinted genes are often observed to replicate asynchronously (Gribnau *et al.* 2003; Kitsberg *et al.* 1993). Interestingly, orthologs of imprinted genes in chickens and platypus, where no imprinting occurs, have also been shown to replicate asynchronously (Dunzinger *et al.* 2005; Wright & Grutzner in prep.), and show random monoallelic expression, where expression only occurs from one allele but silencing is not in a parent-of-origin dependent way (Wright *et al.* in prep.).

1.5.1 - Evolution of genomic imprinting

There are several theories regarding the evolution of genomic imprinting (reviewed by (Renfree *et al.* 2009), however there is more still to be studied regarding the molecular mechanisms of imprinting evolution. There are suggestions that genomic imprinting is a result of parental conflict, whereby the evolutionary benefit to each of the parents is opposing (Moore & Haig 1991). An example of parental conflict would be where elevated growth of the offspring increases the likelihood of the father's genes being passed on to later generations, but due to the energy cost is detrimental to the mother's health and potential future pregnancies. This is considered particularly relevant in the eutherian mammals, where maternal investment is quite significant. This reinforces the need to truly understand the

mechanistic disruption of imprinting in hybrids, where there is a unique opportunity to study the epigenetics of two conflicting genomes, that are otherwise genetically similar, but with pronounced phenotypic differences.

1.5.2 – Altered imprinting regulation in disease

Imprinted clusters are also relevant in disease contexts such as in cancer, and Beckwith-Wiedemann (Brown *et al.* 1996; Poole *et al.* 2012), Prader-Willi, and Angelman syndromes (Buiting *et al.* 1995). In Prader-Willi syndrome, errors involving the imprinted cluster located on chromosome 15q11-q13 are associated with development of the disease (Ohta *et al.* 1999). Imprinting at this region is controlled by an imprinting centre (IC). Deletion of the paternal *Small nuclear ribonucleoprotein polypeptide N (SNRPN)* gene results in Prader-Willi syndrome (Reed & Leff 1994), as the maternal copy is silenced and does not compensate for loss of the paternal transcripts. Similarly, a deletion of the maternal copy of *ubiquitin-protein ligase 3A (UBE3A)* results in Angelman syndrome, as the paternal copy in unable to compensate due to imprinting (Kishino *et al.* 1997). Angelman syndrome can also arise through paternal uniparental disomy, due to the lack of maternal transcripts (Fang *et al.* 1999). In Beckwith-Wiedemann syndrome (BWS), an overgrowth phenotype and predisposition to cancer is common (Weksberg *et al.* 2010). Several mutations have been associated with the disease, but are all located in an imprinted cluster located on chromosome 11p15.5 in humans. Loss of the maternal transcript from imprinted gene $p57^{KIP2}$, a negative regulator of proliferation, is associated with development of the disease (Hatada *et al.* 1996). Altered expression of the growth factor *IGF2* is also thought to contribute to the disease phenotype (Brown *et al.* 1996; Hedborg *et al.* 1994).

Loss of imprinting of *IGF2* is also observed in colorectal (Cheng *et al.* 2010) and ovarian cancers (Chen *et al.* 2000; Hiura *et al.* 2012; Kim *et al.* 1998; Murphy *et al.* 2006), as well as Wilm's Tumour (Hubertus *et al.* 2011) and multiple cancer cell lines (Dammann *et al.* 2010; Vu *et al.* 2010a). Recent studies have shown that a significant change in interaction patterns are observed in breast cancer (Zeitz *et al.* 2013), colorectal cancer (Ling & Hoffman 2011), and multiple cancer cell lines (Vu *et al.* 2010b).

Specifically, altered methylation patterns are observed at CTCF binding sites in cancer. In a study of ovarian cancer cell lines, Dammann *et al* (2010) found different methylation patterns at the CTCF binding sites in the *H19* ICR and *IGF2* DMR (Table 1). It

is likely that the changes in methylation patterns correlate with altered interaction profiles, given the importance of CTCF binding in mediating chromatin interactions.

Table 1: Methylation patterns observed at key *IGF2* and *H19* regulatory sites in ovarian cancer cell lines (Dammann *et al.* 2010).

Cell line	Cell type	Methylation at *IGF2*-DMR0	Methylation at *H19* ICR
Primary cancer cells	Ascites-derived	Hypo	Hyper
SKOV-3	Ascites-derived – ovarian adenocarcinoma	Hypo	Hypo
OVCAR-3	Ascites-derived – ovarian adenocarcinoma	Hypo	Hypo
OAW42	Ascites-derived - ovarian cystadenocarcinoma	Hyper	Hypo
CAOV3	Ovarian adenocarcinoma	Hyper	Hyper

1.6 - Ovarian cancer and investigating the epigenetics of cancer genome organisation

As the most lethal gynaecological cancer (Australian Institute of Health & Welfare 2010), ovarian cancer is a challenging area of medical research that, compared to the more common breast and prostate cancers, is poorly understood. Early diagnosis is extremely challenging due to the commonality of symptoms with other non-malignant gynaecological conditions (Bast *et al.* 2009). There is little known regarding the origins of ovarian cancer (see Figure 7). It has been suggested that cells of the ovarian epithelium or the epithelial cells of the inclusion cysts that form post-ovulation, are transformed due to exposure to pro-inflammatory cytokines and reactive oxygen species (ROS) (Auersperg 2013; Feeley & Wells 2001; Mittal *et al.* 1993). Other studies claim that ovarian cancers arise from epithelial cells of the fimbrial ends of the fallopian tube (Li *et al.* 2011; Li *et al.* 2012). It is thought that these cells are similarly transformed by exposure to the same pro-inflammatory cytokines and ROS as the ovarian surface epithelium. Therefore, the origin of ovarian cancer remains contentious.

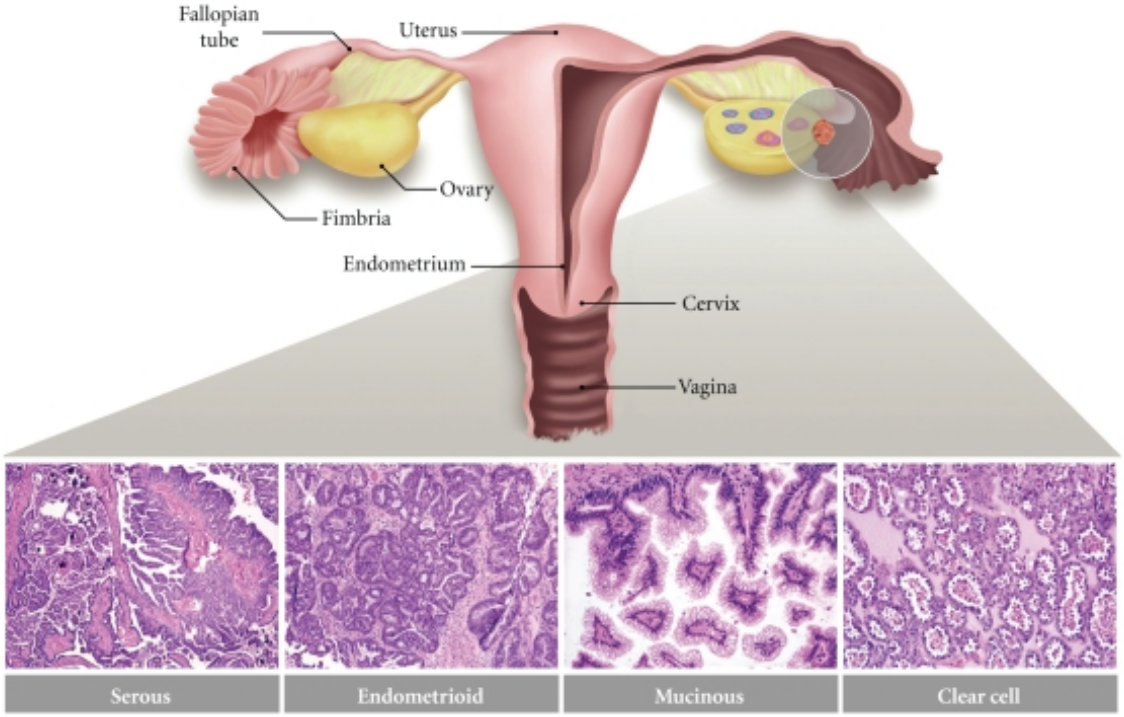

Figure 7: H&E histological stains of the four most common types of ovarian cancer showing varied morphology. The inset white circle indicates the likely position of origin for ovarian cancer, whether the transformed cells are from the fallopian fimbrial epithelium or the surface epithelium of the ovary itself. Figure from (Karst & Drapkin 2010).

A third model has recently been suggested however, involving a stem cell niche surrounding the ovary, where transformation-prone stem cells assist in repair of the ovary epithelium after follicle rupture (Flesken-Nikitin *et al.* 2013). Interestingly, these cells are also found in the ascites (Jiang *et al.* 2012) and are chemo-resistant and retain tumourigenic potential (Hu *et al.* 2010; Tomao *et al.* 2013). As it is thought that epigenetic changes may be fundamental in the initiation of disease (Berry & Bapat 2008), these cancer stem-like cells (CSCs) are a unique target for epigenetic therapies, as many of their stem cell like properties are due to DNA methylation of promoters responsible for maintaining pluripotency (Esteller 2002; Lopez-Serra & Esteller 2012). Due to the importance of the epigenetic mechanisms in maintaining this stem cell pluripotency and as a 'pre-cancerous' population, they provide an opportunity to examine early epigenetic characteristics in a very early stage of disease.

1.7 - Summary

Chromatin interaction networks that play an important role in the regulation of gene expression and other epigenetic mechanisms are a hotspot of current investigation. New technologies allow investigation of how changes in the complex networks might occur in hybridisation and in human disease. The evolution of these interactions is poorly understood, particularly the complex epigenetic regulation in hybrid dysgenesis, and little is known about the impact on expression and growth at early developmental stages in hybrid individuals. Investigation of the dynamics of long-range chromatin interactions from an evolutionary perspective can provide unique insights into the development of aberrant epigenetic regulation in diseases such as cancer, where loss of imprinting is commonly observed. The proposed project aims to advance our knowledge of how chromatin interactions and epigenetic makeup change specifically in the *IGF2/H19* region in bovine hybrids and in ovarian cancer.

Project aims

The major aim of this project is to investigate the dynamics of chromatin interactions in both evolutionary and disease contexts, identifying shared mechanisms and patterns of altered regulation at the critically important *IGF2/H19* locus.

From an evolutionary perspective, we are interested in what happens to these higher order chromatin structures when two potentially conflicting genomes interact during a hybridisation event. To investigate this, we use the *Bos primigenius taurus* and *Bos primigenius indicus* cattle breeds, where hybrid offspring are observed to display foetal overgrowth phenotypes and imprinting discrepancies. A combination of cytogenetic and molecular biology techniques are used to determine patterns of altered regulation in primary embryonic cell lines. We predict that the long-range interactions in the hybrids will be altered, based on the level of expression of the key imprinted genes.

In a disease context, we aim to determine changes in DNA methylation, expression, and long-range interaction frequency at the *IGF2/*H19 locus in early and late stage ovarian cancer using a combination of bisulphite sequencing, qPCR and DNA fluorescence *in situ* hybridisation when possible. We are also interested in how common drug therapies impact or interact with the epigenome in ovarian cancer, specifically how demethylation drugs effect the regulation of the *IGF2/H19* region, and what impact this has on the interactome.

This project looks to develop our understanding of the role of these long-range interactions and chromatin structures in gene expression, and further our knowledge of how this regulatory mechanism works in both evolution and disease.

References

Apostolou, E, Ferrari, F, Walsh, RM, Bar-Nur, O, Stadtfeld, M, Cheloufi, S, Stuart, HT*, et al.* 2013, 'Genome-wide chromatin interactions of the Nanog locus in pluripotency, differentiation, and reprogramming', *Cell Stem Cell*, vol. 12, no. 6, Jun 6, pp. 699-712.

Auersperg, N 2013, 'The origin of ovarian cancers--hypotheses and controversies', *Front Biosci (Schol Ed)*, vol. 5, pp. 709-719.

Australian Institute of Health & Welfare 2010, 'Ovarian cancer in Australia: an overview, 2010', *Cancer Series*, no. 52.

Bartolomei, MS & Ferguson-Smith, AC 2011, 'Mammalian Genomic Imprinting', *Cold Spring Harbor Perspectives in Biology*, vol. 3, no. 7, July 1, 2011.

Bast, RC, Jr., Hennessy, B & Mills, GB 2009, 'The biology of ovarian cancer: new opportunities for translation', *Nat Rev Cancer*, vol. 9, no. 6, Jun, pp. 415-428.

Bell, AC, West, AG & Felsenfeld, G 1999, 'The Protein CTCF Is Required for the Enhancer Blocking Activity of Vertebrate Insulators', *Cell*, vol. 98, no. 3, 8/6/, pp. 387-396.

Berry, NB & Bapat, SA 2008, 'Ovarian cancer plasticity and epigenomics in the acquisition of a stem-like phenotype', *J Ovarian Res*, vol. 1, p. 8.

Bickmore, WA 2013, 'The spatial organization of the human genome', *Annu Rev Genomics Hum Genet*, vol. 14, pp. 67-84.

Botta, M, Haider, S, Leung, IXY, Lio, P & Mozziconacci, J 2010, 'Intra- and inter-chromosomal interactions correlate with CTCF binding genome wide', *Mol Syst Biol*, vol. 6, no. 426.

Branco, MR & Pombo, A 2006, 'Intermingling of Chromosome Territories in Interphase Suggests Role in Translocations and Transcription-Dependent Associations', *PLoS Biol*, vol. 4, no. 5, p. e138.

Brown, KW, Villar, AJ, Bickmore, W, Clayton-Smith, J, Catchpoole, D, Maher, ER & Reik, W 1996, 'Imprinting mutation in the Beckwith-Wiedemann syndrome leads to biallelic IGF2 expression through an H19-independent pathway', *Hum Mol Genet*, vol. 5, no. 12, Dec, pp. 2027-2032.

Brown, MA, Tharel, LM, Brown, AH, Jr., Jackson, WG & Miesner, JR 1993, 'Genotype x environment interactions in preweaning traits of purebred and reciprocal cross Angus and Brahman calves on common bermudagrass and endophyte-infected tall fescue pastures', *J Anim Sci*, vol. 71, no. 2, Feb, pp. 326-333.

Buiting, K, Saitoh, S, Gross, S, Dittrich, B, Schwartz, S, Nicholls, RD & Horsthemke, B 1995, 'Inherited microdeletions in the Angelman and Prader-Willi syndromes define an imprinting centre on human chromosome 15', *Nat Genet*, vol. 9, no. 4, 04//print, pp. 395-400.

Chambers, EV, Bickmore, WA & Semple, CA 2013, 'Divergence of Mammalian Higher Order Chromatin Structure Is Associated with Developmental Loci', *PLoS Comput Biol*, vol. 9, no. 4, p. e1003017.

Chaumeil, J & Skok, JA 2012, 'The role of CTCF in regulating V(D)J recombination', *Curr Opin Immunol*, vol. 24, no. 2, Apr, pp. 153-159.

Chen, CL, Ip, SM, Cheng, D, Wong, LC & Ngan, HYS 2000, 'Loss of imprinting of the IGF-II and H19 genes in epithelial ovarian cancer', *Clinical Cancer Research*, vol. 6, no. 2, pp. 474-479.

Chen, Z, Hagen, DE, Elsik, CG, Ji, T, Morris, CJ, Moon, LE & Rivera, RM 2015, 'Characterization of global loss of imprinting in fetal overgrowth syndrome induced by assisted reproduction', *Proc Natl Acad Sci U S A*, vol. 112, no. 15, Apr 14, pp. 4618-4623.

Cheng, YW, Idrees, K, Shattock, R, Khan, SA, Zeng, ZS, Brennan, CW, Paty, P*, et al.* 2010, 'Loss of imprinting and marked gene elevation are 2 forms of aberrant IGF2 expression in colorectal cancer', *International Journal of Cancer*, vol. 127, no. 3, pp. 568-577.

Chess, A, Simon, I, Cedar, H & Axel, R 1994, 'Allelic inactivation regulates olfactory receptor gene expression', *Cell*, vol. 78, no. 5, Sep 9, pp. 823-834.

Constancia, M, Hemberger, M, Hughes, J, Dean, W, Ferguson-Smith, A, Fundele, R, Stewart, F*, et al.* 2002, 'Placental-specific IGF-II is a major modulator of placental and fetal growth', *Nature*, vol. 417, no. 6892, pp. 945-948.

Cremer, T & Cremer, M 2010, 'Chromosome territories', *Cold Spring Harb Perspect Biol*, vol. 2, no. 3, Mar, p. a003889.

Cui, HM, Onyango, P, Brandenburg, S, Wu, YQ, Hsieh, CL & Feinberg, AP 2002, 'Loss of imprinting in colorectal cancer linked to hypomethylation of H19 and IGF2', *Cancer Research*, vol. 62, no. 22, pp. 6442-6446.

Dammann, RH, Kirsch, S, Schagdarsurengin, U, Dansranjavin, T, Gradhand, E, Schmitt, WD & Hauptmann, S 2010, 'Frequent aberrant methylation of the imprinted IGF2/H19 locus and LINE1 hypomethylation in ovarian carcinoma', *International Journal of Oncology*, vol. 36, no. 1, pp. 171-179.

Dawson, WD 1965, 'Fertility and Size Inheritance in a Peromyscus Species Cross', *Evolution*, vol. 19, no. 1, pp. 44-55.

Deligianni, C & Spilianakis, CG 2012, 'Long-range genomic interactions epigenetically regulate the expression of a cytokine receptor', *EMBO Rep*, vol. 13, no. 9, pp. 819-826.

Dixon, JR, Selvaraj, S, Yue, F, Kim, A, Li, Y, Shen, Y, Hu, M*, et al.* 2012, 'Topological domains in mammalian genomes identified by analysis of chromatin interactions', *Nature*, vol. 485, no. 7398, pp. 376-380.

Dunzinger, U, Nanda, I, Schmid, M, Haaf, T & Zechner, U 2005, 'Chicken orthologues of mammalian imprinted genes are clustered on macrochromosomes and replicate asynchronously', *Trends Genet*, vol. 21, no. 9, pp. 488-492.

Edwards, CA & Ferguson-Smith, AC 2007, 'Mechanisms regulating imprinted genes in clusters', *Curr Opin Cell Biol*, vol. 19, no. 3, 6//, pp. 281-289.

Essien, K, Vigneau, S, Apreleva, S, Singh, LN, Bartolomei, MS & Hannenhalli, S 2009, 'CTCF binding site classes exhibit distinct evolutionary, genomic, epigenomic and transcriptomic features', *Genome Biology*, vol. 10, no. 11.

Esteller, M 2002, 'CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future', *Oncogene*, vol. 21, no. 35, Aug 12, pp. 5427-5440.

Ezkurdia, I, Juan, D, Rodriguez, JM, Frankish, A, Diekhans, M, Harrow, J, Vazquez, J*, et al.* 2014, 'Multiple evidence strands suggest that there may be as few as 19,000 human protein-coding genes', *Hum Mol Genet*, vol. 23, no. 22, Nov 15, pp. 5866-5878.

Fang, P, Lev-Lehman, E, Tsai, T-F, Matsuura, T, Benton, CS, Sutcliffe, JS, Christian, SL*, et al.* 1999, 'The Spectrum of Mutations in UBE3ACausing Angelman Syndrome', *Human Molecular Genetics*, vol. 8, no. 1, January 1, 1999, pp. 129-135.

Feeley, KM & Wells, M 2001, 'Precursor lesions of ovarian epithelial malignancy', *Histopathology*, vol. 38, no. 2, pp. 87-95.

Filippova, GN, Cheng, MK, Moore, JM, Truong, J-P, Hu, YJ, Di Kim, N, Tsuchiya, KD*, et al.* 2005, 'Boundaries between Chromosomal Domains of X Inactivation and Escape Bind CTCF and Lack CpG Methylation during Early Development', *Developmental Cell*, vol. 8, no. 1, pp. 31-42.

Flesken-Nikitin, A, Hwang, C-I, Cheng, C-Y, Michurina, TV, Enikolopov, G & Nikitin, AY 2013, 'Ovarian surface epithelium at the junction area contains a cancer-prone stem cell niche', *Nature*, vol. 495, pp. 241-245.

Fuss, SH, Omura, M & Mombaerts, P 2007, 'Local and cis effects of the H element on expression of odorant receptor genes in mouse', *Cell*, vol. 130, no. 2, Jul 27, pp. 373-384.

Glusman, G, Yanai, I, Rubin, I & Lancet, D 2001, 'The complete human olfactory subgenome', *Genome Res*, vol. 11, no. 5, May, pp. 685-702.

Goodall, JJ & Schmutz, SM 2007, 'IGF2 gene characterization and association with rib eye area in beef cattle', *Animal Genetics*, vol. 38, no. 2, pp. 154-161.

Gribnau, J, Hochedlinger, K, Hata, K, Li, E & Jaenisch, R 2003, 'Asynchronous replication timing of imprinted loci is independent of DNA methylation, but consistent with differential subnuclear localization', *Genes Dev*, vol. 17, no. 6, Mar 15, pp. 759-773.

Guelen, L, Pagie, L, Brasset, E, Meuleman, W, Faza, MB, Talhout, W, Eussen, BH*, et al.* 2008, 'Domain organization of human chromosomes revealed by mapping of nuclear lamina interactions', *Nature*, vol. 453, no. 7197, 06/12/print, pp. 948-951.

Haldane, JBS 1922, 'Sex ratio and unisexual sterility in hybrid animals', *Journal of Genetics*, vol. 12, no. 2, 1922//, pp. 101-109.

Handoko, L, Xu, H, Li, G, Ngan, CY, Chew, E, Schnapp, M, Lee, CWH*, et al.* 2011, 'CTCF-mediated functional chromatin interactome in pluripotent cells', *Nature Genetics*, vol. 43, no. 7, pp. 630-638.

Hao, Y, Crenshaw, T, Moulton, T, Newcomb, E & Tycko, B 1993, 'Tumor-suppressor activity of H19 RNA', *Nature*, vol. 365, no. 6448, pp. 764-767.

Hark, AT, Schoenherr, CJ, Katz, DJ, Ingram, RS, Levorse, JM & Tilghman, SM 2000, 'CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus', *Nature*, vol. 405, no. 6785, May 25, pp. 486-489.

Hatada, I, Ohashi, H, Fukushima, Y, Kaneko, Y, Inoue, M, Komoto, Y, Okada, A*, et al.* 1996, 'An imprinted gene p57KIP2 is mutated in Beckwith-Wiedemann syndrome', *Nat Genet*, vol. 14, no. 2, Oct, pp. 171-173.

Hedborg, F, Holmgren, L, Sandstedt, B & Ohlsson, R 1994, 'The cell type-specific IGF2 expression during early human development correlates to the pattern of overgrowth and neoplasia in the Beckwith-Wiedemann syndrome', *Am J Pathol*, vol. 145, no. 4, pp. 802-817.

Hiura, H, Okae, H, Kobayash, H, Miyauchi, N, Sato, F, Sato, A, Suzuki, F*, et al.* 2012, 'High-throughput detection of aberrant imprint methylation in the ovarian cancer by the bisulphite PCR-Luminex method', *Bmc Medical Genomics*, vol. 5, Mar.

Hou, C, Dale, R & Dean, A 2010, 'Cell type specificity of chromatin organization mediated by CTCF and cohesin', *Proc Natl Acad Sci U S A*, vol. 107, no. 8, Feb 23, pp. 3651-3656.

Hu, L, McArthur, C & Jaffe, RB 2010, 'Ovarian cancer stem-like side-population cells are tumourigenic and chemoresistant', *British Journal of Cancer*, vol. 102, no. 8, pp. 1276-1283.

Huang, Z & Murphy, SK 2013, 'Increased Intragenic IGF2 Methylation is Associated with Repression of Insulator Activity and Elevated Expression in Serous Ovarian Carcinoma', *Front Oncol*, vol. 3, p. 131.

Hubertus, J, Lacher, M, Rottenkolber, M, Muller-Hocker, J, Berger, M, Stehr, M, von Schweinitz, D*, et al.* 2011, 'Altered expression of imprinted genes in Wilms tumors', *Oncol Rep*, vol. 25, no. 3, Mar, pp. 817-823.

Issa, JPJ, Vertino, PM, Boehm, CD, Newsham, IF & Baylin, SB 1996, 'Switch from monoallelic to biallelic human IGF2 promoter methylation during aging and carcinogenesis', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 21, pp. 11757-11762.

Jiang, H, Lin, X, Liu, Y, Gong, W, Ma, X, Yu, Y, Xie, Y*, et al.* 2012, 'Transformation of epithelial ovarian cancer stemlike cells into mesenchymal lineage via EMT results in cellular heterogeneity and supports tumor engraftment', *Molecular Medicine*, vol. 18, pp. 1197-1208.

Jin, F, Li, Y, Dixon, JR, Selvaraj, S, Ye, Z, Lee, AY, Yen, CA*, et al.* 2013, 'A high-resolution map of the three-dimensional chromatin interactome in human cells', *Nature*, vol. 503, no. 7475, Nov 14, pp. 290-294.

Kalhor, R, Tjong, H, Jayathilaka, N, Alber, F & Chen, L 2012, 'Genome architectures revealed by tethered chromosome conformation capture and population-based modeling', *Nat Biotechnol*, vol. 30, no. 1, Jan, pp. 90-98.

Kanduri, C, Pant, V, Loukinov, D, Pugacheva, E, Qi, CF, Wolffe, A, Ohlsson, R*, et al.* 2000, 'Functional association of CTCF with the insulator upstream of the H19 gene is parent of origin-specific and methylation-sensitive', *Current Biology*, vol. 10, no. 14, pp. 853-856.

Karst, AM & Drapkin, R 2010, 'Ovarian cancer pathogenesis: a model in evolution', *J Oncol*, vol. 2010, p. 932371.

Kemp, Christopher J, Moore, James M, Moser, R, Bernard, B, Teater, M, Smith, Leslie E, Rabaia, Natalia A*, et al.* 2014, 'CTCF Haploinsufficiency Destabilizes DNA Methylation and Predisposes to Cancer', *Cell Reports*, no. 0.

Kim, HT, Choi, BH, Niikawa, N, Lee, TS & Chang, SI 1998, 'Frequent loss of imprinting of the H19 and IGF-II genes in ovarian tumors', *American Journal of Medical Genetics*, vol. 80, no. 4, pp. 391-395.

Kim, TH, Abdullaev, ZK, Smith, AD, Ching, KA, Loukinov, DI, Green, RD, Zhang, MQ*, et al.* 2007, 'Analysis of the vertebrate insulator protein CTCF-binding sites in the human genome', *Cell*, vol. 128, no. 6, pp. 1231-1245.

Kishino, T, Lalande, M & Wagstaff, J 1997, 'UBE3A/E6-AP mutations cause Angelman syndrome', *Nat Genet*, vol. 15, no. 1, 01//print, pp. 70-73.

Kitsberg, D, Selig, S, Brandeis, M, Simon, I, Keshet, I, Driscoll, DJ, Nicholls, RD*, et al.* 1993, 'Allele-specific replication timing of imprinted gene regions', *Nature*, vol. 364, no. 6436, pp. 459-463.

Kulis, M & Esteller, M 2010, 'DNA methylation and cancer', *Adv Genet*, vol. 70, pp. 27-56.

Kurukuti, S, Tiwari, VK, Tavoosidana, G, Pugacheva, E, Murrell, A, Zhao, Z, Lobanenkov, V*, et al.* 2006, 'CTCF binding at the H19 imprinting control region mediates maternally inherited higher-order chromatin conformation to restrict enhancer access to Igf2', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 28, Jul 11, pp. 10684-10689.

Li, J, Abushahin, N, Pang, SJ, Xiang, L, Chambers, SK, Fadare, O, Kong, BH*, et al.* 2011, 'Tubal origin of 'ovarian' low-grade serous carcinoma', *Modern Pathology*, vol. 24, no. 11, Nov, pp. 1488-1499.

Li, J, Fadare, O, Xiang, L, Kong, BH & Zheng, WX 2012, 'Ovarian serous carcinoma: recent concepts on its origin and carcinogenesis', *Journal of Hematology & Oncology*, vol. 5, no. 8.

Li, T, Hu, J-F, Qiu, X, Ling, J, Chen, H, Wang, S, Hou, A*, et al.* 2008, 'CTCF regulates allelic expression of Igf2 by orchestrating a promoter-polycomb repressive complex 2 intrachromosomal loop', *Molecular and Cellular Biology*, vol. 28, no. 20, pp. 6473-6482.

Ling, J & Hoffman, AR 2011, 'Associated chromosome trap for identifying long-range DNA interactions', *Journal of visualized experiments : JoVE*, no. 50.

Ling, JQ, Li, T, Hu, JF, Vu, TH, Chen, HL, Qiu, XW, Cherry, AM*, et al.* 2006, 'CTCF Mediates Interchromosomal Colocalization Between Igf2/H19 and Wsb1/Nf1', *Science*, vol. 312, no. 5771, pp. 269-272.

Lomvardas, S, Barnea, G, Pisapia, DJ, Mendelsohn, M, Kirkland, J & Axel, R 2006, 'Interchromosomal interactions and olfactory receptor choice', *Cell*, vol. 126, no. 2, Jul 28, pp. 403-413.

Long, JE & Cai, X 2007, 'Igf-2r expression regulated by epigenetic modification and the locus of gene imprinting disrupted in cloned cattle', *Gene*, vol. 388, no. 1-2, Feb 15, pp. 125-134.

Lopez-Serra, P & Esteller, M 2012, 'DNA methylation-associated silencing of tumor-suppressor microRNAs in cancer', *Oncogene*, vol. 31, no. 13, Mar 29, pp. 1609-1622.

Lu, L, Katsaros, D, Wiley, A, de la Longrais, IAR, Puopolo, M, Schwartz, P & Yu, H 2006, 'Promoter-specific transcription of insulin-like growth factor-II in epithelial ovarian cancer', *Gynecologic Oncology*, vol. 103, no. 3, pp. 990-995.

Mahy, NL, Perry, PE & Bickmore, WA 2002, 'Gene density and transcription influence the localization of chromatin outside of chromosome territories detectable by FISH', *J Cell Biol*, vol. 159, no. 5, Dec 9, pp. 753-763.

Malnic, B, Godfrey, PA & Buck, LB 2004, 'The human olfactory receptor gene family', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 8, February 24, 2004, pp. 2584-2589.

Malnic, B, Hirono, J, Sato, T & Buck, LB 1999, 'Combinatorial receptor codes for odors', *Cell*, vol. 96, no. 5, Mar 5, pp. 713-723.

Martin, D, Pantoja, C, Miñán, AF, Valdes-Quezada, C, Moltó, E, Matesanz, F, Bogdanović, O*, et al.* 2011, 'Genome-wide CTCF distribution in vertebrates defines equivalent sites that aid the identification of disease-associated genes', *Nat Struct Mol Biol*, vol. 18, no. 6, 06//print, pp. 708-714.

Mittal, KR, Zeleniuchjacquotte, A, Cooper, JL & Demopoulos, RI 1993, 'Contralateral ovary in unilateral ovarian-carcinoma - A search for preneoplastic lesions', *International Journal of Gynecological Pathology*, vol. 12, no. 1, pp. 59-63.

Moon, H, Filippova, G, Loukinov, D, Pugacheva, E, Chen, Q, Smith, ST, Munhall, A*, et al.* 2005, 'CTCF is conserved from Drosophila to humans and confers enhancer blocking of the Fab-8 insulator', *EMBO Rep*, vol. 6, no. 2, Feb, pp. 165-170.

Moore, T & Haig, D 1991, 'Genomic imprinting in mammalian development: a parental tug-of-war', *Trends Genet*, vol. 7, no. 2, Feb, pp. 45-49.

Murphy, SK, Huang, Z, Wen, Y, Simel, LR, Price, TM & Berchuck, A 2009, 'Intragenic methylation at a novel CTCF binding site is associated with elevated IGF2 expression in serous epithelial ovarian cancer', *Gynecologic Oncology*, vol. 112, no. 2, pp. S138-S138.

Murphy, SK, Huang, ZQ, Wen, YQ, Spillman, MA, Whitaker, RS, Simel, LR, Nichols, TD*, et al.* 2006, 'Frequent IGF2/H19 domain epigenetic alterations and elevated IGF2

expression in epithelial ovarian cancer', *Molecular Cancer Research*, vol. 4, no. 4, pp. 283-292.

Murrell, A, Heeson, S & Reik, W 2004, 'Interaction between differentially methylated regions partitions the imprinted genes Igf2 and H19 into parent-specific chromatin loops', *Nat Genet*, vol. 36, no. 8, Aug, pp. 889-893.

Nakahashi, H, Kwon, KR, Resch, W, Vian, L, Dose, M, Stavreva, D, Hakim, O*, et al.* 2013, 'A genome-wide map of CTCF multivalency redefines the CTCF code', *Cell Rep*, vol. 3, no. 5, May 30, pp. 1678-1689.

Nowack, MK, Shirzadi, R, Dissmeyer, N, Dolf, A, Endl, E, Grini, PE & Schnittger, A 2007, 'Bypassing genomic imprinting allows seed development', *Nature*, vol. 447, no. 7142, May 17, pp. 312-315.

Ohlsson, R, Renkawitz, R & Lobanenkov, V 2001, 'CTCF is a uniquely versatile transcription regulator linked to epigenetics and disease', *Trends Genet*, vol. 17, no. 9, Sep, pp. 520-527.

Ohta, T, Gray, TA, Rogan, PK, Buiting, K, Gabriel, JM, Saitoh, S, Muralidhar, B*, et al.* 1999, 'Imprinting-Mutation Mechanisms in Prader-Willi Syndrome', *The American Journal of Human Genetics*, vol. 64, no. 2, pp. 397-413.

Parelho, V, Hadjur, S, Spivakov, M, Leleu, M, Sauer, S, Gregson, HC, Jarmuz, A*, et al.* 2008, 'Cohesins functionally associate with CTCF on mammalian chromosome arms', *Cell*, vol. 132, no. 3, Feb 8, pp. 422-433.

Phillips, JE & Corces, VG 2009, 'CTCF: master weaver of the genome', *Cell*, vol. 137, no. 7, Jun 26, pp. 1194-1211.

Plasschaert, RN, Vigneau, S, Tempera, I, Gupta, R, Maksimoska, J, Everett, L, Davuluri, R*, et al.* 2014, 'CTCF binding site sequence differences are associated with unique regulatory and functional trends during embryonic stem cell differentiation', *Nucleic Acids Res*, vol. 42, no. 2, Jan, pp. 774-789.

Poole, RL, Leith, DJ, Docherty, LE, Shmela, ME, Gicquel, C, Splitt, M, Temple, IK*, et al.* 2012, 'Beckwith-Wiedemann syndrome caused by maternally inherited mutation of an OCT-binding motif in the IGF2/H19-imprinting control region, ICR1', *Eur J Hum Genet*, vol. 20, no. 2, 02//print, pp. 240-243.

Quitschke, WW, Taheny, MJ, Fochtmann, LJ & Vostrov, AA 2000, 'Differential effect of zinc finger deletions on the binding of CTCF to the promoter of the amyloid precursor protein gene', *Nucleic Acids Research*, vol. 28, no. 17, September 1, 2000, pp. 3370-3378.

Reddy, KL, Zullo, JM, Bertolino, E & Singh, H 2008, 'Transcriptional repression mediated by repositioning of genes to the nuclear lamina', *Nature*, vol. 452, no. 7184, 03/13/print, pp. 243-247.

Reed, ML & Leff, SE 1994, 'Maternal imprinting of human SNRPN, a gene deleted in Prader-Willi syndrome', *Nat Genet*, vol. 6, no. 2, Feb, pp. 163-167.

Renfree, MB, Hore, TA, Shaw, G, Marshall Graves, JA & Pask, AJ 2009, 'Evolution of Genomic Imprinting: Insights from Marsupials and Monotremes', *Annual Review of Genomics and Human Genetics*, vol. 10, no. 1, pp. 241-262.

Ryba, T, Hiratani, I, Lu, J, Itoh, M, Kulik, M, Zhang, J, Schulz, TC*, et al.* 2010, 'Evolutionarily conserved replication timing profiles predict long-range chromatin interactions and distinguish closely related cell types', *Genome Res*, vol. 20, no. 6, Jun, pp. 761-770.

Schmidt, D, Schwalie, PC, Wilson, MD, Ballester, B, Goncalves, A, Kutter, C, Brown, GD*, et al.* 2012, 'Waves of retrotransposon expansion remodel genome organization and CTCF binding in multiple mammalian lineages', *Cell*, vol. 148, no. 1-2, Jan 20, pp. 335-348.

Schütt, S, Florl, AR, Shi, W, Hemberger, M, Orth, A, Otto, S, Schulz, WA*, et al.* 2003, 'DNA Methylation in Placentas of Interspecies Mouse Hybrids', *Genetics*, vol. 165, no. 1, p. 223.

Spilianakis, CG, Lalioti, MD, Town, T, Lee, GR & Flavell, RA 2005, 'Interchromosomal associations between alternatively expressed loci', *Nature*, vol. 435, no. 7042, 06/02/print, pp. 637-645.

Stringer, JM, Suzuki, S, Pask, AJ, Shaw, G & Renfree, MB 2012, 'Promoter-specific expression and imprint status of marsupial IGF2', *PLoS ONE*, vol. 7, no. 7, p. e41690.

Szabo, PE, Tang, SHE, Silva, FJ, Tsark, WMK & Mann, JR 2004, 'Role of CTCF binding sites in the Igf2/H19 imprinting control region', *Molecular and Cellular Biology*, vol. 24, no. 11, pp. 4791-4800.

Tennant, MK, Thrasher, JB, Twomey, PA, Drivdahl, RH, Birnbaum, RS & Plymate, SR 1996, 'Protein and messenger ribonucleic acid (mRNA) for the type 1 insulin-like growth factor (IGF) receptor is decreased and IGF-II mRNA is increased in human prostate carcinoma compared to benign prostate epithelium', *Journal of Clinical Endocrinology & Metabolism*, vol. 81, no. 10, pp. 3774-3782.

Tomao, F, Papa, A, Rossi, L, Strudel, M, Vici, P, Lo Russo, G & Tomao, S 2013, 'Emerging role of cancer stem cells in the biology and treatment of ovarian cancer: basic knowledge and therapeutic possibilities for an innovative approach', *Journal of Experimental & Clinical Cancer Research*, vol. 32, no. 1, p. 48.

Tycko, B & Morison, IM 2002, 'Physiological functions of imprinted genes', *J Cell Physiol*, vol. 192, no. 3, Sep, pp. 245-258.

Ulaner, GA, Vul, TH, Li, T, Hu, JF, Yao, XM, Yang, YW, Gorlick, R*, et al.* 2003, 'Loss of imprinting of IGF2 and H19 in osteosarcoma is accompanied by reciprocal methylation changes of a CTCF-binding site', *Human Molecular Genetics*, vol. 12, no. 5, pp. 535-549.

Vandijk, MA, Vanschaik, FMA, Bootsma, HJ, Holthuizen, P & Sussenbach, JS 1991, 'Initial characterisation of the 4 promoters of the human insulin-like growth factor-II gene', *Molecular and Cellular Endocrinology*, vol. 81, no. 1-3, pp. 81-94.

Vrana, PB, Fossella, JA, Matteson, P, del Rio, T, O'Neill, MJ & Tilghman, SM 2000, 'Genetic and epigenetic incompatibilities underlie hybrid dysgenesis in Peromyscus', *Nat Genet*, vol. 25, no. 1, May, pp. 120-124.

Vrana, PB, Guan, XJ, Ingram, RS & Tilghman, SM 1998, 'Genomic imprinting is disrupted in interspecific Peromyscus hybrids', *Nat Genet*, vol. 20, no. 4, Dec, pp. 362-365.

Vu, TH & Hoffman, AR 1994, 'Promoter-specific imprinting of the human insulin-like growth factor-II gene', *Nature*, vol. 371, no. 6499, pp. 714-717.

Vu, TH, Nguyen, AH & Hoffman, AR 2010a, 'Loss of IGF2 imprinting is associated with abrogation of long-range intrachromosomal interactions in human cancer cells', *Hum Mol Genet*, vol. 19, no. 5, Mar 1, pp. 901-919.

Vu, TH, Nguyen, AH & Hoffman, AR 2010b, 'Loss of IGF2 imprinting is associated with abrogation of long-range intrachromosomal interactions in human cancer cells', *Human Molecular Genetics*, vol. 19, no. 5, March 1, 2010, pp. 901-919.

Wang, H, Maurano, MT, Qu, H, Varley, KE, Gertz, J, Pauli, F, Lee, K*, et al.* 2012b, 'Widespread plasticity in CTCF occupancy linked to DNA methylation', *Genome Research*, vol. 22, no. 9, September 1, 2012, pp. 1680-1688.

Wang, J, Valo, Z, Smith, D & Singer-Sam, J 2007, 'Monoallelic Expression of Multiple Genes in the CNS', *PLoS ONE*, vol. 2, no. 12, p. e1293.

Weksberg, R, Shuman, C & Beckwith, JB 2010, 'Beckwith-Wiedemann syndrome', *Eur J Hum Genet*, vol. 18, no. 1, Jan, pp. 8-14.

Wright, ML 2014, 'Investigating the evolution of replication timing and monoallelic expression in mammals and birds', PhD thesis, University of Adelaide.

Wright, ML, Casey, A, Necsulea, A, Julien, P, Kaessmann, H & Grutzner, F in prep., 'Imprinted orthologs in platypus display an amount of monoallelic expression, which is not exclusive to one allele.', *Unpublished*.

Wright, ML & Grutzner, F in prep., 'Asynchronous replication of imprinted-orthologs in platypus, and the conservation of replication timing initiation in imprinted orthologs across amniote evolution.', *Unpublished*.

Xiao, T, Wallace, J & Felsenfeld, G 2011, 'Specific sites in the C terminus of CTCF interact with the SA2 subunit of the cohesin complex and are required for cohesin-dependent insulation activity', *Mol Cell Biol*, vol. 31, no. 11, Jun, pp. 2174-2183.

Zakharova, IS, Shevchenko, AI & Zakian, SM 2009, 'Monoallelic gene expression in mammals', *Chromosoma*, vol. 118, no. 3, Jun, pp. 279-290.

Zeitz, MJ, Ay, F, Heidmann, JD, Lerner, PL, Noble, WS, Steelman, BN & Hoffman, AR 2013, 'Genomic Interaction Profiles in Breast Cancer Reveal Altered Chromatin Architecture', *PLoS ONE*, vol. 8, no. 9, p. e73974.

Zhang, H, Niu, BB, Hu, JF, Ge, SF, Wang, HB, Li, T, Ling, JQ*, et al.* 2011, 'Interruption of intrachromosomal looping by CCCTC binding factor decoy proteins abrogates genomic imprinting of human insulin-like growth factor II', *Journal of Cell Biology*, vol. 193, no. 3, May, pp. 475-487.

Zhao, Z, Tavoosidana, G, Sjolinder, M, Gondor, A, Mariano, P, Wang, S, Kanduri, C*, et al.* 2006, 'Circular chromosome conformation capture (4C) uncovers extensive networks of epigenetically regulated intra- and interchromosomal interactions', *Nature Genetics*, vol. 38, no. 11, Nov, pp. 1341-1347.

Chapter 2 – Individual differences in *IGF2-WSB1* interaction in Brahman and Angus cattle are not affected by hybridisation

This chapter consists of an unpublished manuscript.

Chapter Overview

This chapter describes our work investigating long range interactions at the imprinted *IGF2* locus in bovine hybrids. Previous work has mainly focussed in murine models, so this is some of the first research into this area in other species. The bovine system has unique advantages for this study, particularly that cattle hybrids are typically from highly genetically similar subspecies, yet show distinct hybrid phenotypes. This manuscript outlines results showing the conservation of the long-range interaction between the *IGF2/H19* and *WSB1* loci in bovine species.

We also investigated these long-range interactions in bovine hybrids, where it is thought that aberrant imprinting regulation causes foetal overgrowth phenotypes in hybrids. We found that the interactions appeared to differ at an individual level, rather than by breed, suggesting some other mechanism is causing differential expression of key genes. We found key gene expression in embryonic cells still differed significantly between breeds, and differed to existing results in foetal tissues. We suggest that the overgrowth phenotype may become evident at a later developmental stage.

This research required significant troubleshooting from growing the embryonic fibroblasts in culture, finding robust, reliable probes for fluorescence *in situ* experiments and finding appropriate probes to quantitate expression.

This manuscript represents a large body of novel work where we have investigated the role of long range interactions of *IGF2/H19* and changes in gene expression in embryonic bovine hybrids.

Statement of Authorship

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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Nicole Williams
Contribution to the paper	Design of project and experiments Preparation of samples Counting between bovine crosses. Generated all expression data. Major analysis Writing of manuscript.
	Date 02.12.16

Name of Co-Author	Megan Wright
Contribution to the paper	Design of project and FISH experiment Preparation of samples Identification of BACs Experimentally shown the conservation of interaction (FISH and 3C). – Figure 1 Counting between bovine crosses. Analysis
Signature	**Date**

Name of Co-Author	Stephen Pederson
Contribution to the paper	Statistical modelling of expression data.
Signature	**Date** 2/12/16

Name of Co-Author	Stefan Hiendleder
Contribution to the paper	Design of project and experiments Provided reagents and materials
	Date 28/11/2016

Name of Co-Author	Frank Grützner
Contribution to the paper	Design of project and experiments Funding of project Editing of manuscript
	Date 2/12/16

Manuscript Title: Individual differences in *IGF2-WSB1* interaction in Brahman and Angus cattle are not affected by hybridisation

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Abstract

Regulation of the *IGF2/H19* region is multilayered, involving differential DNA methylation, CTCF binding and long-range interactions, and the region is thought to play a role in hybrid dysgenesis. However, the role of long-range interactions in *IGF2/H19* expression at different developmental stages is poorly understood. Hybrid dysgenesis leading to differing birthweights in bovine crosses is thought to be due to altered epigenetic regulation. We investigated the interactions and expression of the *IGF2/H19* locus in embryonic bovine fibroblasts in purebred and hybrid animals of the domestic cattle breeds *Bos primigenius indicus* (Brahman) and *Bos primigenius taurus* (Angus). We found that the frequency of long-range interactions involving the *IGF2/H19* region were variable between individuals and did not show significant differences between the two breeds. However, we observed significant expression differences of both *IGF2* and *H19* between the purebred and hybrid crosses which did not appear to explain differences in embryo weight. Interestingly, previous studies in foetal tissues have found similar changes, particularly in *H19* expression. We suggest that the long-range interaction frequency of *IGF2/H19* is not affected by hybridisation and are variable between individual animals. Expression differences in *IGF2* and *H19* in embryonic cell lines seem independent of interaction patterns, and do not entirely explain the weight differences observed, although this may differ by developmental stage.

Key words

Imprinting, chromatin organization, development, hybrid dysgenesis, *IGF2, H19,* CTCF

Introduction

Hybrid vigour or heterosis is a phenomenon where hybrid animals display enhanced traits over either parent. However hybrids can also show hybrid dysgenesis effects, such as sterility, which is often observed to be more pronounced in the heterogametic sex (Haldane 1922). Increasingly, differences between parents and hybrid offspring are thought to have potential epigenetic causes, due to the incompatibility of the genomes. One example is loss of parent of origin monoallelic expression (genomic imprinting) in hybrids, causing abnormal growth phenotypes resulting from the incorrect expression of potent imprinted genes (Schütt *et al.* 2003; Vrana *et al.* 2000). In the example of the hybrid offspring of *Peromyscus polionotus* and *Peromyscus maniculatus*, whilst the parents are about equal in size, one hybrid is larger than either parent, the reciprocal cross leads to offspring around 40% smaller than either parent (Dawson 1965; Vrana *et al.* 1998).

In the agricultural industry, the *B. p. taurus* (Angus) and *B. p. indicus* (Brahman) bovine subspecies have been extensively bred to blend the most desirable characteristics of each breed into a superior hybrid animal. The two breeds, separated by 1.7-2.0M years of divergence, were categorized as subspecies on the basis of mitochondrial studies (Hiendleder *et al.* 2008; MacHugh *et al.* 1997) or satellite markers (Nijman *et al.* 1999). However, the hybrid offspring also display birthweight heterosis effects, where one hybrid displays an increase in birthweight relative to the parents, which may be due to maternal effects (Brown *et al.* 1993).

Much of the literature around hybrid overgrowth phenotypes implicate a loss of imprinting or change in expression of the *IGF2/H19* region (Chen *et al.* 2015; Goodall & Schmutz 2007) or the receptor *IGF2R,* all of which have been found to be imprinted in cattle (Dindot *et al.* 2004). *IGF2R* is an imprinted gene that has been shown to be deregulated in cloned cattle (Long & Cai 2007). Imprinting of *IGF2R* does show individual variation and tissue specificity (Bebbere *et al.* 2013), however IVF foetuses displaying overgrowth phenotypes did not display loss of imprinting or changes in DNA methylation at the *IGF2R* DMR2. *IGF2* and *H19* are similarly imprinted, where *IGF2* is exclusively expressed from the paternally derived allele, and is a potent mitogenic growth factor important in foetal growth. *H19* is a maternally expressed long non-coding RNA that acts as a growth inhibitor. In mice, the imprinting at this region is controlled by DNA methylation at the imprinting control region (ICR) which harbors several CTCF binding sites, just upstream of *H19.* Methylation of these binding sites on the paternal allele prevents CTCF from binding, which allows an intrachromosomal loop to form bringing enhancer elements to the *Igf2* promoter. The unmethylated ICR on the maternal allele is bound by CTCF, blocking the intrachromosomal loop from forming, and facilitating long-range interactions with other regions, such as with the paternal ICR-associated site 1 (IAS1) of the *Wsb1/Nf1* region as observed in mice (Ling *et al.* 2006). These long range interactions are thought to be important in the imprinted regulation of the *Igf2/H19* region (Ling & Hoffman 2007). In mice, if the interaction is disrupted by knockdown of CTCF, *Wsb1/Nf1* expression is reduced and there is a loss of imprinting at *Igf2* and biallelic expression of *H19* (Ling *et al.* 2006). Importantly, these long-range interactions role in imprinted regulation have not been validated in bovine species.

Much of the current research into *IGF2/H19/Igf2R* expression and imprinted regulation in cattle has focussed on foetal tissues and investigating the molecular repercussions of different reproductive technologies. Our research looks to fill a gap in the literature

investigating the role of these long-range interactions and imprinted gene expression in cattle hybrids at the embryonic stage.

Materials and Methods

Cattle breeding

The process of breeding has been described previously (Anand-Ivell *et al.* 2011). Briefly, nulliparous Angus and Brahman females, approximately 16–20 months of age, received estrous cycle synchronization (Cidirol - Timed Insemination, TI). This consisted of an initial injection of 1 ml of 1 mg/ml estradiol benzoate (Cidirol, Genetics Australia Co-operative Ltd., Bacchus Marsh, Australia) and insertion of a progesterone-releasing vaginal insert (Eazi-Breed CIDR, DEC International, Hamilton, New Zealand). After 7-9 days, the inserts were removed and injections of 2 ml of a prostaglandin analogue were given (0.26 mg of cloprostenol sodium/ml (Estrumate), Schering-Plough Animal Health, Baulkam Hills, Australia). Estrus detection devices (Kamar, Agrigene, Wangaratta, Australia) were placed on all animals. Animals were given 0.7 ml estradiol benzoate the day after removal of vaginal inserts and were inseminated 24 h later. Synchronization/insemination was repeated with estradiol benzoate injection of all animals after removal of vaginal inserts. All procedures involving cattle in this study were approved by The University of Adelaide Animal Ethics Committee (S-094-2005 and S-094-2005A).

Sample preparation

Fibroblast cell lines from front-leg tissue of 48-day old purebred and hybrid embryos (Table 1), were cultured in Dulbecco's Eagle Medium with HEPES (DMEM) with 10% FCS, 1% L-Glutamine and 1% Penicillin/Streptomycin at 37^{o}C in an atmosphere of 5% CO_2. The cells were grown to 70% confluence before being harvested or passaged. RNA was extracted using TRIzol (ThermoFisher Scientific, Waltham, MA US) as per the manufacturer's instructions.

Table 1: Breed status, cross and sex of cattle embryonic cell lines.

Breed Status	Parental Cross (male presented first)	Sex and Name of embryonic cell line	Embryo weight (g) before dissection
Purebred	Angus X Angus	Male F40-21	3.78
		Male F40-27	3.92
	Brahman X Brahman	Male F40-31	3.00
		Male F40-14	2.55
Hybrid	Angus X Brahman	Male F40-8	3.36
		Male F40-41	2.98
	Brahman X Angus	Male F40-9	3.73
		Male F40-57	2.93

3C experiment

The 3C protocol was followed for the 3C library establishment (Hagege *et al.* 2007b). Around 1 x 10^7 cells were crosslinked with 2% formaldehyde and stored at -80^{o}C before use. The cells were then lysed and the DNA was digested overnight in 400 units of the BanII restriction enzyme at 37 ^{o}C, before being ligated at low concentration for 4 hours at 16^{o}C. Cross-links were removed with an overnight proteinase K (300 µg) digestion at 65^{o}C. The DNA was then phenol/chloroform extracted, precipitated in ethanol, and the pellet was dissolved in 10 mM Tris pH 7.5. PCR Primers were designed to flank the BanII restriction sites within the ICR and IAS1 regions, and some were also designed internally in fragments to act as loading controls. Nested primers were also designed to closely flank ligation sites within 3C PCR ligation products to aid in the final quantification. Thus, 3C primer sets were tested on 3C libraries using two rounds of 35 cycles for amplification (see Wright, Thesis 2008 for details).

BAC identification and isolation

BAC clones were found using the *Bovine BAC Map* database (http://www.livestockgenomics.csiro.au/perl/gbrowse.cgi/bacmap/) which required searching this database with Ensembl contigs containing the genes of interest (Table 2). Mouse BACs for *Igf2* (RP23-51J2), *Wsb1* (RP24-256H2) and *MEST* (RP24-211G11) were purchased from the Children's Hospital Oakland Research Institute (CHORI, Oakland, CA, USA). BACs were streak plated and grown overnight at 37^{o}C. Single BAC colonies were selected and grown in LB broth culture overnight at 37^{o}C, before being pelleted and extracted using a standard phenol chloroform extraction protocol. Genes of interest were

then confirmed on the BACs through PCR and sequencing (primers used for gene confirmation are available on request). BAC DNA was stored at -20^{o}C until required.

Table 2: Ensemble contigs and Bovine BAC Map clones

Gene/s	Ensemble/NCBI gene ID.	General genomic region	Ensemble contigs covering region	Bovine BAC Map clones
Wsb1	ENSBTAG00000005008	Chr19: 18.83-18.84 Mb	AAFC03016249	CH240-176O21
Igf2	ENSBTAG00000013066	Chr29: 51.25-51.28 Mb	AAFC03103584 AAFC03103585	CH240-21F17
H19	NR_003958	Chr29: 51.365-51.366 Mb	AAFC03071568	CH240-21F17
Actb	ENSBTAG00000026199	Chr 25: 40.635-40.639 Mb	AAFC03011951 AAFC03072713	CH240-474H10

DNA FISH

Cattle embryonic fibroblasts were trypsinised (0.05% trypsin-EDTA), treated with 0.075M KCl and fixed using a 3:1 methanol acetic acid solution. Fixed cells were dropped onto cleaned slides and slowly dried in humid conditions. Slides were pre-treated with pepsin, fixed with 1% formaldehyde and dehydrated in an ethanol series (70%, 90%, 100%). The slides were denatured in 70% formamide/2X SSC at 70^{o}C for 90 secs, dehydrated again before hybridising with labelled BAC probes.

BAC DNA was fluorescently labelled with Vysis SpectrumOrange or SpectrumGreen dUTPs (Abbott Molecular, Abbott Park, IL) using Exo-Klenow (New England Biolabs, Ipswich, MA) and 9-mer random primers (Geneworks, Adelaide, SA). Labelled BACs were ethanol precipitated with sonicated bovine genomic DNA as a carrier and salmon sperm before being dissolved in deionised formamide and hybridisation mix (10% dextran sulphate in 2X SSC). The probe was then denatured at 80^{o}C for 10 mins and re-annealed at 37^{o}C for 30 mins before hybridising overnight on pre-treated slides.

Hybridised slides were thoroughly washed three times with 2X SSC and 50% formamide for 5 minutes at 42°C, 2X SSC for 5 minutes at 42°C, 0.1X SSC for 5 mins at 60°C and finally 2X SSC for 5 minutes at 42°C. Slides were airdried and mounted with coverslips using Vectashield mounting medium (Vector, Burlingame, CA). Nuclei were visualised using AxioVision 4 software on an AxioImager 2.1 epifluorescence microscope (Zeiss, Oberkochen, BW) using filters for GFP green, DS Red and DAPI. All images were taken using the 100x magnification lens. A control interaction pair such as *MEST* or *ACTB* and *WSB1*, where no interaction is known to occur, is used an estimate of the random chance of two signals overlapping and distinguish non-random interaction frequencies. For the first set of individuals more cells were counted (n=300 for each individual) than for the second set (n=50 for each individual).

qPCR

RNA was converted using the BioRad iScript cDNA synthesis kit as per the manufacturer's instructions. The 20µl reaction was diluted to 100µl using DEPC treated MQ, and 5µl was used per well. Each sample was tested in technical triplicate and efficiency determined by the standard curve method. MicroAmp Fast Optical 96-well Reaction qPCR plates (Applied BioSystems) were read by the StepOne Plus Real-time PCR system (Applied BioSystems) using a standard protocol of 95°C for 10 minutes, followed by cycles of 95°C for 15 seconds and 60°C for 1 minute for 40 cycles. The melt curve protocol was 95°C for 15 seconds, 60°C for 1 minute, increasing in 0.3°C increments, then 95°C for 15 seconds. Results were analysed using the StepOne Software v2.3 (Applied BioSystems).

As the amplification efficiencies were equal for all primers, we used the ΔΔCt method to compare results. The following nested model was applied:

$$y_{ijkl} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_k + (\beta\gamma)_{jk} + \varepsilon_{ijkl}$$

Where y_{ijkl} is the Ct value in gene (i), for breed (j) in replicate (k) with triplicate measurements (l). The factors considered are μ as the average Ct value of β-actin in purebred Angus, α_i as the change in Ct value for each gene/primer set, β_j as the change for each breed, $(\alpha\beta)_{ij}$ for any change not explained by the previous terms, γ_k as a measure of error between replicate samples, $(\beta\gamma)_{jk}$ representing the variability within each breed and replicate to

assist in normalising Ct values and ε_{ijkl} representing variability within a plate. Expression differences were compared pair-wise using Tukey's Honest Significance test

Results

IGF2/H19-WSB1 interaction in cattle fibroblasts using 3C and DNA FISH

First we sought to establish if the interaction between the *IGF2/H19* and *WSB1* region, originally observed in mouse, is conserved in cattle using DNA FISH (Figure 1A). The frequency of the interaction was determined by the number of cells with the two overlapping signals. Of the 150 cells counted, 8% ± 1% of cattle nuclei showed an interaction between *IGF2/H19* and *WSB1/NF1* compared to 0.67% ± 1% where control interactions were observed (Figure 1B). In the mouse this frequency was more pronounced, with 19% ± 7% of nuclei showing interaction, with the same control frequency as cattle.

Next we validated the interaction in bovine using the 3C technique. Crosslinked DNA was harvested, PCR performed across the linked chromosomes using primers specific to each interaction partner and the product sequenced (Figure 1C). Together this confirmed the conservation of the interaction in cattle.

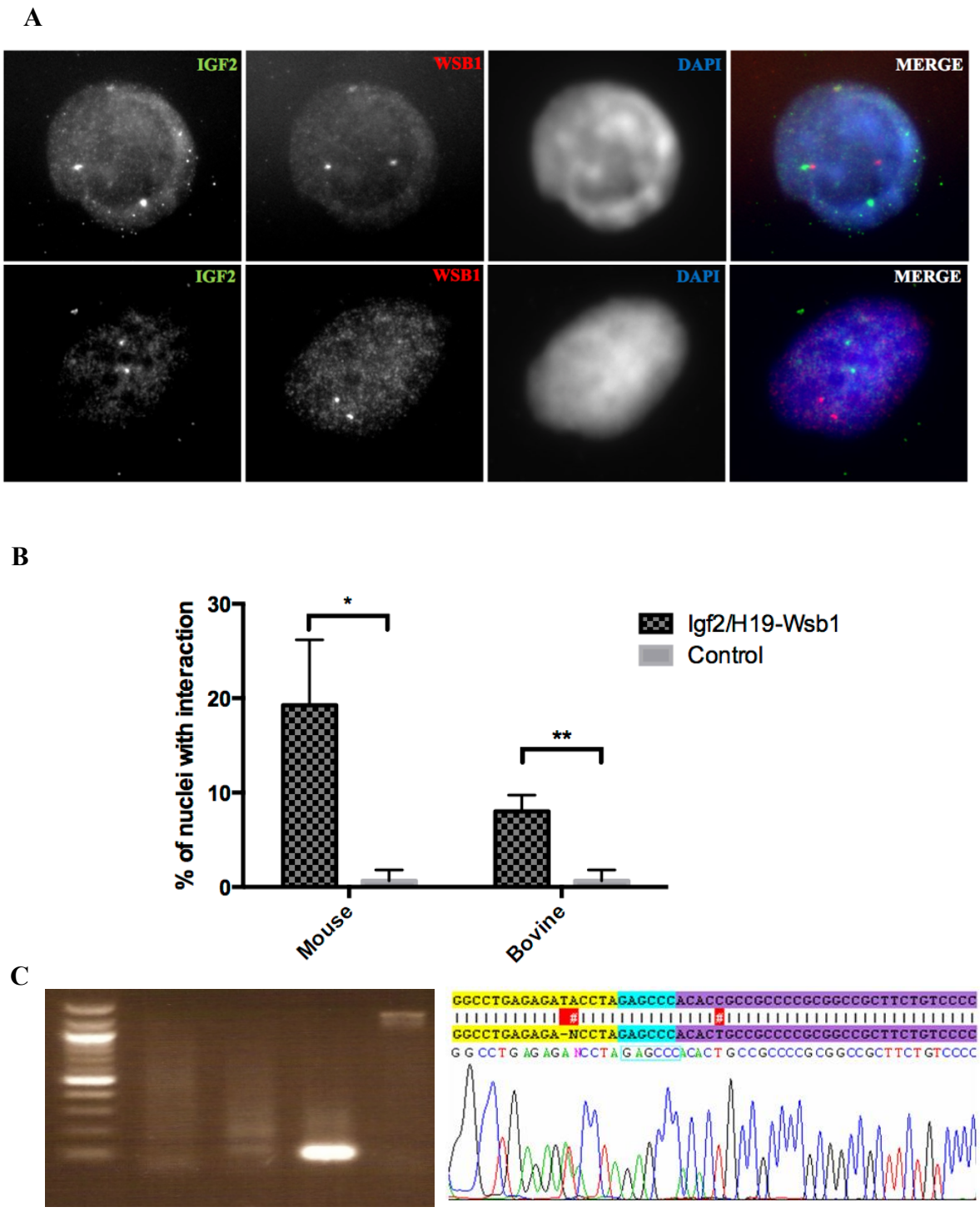

Figure 1A: Two colour DNA FISH dot assay on cattle embryonic fibroblasts. Fluorescently labelled BAC probes were used to determine the frequency of interaction between the *WSB1* and *IGF2/H19* regions by counting the number of cells where the signals were observed to overlap, as depicted in the cell in the top row of images. The bottom row of images shows a cell where no interaction is occurring between the fluorescently tagged regions. The frequency of interaction between the *ACTB* and *WSB*1 probes was used as a control for how often signals would overlap by chance. **B:** Frequency of interactions observed in DNA FISH dot assays of fibroblasts from mouse and cattle samples. The control interaction for mouse

was between *MEST* and *Wsb1*, in cattle the control interaction was between *ACTB* and *WSB1*. The error bars depict the standard deviation between the samples for each species. **C:** 3C results of the *IGF2/H19-WSB1* interaction in cattle. The top sequence is the predicted ligation product (with DNA sequence from the Ensemble database), whilst the bottom sequence is the nested 3C PCR product sequence from cattle. The BanII site (light blue) is flanked by a fragment from the IAS1 (*WSB1/NF1*) region (yellow sequence), and a fragment from the ICR (*IGF2/H19*) region (purple sequence).

Interaction frequency in Angus/Brahman purebreds and hybrids

Next we used the cytological approach to compare the frequency of interaction within the fibroblast cells derived from *B. p. indicus* and *B. p. taurus* purebreds and the reciprocal hybrids. We have exclusively used male embryos as the heterogametic sex shows more pronounced hybrid dysgenesis effects such as birthweight differences which are more pronounced in male calves (Brown *et al.* 1993; Haldane 1922). All individuals of all crosses were found to show *IGF2/H19-WSB1* interaction frequencies above the baseline frequency of the control (Figure 2A). Interaction frequency showed strong variability between individuals (n=2), particularly in the Angus purebred, and Brahman X Angus hybrid. Furthermore, the average of the interaction frequencies for both individuals showed no difference between the breeds (Figure 2B).

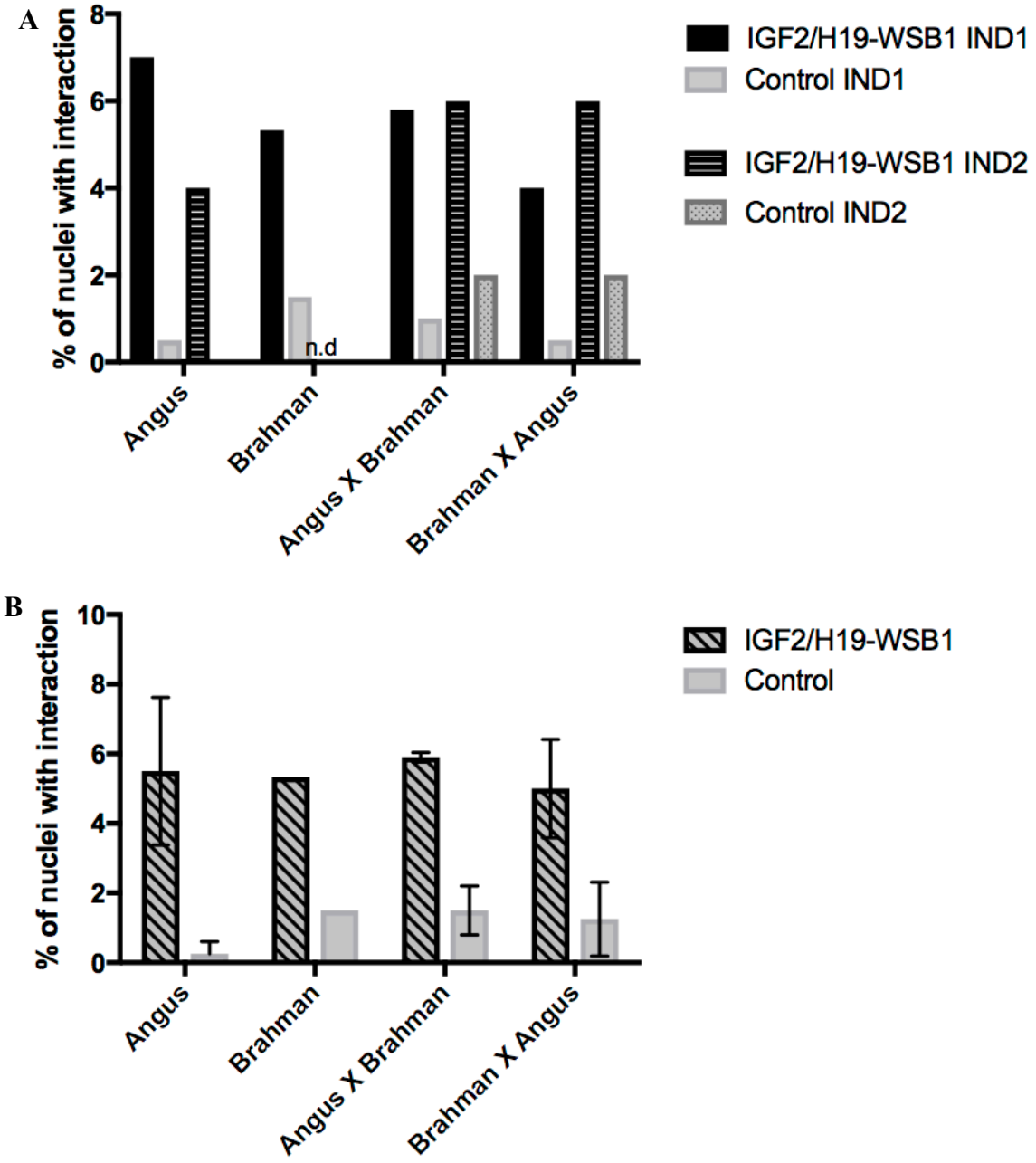

Figure 2A: Interaction frequency of *IGF2/H19-WSB1* as measured by DNA FISH in cattle hybrids and purebreds. Males are listed first. Control interactions between *ACTB* and *WSB1/NF1* probes were used for all individuals. The frequency of *IGF2/H19-WSB1* interaction was deemed to be significantly higher than the control interaction by two-tailed t-test. Two sets of individuals with the same genetic background were counted in separate FISH experiments. 50 cells per individual were counted for each experiment. **B:** Average interaction frequencies of two individuals for each cattle cross. The error bars represent the standard deviation between the counts for each individual.

IGF2 and H19 expression differences between Angus/Brahman purebreds and hybrids

Using the same animals tested in the FISH experiment, we investigated the expression of the *IGF2, H19* and *IGF2R*. *β-actin* was found to be a suitable housekeeper, with no significant differences observed between the crosses or replicate samples. Relative to *β-actin* expression, our analysis indicated no difference in *IGF2R* expression between the breeds (Table 3). *IGF2* expression was significantly higher in both purebreds relative to the hybrids, however no difference was found within those groups, despite a small (but statistically insignificant) increase in the Brahman X Angus hybrid. We also observed significantly higher *H19* expression in the Brahman purebred than all the other crosses, however no difference was observed between the hybrids or the Angus purebred.

Table 3: Pairwise comparisons of *IGF2R, IGF2* and *H19* expression in embryonic fibroblasts of bovine crosses using Tukey's Honest Significance Test. Positive estimate values indicate higher expression in the first listed cross.

Gene	Comparison	Estimate	Std Error	Z	P-value	
IGF2R	Angus-Brahman	-0.187	0.502	-0.373	0.982	
	Angus-AxB	-0.354	0.502	-0.705	0.895	
	Angus-BxA	-0.682	0.502	-1.358	0.526	
	Brahman-AxB	-0.167	0.502	-0.332	0.987	
	Brahman-BxA	-0.494	0.502	-0.985	0.758	
	AxB-BxA	-0.328	0.502	-0.653	0.914	
IGF2	Angus-Brahman	-0.752	0.592	-1.271	0.582	
	Angus-AxB	2.151	0.592	3.634	1.58e-03	**
	Angus-BxA	1.646	0.592	2.781	0.028	*
	Brahman-AxB	2.903	0.592	4.944	3.23e-06	***
	Brahman-BxA	2.398	0.592	4.084	2.83e-04	***
	AxB-BxA	-0.505	0.592	-0.86	0.825	
H19	Angus-Brahman	-3.585	0.643	-5.573	2.71e-07	***
	Angus-AxB	-1.365	0.643	-2.123	0.146	
	Angus-BxA	-1.139	0.643	-1.77	0.288	
	Brahman-AxB	2.219	0.643	3.45	3.15e-03	**
	Brahman-BxA	2.446	0.643	3.803	9.54e-04	***
	AxB-BxA	0.227	0.643	0.353	0.985	

Discussion

Very little is known about interchromosomal interaction networks in different mammals, and long-range interactions involving *IGF2/H19* have not been investigated in hybrids. These interactions may contribute to changes observed in imprinting and gene expression in hybrids.

Conservation of the IGF2/H19-WSB1 interaction in cattle

We found that the *IGF2/H19-WSB1* interaction was conserved in fibroblasts established from 48-day cattle embryos (Figure 1B). However, the interaction frequency in mouse was remarkably higher. This may be due to different replication timing (Ryba *et al.* 2010), possibly different developmental stages in the cells used (Apostolou *et al.* 2013) or potentially some other unknown mechanism influences the frequency at which these interactions occur. As *IGF2/H19* is imprinted in cattle (Gebert *et al.* 2006; Zhang *et al.* 2004), it would be logical that it is the maternal *IGF2/H19* chromosome that is undergoing the interaction as it does in mouse (Ling *et al.* 2006). Further research may be able to identify SNPs that could be used to identify the parental origin of the interaction partners. As shown in Figure 1C, the interacting segments of DNA, the ICR of *H19* and the IAS1 of *WSB1* can be confirmed from the sequencing result of the 3C. This approach, combined with our cytological results, confirmed the conservation of *IGF2/H19-WSB1* interaction in fibroblasts derived from cattle embryos.

Interaction frequency is not affected by hybridisation in cattle

We then wanted to investigate if there were differences in the frequency of this interaction between the cattle crosses and their hybrids. However, the results presented here suggest that there is no difference in the frequency of interaction between either the purebreds or hybrid individuals (Figure 2). Furthermore, our data suggest that the frequency of interaction is individually variable, rather than determined by the breed. This was unexpected, as it suggests the interaction of the two regions is not affected by hybridisation, although imprinting of the region is. This then raises questions of whether methylation differences exist between the crosses at the embryonic stage, or if there are other differences affecting CTCF occupancy in the ICR affecting interaction frequency. Further research using a larger sample of individuals would provide greater insight into how these individual differences impact on embryo development.

Interestingly, there was no difference between the Angus and Brahman purebred samples. This could be an indication that interactions do not vary between subspecies, but are conserved similarly across a species. It would be interesting to know if the patterns that characterise cell developmental stages (Apostolou *et al.* 2013) are conserved between subspecies. Critically, no difference in interaction frequency was observed between the reciprocal hybrids, where large weight discrepancies are later observed (Brown *et al.* 1993), begging the question of whether there are expression changes in *IGF2* or *H19* in the hybrids which are independent of the long-range interactions.

Expression differences in IGF2/H19 between purebred and hybrid animals

In our experiments, no difference in *IGF2R* expression was observed between any of the breeds (Table 4). This implies that similar receptor availability for *IGF2* is found in all the breeds. However, this data does not confirm the imprinted expression of *IGF2R* in these fibroblasts. Given the tissue-specific nature of *IGF2R* imprinting in cattle (Bebbere *et al.* 2013), it would be interesting to investigate any allelic bias in embryonic stage cells.

Elevated *IGF2* expression was observed in the two purebred lines compared to the two hybrids. Considering the loss of imprinting in hybrids (Chen *et al.* 2015) and the pronounced birthweight differential (Brown *et al.* 1993), we would expect higher expression levels in at least one of the hybrids. It is unclear if there is a switch between monoallelic and biallelic expression status in these cells, rather than expression level changes or what the methylation status of the ICR is. Investigating these other factors in *IGF2/H19* expression would help shed light on why we observe such early expression differences.

Interestingly, significantly increased *H19* expression was observed in the Brahman sample compared to all other breeds. Other studies have found that in midgestation foetuses, the maternal genome had a pronounced effect on muscle weight, and that this effect was through expression of the *H19* gene (Xiang *et al.* 2013). If this extends to the earlier embryonic stage, we would expect that the Brahman embryos to be smaller than embryos of other crosses, however as the process of myogenesis is less progressed compared to foetal stages, it is unlikely any weight difference in embryos would be as pronounced.

Interaction frequency and expression of IGF2/H19 do not explain weight differences in embryos

Finally, we investigated the links between interaction frequency, gene expression and weight differences in embryos. In terms of *IGF2* regulation, the long-range interaction occurs at the

maternal locus, so it is unclear how changes in interaction would affect paternal *IGF2* expression. However, a decrease in interaction at the maternal allele could suggest lower CTCF occupancy, allowing some expression from the maternal *IGF2.* Although there was no overall difference in interaction frequency between the breeds, it is possible that the differences between individuals would impact on the expression in that individual. As our expression data so far has only looked at one set of individuals for each breed, more individuals need to be investigated. The weight of the Angus embryos (taken before dissection) were higher than all other breeds, whereas the Brahman was consistently lower than other breeds (Table 1). It is difficult to assess how significant the individual weight differences are, however the weight trends generally do not agree with our expected outcomes from the expression data. It is possible that *IGF2* expression is not significantly impacting on the growth of the embryo at this early stage.

Regarding *H19*, there could be an association between the elevated expression in the Brahman sample and the smaller embryo weights observed. We would then expect the lowest expression of *H19* to be observed in the Angus, due to its greater weight. However, the Angus was observed to have similar expression levels to the hybrid samples, with intermediate weights. Mechanistically, it could be that the *H19* gene is being activated strongly by the downstream enhancers via intra-chromosomal looping in the Brahman, as observed in mice (Ling *et al.* 2006), however the presence of these enhancer elements and the conservation of intra-chromosomal looping in bovine has not been shown.

Conclusion

We have for the first time investigated long-range interaction and expression in 48-day embryo derived fibroblasts. We have shown the conservation of *IGF2/H19* long range interactions in bovine and have found that interaction frequency is individually variable between *B. p. indicus* and *B. p. taurus* purebred and hybrid animals, rather than linked to a breed specific form of regulation. We found that in embryonic fibroblasts, *IGF2* expression was significantly higher in both purebred breeds compared to the hybrids. *H19* expression was also much higher in Brahman purebreds compared to Angus purebred and hybrid crosses. Due to its effects in muscle development, we suggest that if hybrid overgrowth phenotypes are linked to *H19* in muscle, this difference becomes pronounced at a later stage of growth. Importantly, as this work was carried out in cultured embryonic cells, these results should ideally be reproduced using tissue, to more closely resemble primary biology. Further

study is needed into the methylation patterns of the ICR in embryonic stage tissues in hybrids to truly understand the epigenetics of these hybrid differences.

Conflict of Interest

The authors declare they have no conflict of interest.

References

Anand-Ivell, R, Hiendleder, S, Viñoles, C, Martin, GB, Fitzsimmons, C, Eurich, A, Hafen, B*, et al.* 2011, 'INSL3 in the Ruminant: A Powerful Indicator of Gender- and Genetic-Specific Feto-Maternal Dialogue', *PLoS ONE*, vol. 6, no. 5, p. e19821.

Apostolou, E, Ferrari, F, Walsh, RM, Bar-Nur, O, Stadtfeld, M, Cheloufi, S, Stuart, HT*, et al.* 2013, 'Genome-wide chromatin interactions of the Nanog locus in pluripotency, differentiation, and reprogramming', *Cell Stem Cell*, vol. 12, no. 6, Jun 6, pp. 699-712.

Bebbere, D, Bauersachs, S, Fürst, RW, Reichenbach, H-D, Reichenbach, M, Medugorac, I, Ulbrich, SE*, et al.* 2013, 'Tissue-Specific and Minor Inter-Individual Variation in Imprinting of <italic>IGF2R</italic> Is a Common Feature of <italic>Bos taurus</italic> Concepti and Not Correlated with Fetal Weight', *PLoS ONE*, vol. 8, no. 4, p. e59564.

Brown, MA, Tharel, LM, Brown, AH, Jr., Jackson, WG & Miesner, JR 1993, 'Genotype x environment interactions in preweaning traits of purebred and reciprocal cross Angus and Brahman calves on common bermudagrass and endophyte-infected tall fescue pastures', *J Anim Sci*, vol. 71, no. 2, Feb, pp. 326-333.

Chen, Z, Hagen, DE, Elsik, CG, Ji, T, Morris, CJ, Moon, LE & Rivera, RM 2015, 'Characterization of global loss of imprinting in fetal overgrowth syndrome induced by assisted reproduction', *Proc Natl Acad Sci U S A*, vol. 112, no. 15, Apr 14, pp. 4618-4623.

Dawson, WD 1965, 'Fertility and Size Inheritance in a Peromyscus Species Cross', *Evolution*, vol. 19, no. 1, pp. 44-55.

Dindot, SV, Farin, PW, Farin, CE, Romano, J, Walker, S, Long, C & Piedrahita, JA 2004, 'Epigenetic and Genomic Imprinting Analysis in Nuclear Transfer Derived Bos gaurus/Bos taurus Hybrid Fetuses', *Biology of Reproduction*, vol. 71, no. 2, August 1, 2004, pp. 470-478.

Gebert, C, Wrenzycki, C, Herrmann, D, Gröger, D, Reinhardt, R, Hajkova, P, Lucas-Hahn, A*, et al.* 2006, 'The bovine IGF2 gene is differentially methylated in oocyte and sperm DNA', *Genomics*, vol. 88, no. 2, 8//, pp. 222-229.

Goodall, JJ & Schmutz, SM 2007, 'IGF2 gene characterization and association with rib eye area in beef cattle', *Animal Genetics*, vol. 38, no. 2, pp. 154-161.

Hagege, H, Klous, P, Braem, C, Splinter, E, Dekker, J, Cathala, G, de Laat, W*, et al.* 2007b, 'Quantitative analysis of chromosome conformation capture assays (3C-qPCR)', *Nat. Protocols*, vol. 2, no. 7, pp. 1722-1733.

Haldane, JBS 1922, 'Sex ratio and unisexual sterility in hybrid animals', *Journal of Genetics*, vol. 12, no. 2, 1922//, pp. 101-109.

Hiendleder, S, Lewalski, H & Janke, A 2008, 'Complete mitochondrial genomes of Bos taurus and Bos indicus provide new insights into intra-species variation, taxonomy and domestication', *Cytogenet Genome Res*, vol. 120, no. 1-2, pp. 150-156.

Ling, JQ & Hoffman, AR 2007, 'Epigenetics of long-range chromatin interactions', *Pediatric Research*, vol. 61, no. 5, pp. 11R-16R.

Ling, JQ, Li, T, Hu, JF, Vu, TH, Chen, HL, Qiu, XW, Cherry, AM*, et al.* 2006, 'CTCF Mediates Interchromosomal Colocalization Between Igf2/H19 and Wsb1/Nf1', *Science*, vol. 312, no. 5771, pp. 269-272.

Long, JE & Cai, X 2007, 'Igf-2r expression regulated by epigenetic modification and the locus of gene imprinting disrupted in cloned cattle', *Gene*, vol. 388, no. 1-2, Feb 15, pp. 125-134.

MacHugh, DE, Shriver, MD, Loftus, RT, Cunningham, P & Bradley, DG 1997, 'Microsatellite DNA variation and the evolution, domestication and phylogeography of taurine and zebu cattle (Bos taurus and Bos indicus)', *Genetics*, vol. 146, no. 3, Jul, pp. 1071-1086.

Nijman, IJ, Bradley, DG, Hanotte, O, Otsen, M & Lenstra, JA 1999, 'Satellite DNA polymorphisms and AFLP correlate with Bos indicus-taurus hybridization', *Animal Genetics*, vol. 30, no. 4, pp. 265-273.

Ryba, T, Hiratani, I, Lu, J, Itoh, M, Kulik, M, Zhang, J, Schulz, TC*, et al.* 2010, 'Evolutionarily conserved replication timing profiles predict long-range chromatin interactions and distinguish closely related cell types', *Genome Res*, vol. 20, no. 6, Jun, pp. 761-770.

Schütt, S, Florl, AR, Shi, W, Hemberger, M, Orth, A, Otto, S, Schulz, WA*, et al.* 2003, 'DNA Methylation in Placentas of Interspecies Mouse Hybrids', *Genetics*, vol. 165, no. 1, p. 223.

Vrana, PB, Fossella, JA, Matteson, P, del Rio, T, O'Neill, MJ & Tilghman, SM 2000, 'Genetic and epigenetic incompatibilities underlie hybrid dysgenesis in Peromyscus', *Nat Genet*, vol. 25, no. 1, May, pp. 120-124.

Vrana, PB, Guan, XJ, Ingram, RS & Tilghman, SM 1998, 'Genomic imprinting is disrupted in interspecific Peromyscus hybrids', *Nat Genet*, vol. 20, no. 4, Dec, pp. 362-365.

Xiang, R, Ghanipoor-Samami, M, Johns, WH, Eindorf, T, Rutley, DL, Kruk, ZA, Fitzsimmons, CJ*, et al.* 2013, 'Maternal and Paternal Genomes Differentially Affect Myofibre Characteristics and Muscle Weights of Bovine Fetuses at Midgestation', *PLoS ONE*, vol. 8, no. 1, p. e53402.

Zhang, S, Kubota, C, Yang, L, Zhang, Y, Page, R, O'Neill, M, Yang, X*, et al.* 2004, 'Genomic imprinting of H19 in naturally reproduced and cloned cattle', *Biol Reprod*, vol. 71, no. 5, Nov, pp. 1540-1544.

Chapter 3 – Differential methylation of CTCF binding sites in ovarian cancer

This chapter consists of an unpublished manuscript.

Chapter Overview

This manuscript is formatted for submission to the journal Molecular Cancer Research. This chapter describes our research into methylation patterns at CTCF binding sites in the *IGF2/H19* ICR in ovarian cancer serous tumours. It is well established that there is aberrant *IGF2* expression in ovarian cancers that occurs without the loss of imprinting. This pilot study uses targeted bisulphite amplicon sequencing of the seven CTCF binding sites within the ICR.

We successfully sequenced six of the seven CTCF binding sites, except site 3, as the amplicon narrowly missed the target binding site. Site 7 had insufficient read depth to perform adequate statistical analysis.

This work shows that in the samples tested, two early stage and two late stage tumours, there is no correlation between tumour stage and methylation. However, two tumours were found to be similar to normal fallopian tissue and peripheral blood lymphocytes, and two very different. Future work including more tumour samples may explore the use of similar DNA methylation analysis to inform potential resistance to DNA methylation sensitive chemotherapy such as Carboplatin

Statement of Authorship

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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

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Manuscript Title: Differential methylation of CTCF binding sites in ovarian cancer

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Abstract

Loss of imprinting of the important growth factor *insulin-like growth factor II (IGF2)* correlates with poor prognosis in ovarian cancer, however it is not yet known how this loss of imprinting occurs. Previous studies have identified hypo- and hyper-methylation of the imprinting control region (ICR) of *IGF2* in ovarian cancer cell lines and hyper-methylation of two CCCTC-binding factor (CTCF) binding sites in serous ovarian tumours. We investigated seven CTCF binding sites within the ICR using targeted amplicon bisulphite sequencing in four primary ovarian tumours, fallopian tube and peripheral blood lymphocytes. We found that each of the sites could exhibit a very different methylation state to other sites in the same sample, and therefore methylation is not uniform across the ICR. There was no evidence of a methylation status unique to either early or late stages of tumour development. We believe that further study into the impact of having hypo- and hyper-methylated sites within the ICR on the imprinted regulation in cancer could bring further insights into this region's role in cancer.

Key words

IGF2, H19, DNA methylation, Ovarian Cancer

Introduction

Despite its status as the most lethal gynaecological malignancy, ovarian cancer is relatively poorly understood, particularly the early unpinning mechanisms of disease, as diagnosis generally occurs at late stages. This bias has meant little is known, particularly about the epigenetic changes that could underlie early oncogenic events. The origin of ovarian cancer is still disputed, however evidence currently suggests the fallopian epithelium is the initial point of transformation (Li *et al.* 2011; Li *et al.* 2012).

The important mitogenic growth factor *IGF2* has been found to be overexpressed and show loss of imprinting (LOI) in multiple cancers, including colorectal (Cheng *et al.* 2010), ovarian (Chen *et al.* 2000; Hiura *et al.* 2012; Kim *et al.* 1998; Murphy *et al.* 2006) and multiple cancer cell lines (Vu, Nguyen & Hoffman 2010a). Furthermore, elevated *IGF2* expression has been correlated with poor prognosis (Lu *et al.* 2006). *IGF2* is co-regulated with the non-coding RNA gene *H19*, which is thought to have tumour-suppressor activity (Hao *et al.* 1993). A critical factor in foetal development, *IGF2* and *H19* expression is imprinted, meaning under normal regulatory conditions only the paternal *IGF2* and maternal *H19* are expressed. This is controlled through DNA methylation of the ICR upstream of H19,

which harbours seven binding sites for the zinc-finger protein CTCF. If the ICR is unmethylated, as on the maternal chromosome, the CTCF binding sites are free to access by CTCF, and when occupied, prevents long range chromatin loops forming, blocking downstream enhancers to act on the *IGF2* promoters (Ling *et al.* 2006).

The start of the CTCF sites within the *H19* ICR is roughly 5kb upstream from the start of the *H19* gene, with the closest, seventh site just ~600bp away. They are arranged as two clusters of three sites (each cluster ~800bp) with the seventh site not clustered. CTCF sites genome wide can be classed into high, medium and low occupancy sites (Essien *et al.* 2009), with developmental gene regulatory sequences commonly appearing as low occupancy sites, where occupancy varies with cell differentiation (Plasschaert *et al.* 2014). Interestingly, CTCF binding is not always methylation sensitive (Wang *et al.* 2012a), however binding of CTCF has been suggested to lead to localised reduction of methylation (Stadler *et al.* 2011).

We wanted to investigate methylation in the ICR within each binding site, rather than as a region, to see if the previously observed hypo- and hyper-methylation patterns were consistent across the binding sites. Previous studies investigated the first and sixth CTCF binding sites in the ICR, and determined site one was more frequently hypermethylated than site six in the tumours tested (Murphy *et al.* 2006). Interestingly, they found that LOI did not correlate with increased *IGF2* expression. It would be assumed that accumulating methylation on the maternal ICR would lead to increased or biallelic *IGF2* expression, as is observed in Wilm's tumour (Cui *et al.* 2001). Conversely, hypomethylation of the paternal allele can lead to biallelic expression of *H19*.

Materials and Methods

Sample preparation

Fallopian tube tissue and ovarian tumour samples were taken during debulking surgery at the Royal Adelaide Hospital (RAH) and stored at -80^{o}C before DNA extraction. All patient samples were taken under approval of the Royal Adelaide Hospital Human Research Ethics Committee (RAH Protocol No. 140101). All DNA was extracted using a standard phenol-chloroform protocol and stored at -20^{o}C. RNA was extracted using TRIzol (ThermoFisher Scientific, Waltham, MA US) as per the manufacturer's instructions.

Bisulphite treatment and PCR

Genomic DNA was treated and cleaned using the EpiTect Bisulphite Kit (Qiagen, Melbourne, VIC) as per the manufacturer's instructions. Primers were designed using the

MethPrimer program (Li & Dahiya 2002). PCRs were set up as 20µl reactions using 0.1µM primers (Table 1), 10µl Kapa Uracil+ enzyme and 2X Ready Mix (Geneworks, Adelaide, SA) and ~2µl template DNA using a BioRad thermocycler with the program 95^{o}C for 4 minutes, 95^{o}C for 45 seconds, 60^{o}C for 1.5 minutes, 72^{o}C for 2 minutes for 5 cycles, then 95^{o}C for 45 seconds, 60^{o}C for 1.5 minutes, 72^{o}C for 1.5 minutes for 25 cycles and 72^{o}C for 4 minutes. PCR reactions were cleaned using an Ampure Bead clean up kit and were sequenced using an Illumina MiSeq Reagent Nano Kit v2 at 2x 150bp paired end reads on the Illumina MiSeq platform (ACRF Cancer Genomics Facility, Adelaide, SA).

Table 1: Primers, product sizes and temperature used to bisulphite sequence each of the seven CTCF sites in the human *H19* ICR.

Target	Forward primer (5'-3')	Reverse Primer (5'-3')	Annealing Temp (^{o}C)	Product size
CTCF1	TTTGTTGATTTTATTAAGGGAGGTT	TTCTATAAATAAACCCCAACCAAAC	60	147
CTCF2	ATGTGTATTTTTGGAGGTTTTTTTT	AACTCCCATAAATATTCTATCCCTCA	60	223
CTCF3	GTTTAAGTTTTTTTTGGATGGGG	CAAACCATAACACTAAAACCCTCA	60	190
CTCF4	ATGAATATTTTTGGAGGTTTTTTTT	ATAAATATCCTATCCCTAATAACCCC	60	216
CTCF5	ATGTGTATTTTTGGAGGTTTTTTTT	ACTCCCATAAATATCCTATACCTCAC	60	222
CTCF6	TATGGGTATTTTTGGAGGTTTTTT	ACTCCCATAAATATCCTATTCCCA	60	224
CTCF7	TTTTATTAAAGGTTAAGGTGGTGAT	CAAAACAAAATCCCCACAAC	60	254

The DNA sequences were merged, and mapped against the GRCh37/hg19 assembly using Bismark (Krueger & Andrews 2011). Methylation base calls were performed using the PileOMeth tool (Ryan *et al.* 2016) and sorted using SAMtools (Li *et al.* 2009a). The sorted sequences were then fitted to a logistic regression model as follows:

$$logit\pi_{ijk}=y_{ijk}=\mu+\alpha_i+\beta_j+\gamma_k+\delta_{ij}+\lambda_{jk}+\zeta_{ik}$$

Where *yijk* is the logit transformed proportion of methylated bases in sample *i,* at position *j,* with 5aza staus *k*. The factors considered are μ, the overall average of the pooled peripheral blood lymphocytes and fallopian samples at the site of the first CpG motif, αi the change in average value between samples, βj as a measure of positional effects between CpGs within one binding site, γk representing the effects of 5aza (modelling of two experiments was done simultaneously. See Williams, Thesis 2016), δij measuring any site-specific effects between samples, λjk to determine site-specific effects due to 5aza treatment and ζik to capture cell-

line specific effects due to 5aza treatment that have not been covered previously. The methylation data was then graphed using Methylation plotter (Mallona *et al.* 2014).

qPCR

RNA was converted using the BioRad iScript cDNA synthesis kit as per the manufacturer's instructions. The 20µl reaction was diluted to 100µl using DEPC treated sterile water, and 5µl was used per well. KiCqStart SYBR green primers for *ACTB, H19* and *WSB1* were purchased from Sigma Aldrich (Castle Hill, NSW) and Power SYBR Green Master Mix (Life Technologies) were used as per the manufacturer's instructions. Primers for *IGF2* (F: CCCCTCCGACCGTGCT; R: TGGACTGCTTCCAGGTGTCAT) were kindly lent by Assoc. Prof Briony Forbes (Flinders University, Adelaide). Each sample was tested in technical triplicate and efficiency determined by the standard curve method. MicroAmp Fast Optical 96-well Reaction qPCR plates (Applied BioSystems) were read by the StepOne Plus Real-time PCR system (Applied BioSystems) using a standard protocol of 95^{o}C for 10 minutes, followed by cycles of 95^{o}C for 15 seconds and 60^{o}C for 1 minute for 40 cycles. The melt curve protocol was 95^{o}C for 15 seconds, 60^{o}C for 1 minute, increasing in 0.3^{o}C increments, then 95^{o}C for 15 seconds. Results were analysed using the StepOne Software v2.3 (Applied BioSystems).

Results

Sample selection

We chose a small selection of tumours (identified by alpha-numeric code) to cover both early and late stage disease, where 1354A (Stage IA), 277B (Stage IC), 971A and 620A (Stage IIIC). All tumours were identified as grade 3, and are therefore poorly differentiated and likely to be aggressive. All samples were pre-chemotherapy, taken during debulking surgery.

Methylation status of individual CpG residues in CTCF binding sites

We investigated the methylation status of each of the seven CTCF binding sites within the *H19* ICR using targeted amplicon bisulphite sequencing. All but the third CTCF binding site was successfully amplified and bisulfite sequenced. The third site was not covered by the amplicon due to primer design restrictions. Site seven is excluded due to insufficient read depth to perform analysis (Appendix A). Sites one, two, and six contained four CpG residues, while sites four, five, and seven had three (Figure 1). We examined if

there were any differences in methylation state between individual residues within the one site. Each residue was labelled according to its location, position two, four, six or nine, within the binding motif.

Generally, the methylation levels of each CpG within one binding site were very similar to others within the same sample. However, CpG positions six and nine were flagged in a range of tumours as significantly different from both fallopian or peripheral blood lymphocytes (Table 2). These positional differences were only observed at CTCF binding sites two, five and six.

Table 2: Methylation differences at individual CpG positions between normal samples and serous tumour samples.

Coefficient	CTCF site	Estimate	Std. Error	z value	Pr(>|z|)	adjP	
Normal	2	-0.21	0.01	-20.76	1.05E-95	3.14E-94	
1354A:pos9	2	0.01	0.03	0.43	0.6697	1	
277B:pos9	2	0.03	0.03	1.03	0.3012	1	
620A:pos9	2	-0.05	0.05	-1.10	0.2727	1	
971A:pos9	2	-0.91	0.03	-36.18	1.09E-286	3.27E-285	***
Normal	5#	1.47	0.03	56.70	0	0	***
1354A:pos6	5#	-0.33	0.05	-6.66	2.78E-11	6.40E-10	***
277B:pos6	5#	-0.18	0.07	-2.45	0.01438	0.3308	
620A:pos6	5#	-0.27	0.08	-3.19	0.001438	0.03307	
971A:pos6	5#	-0.01	0.10	-0.12	0.9008	1	
1354A:pos9	5#	-0.47	0.05	-9.26	2.01E-20	4.62E-19	***
277B:pos9	5#	-0.29	0.07	-3.97	7.14E-05	0.001643	
620A:pos9	5#	0.00	0.09	0.02	0.9839	1	
971A:pos9	5#	-0.60	0.09	-6.38	1.77E-10	4.07E-09	***
Normal	6#	-0.16	0.02	-9.95	2.51E-23	8.52E-22	***
1354A:pos6	6#	0.05	0.03	1.35	0.1774	1	
277B:pos6	6#	-7.05	0.21	-34.15	1.16E-255	3.93E-254	***
620A:pos6	6#	-2.99	0.05	-57.99	0	0	***
971A:pos6	6#	-0.37	0.05	-8.07	6.88E-16	2.34E-14	***
PBL:pos6	6#	-2.20	0.04	-55.91	0	0	***

#Normal samples were not pooled and treated separately due to discrepancies between peripheral blood lymphocytes and fallopian tube tissue. The tumour samples were compared only to fallopian tube. Estimate, Std Error and z value columns presented at 2dp. Bonferroni's method was used to obtain adjusted p-values. Significant results, shown by asterisk, were determined as those with estimates ±0.3 different to normal samples.

Methylation status of each CTCF binding site in serous ovarian tumours

We then compared the average methylation levels across each binding site in our tumour samples to the values observed in PBLs and fallopian tissue (Figure 2). The 277B and 620A tumours were consistently different to the pooled normal samples at nearly all binding sites, excluding site six for 620A (Table 3). Tumour 620A displayed the most varied methylation patters, with hypo-methylation of sites one, two and six, and hyper-methylation of sites four and five. Tumour 277B displayed relatively consistent hyper-methylation at all sites. Interestingly, tumours 1354A and 971A displayed normal methylation levels at sites one, two and four, however showed hypo-methylation at site five. 971A had increased methylation at site six.

Table 3: Methylation differences between normal samples and serous tumour samples.

Coefficient	CTCF site	Estimate	Std. Error	z value	Pr(>|z|)	adjP	
Normal	1	0.05	0.10	0.48	0.6336	1	
1354A	1	0.33	0.17	1.88	0.06066	0.4852	
277B	1	0.94	0.18	5.31	1.07E-07	8.58E-07	***
620A	1	-2.15	0.23	-9.54	1.39E-21	1.11E-20	***
971A	1	-0.11	0.18	-0.63	0.5286	1	
Normal	2	-0.21	0.01	-20.76	1.05E-95	3.14E-94	
1354A	2	0.02	0.02	0.74	0.4608	1	
277B	2	0.76	0.02	40.84	0	0	***
620A	2	-2.73	0.03	-81.60	0	0	***
971A	2	-0.09	0.02	-5.43	5.71E-08	1.71E-06	
Normal	4	-0.23	0.01	-25.95	1.78E-148	1.96E-147	
1354A	4	-0.07	0.01	-5.60	2.18E-08	2.40E-07	
277B	4	0.87	0.02	51.97	0	0	***
620A	4	2.74	0.03	107.20	0	0	***
971A	4	-0.06	0.01	-5.38	7.65E-08	8.42E-07	
Normal	5[#]	1.47	0.03	56.70	0	0	***
1354A	5[#]	-1.34	0.03	-39.70	0	0	***
277B	5[#]	0.35	0.05	7.28	3.33E-13	7.65E-12	***
620A	5[#]	1.76	0.06	31.11	1.58E-212	3.63E-211	***
971A	5[#]	-0.47	0.07	-7.13	9.99E-13	2.30E-11	***
Normal	6[#]	-0.16	0.02	-9.95	2.51E-23	8.52E-22	***
1354A	6[#]	0.06	0.02	2.46	0.01398	0.4754	
277B	6[#]	0.92	0.02	37.47	3.39E-307	1.15E-305	***
620A	6[#]	-0.05	0.02	-2.08	0.03727	1	
971A	6[#]	0.13	0.03	4.21	2.54E-05	0.0008622	***
PBL	6[#]	0.26	0.02	11.10	1.19E-28	4.06E-27	***

[#]Normal samples were not pooled and treated separately due to discrepancies between peripheral blood lymphocytes and fallopian tube tissue. The tumour samples were compared only to fallopian tube. Estimate, Std Error and z value columns presented at 2dp. Bonferroni's method was used to obtain adjusted p-values. Significant results, shown by asterisk, were determined as those with estimates ±0.3 different to normal samples.

Expression of Igf2 and H19 in serous tumours

We investigated the expression of *Igf2* and *H19* in these tumours to see if there were any pronounced differences. However, reliable expression data for these samples is not included due to technical issues with the samples. See Appendix A for current results.

Discussion

Individual CpG methylation states in CTCF binding sites

Of the sites examined, three or four CpG residues were identified in each binding site (Figure 1), which differs to observations in human-mouse somatic hybrid cells, where one extra residue was found in every site, except for site seven (Takai *et al.* 2001). As CTCF sites are frequently mutated in cancer (Katainen *et al.* 2015), it may be that the number of residues has changed in these samples. Our results highlighted that individual CpG residues within CTCF binding sites generally show similar methylation levels to nearby residues (Table 2). This is consistent with the model of DNA methylation maintenance, whereby a CpG is more likely to show a particular methylation state if those around it also present in the same state due to recruitment and the close presence of methylating or demethylating enzymes (Lövkvist *et al.* 2016). However, there were exceptions to this, mainly at position six and nine in some tumours. It is not clear what the biological consequences of this would be, as it is not known what configuration CTCF uses to bind these particular motifs.

Average methylation of each CTCF binding site differs in serous ovarian tumours

When we compared the average methylation of all residues for each CTCF site, we saw pronounced differences between the tumours (Figure 2). Two of the four tumours were consistently different to normal tissues, however the methylation levels were not consistently affected in the same way. Previous studies indicated that in embryonic uterine tissue, all CTCF binding sites except site six were methylated (78%-90%), while site six displayed allele specific methylation levels of ~50%, and was hypo-methylated in bladder cancer (Takai *et al.* 2001). In serous epithelial ovarian tumours, sites one and six were observed to be consistently hyper-methylated (Murphy *et al.* 2006). Our results suggest that in serous ovarian tumours, there is more variability in methylation levels between the different binding sites. This was particularly apparent in tumour 620A, where two sites were hypo-methylated, two hyper-methylated and one displayed normal methylation levels (Table 3). By contrast the earlier stage tumour 277B was consistently hyper-methylated. Whether this outcome is random or certain sites are more likely to be hyper-methylated than not would need further study with many more patient samples.

Expression of Igf2 and H19 in serous ovarian tumours

According to the CSIOVDB (Tan *et al.* 2015), high *IGF2* expression correlates with decreased overall survival ($p=9.9x10^{-3}$) and decreased disease-free survival ($p=3.8x10^{-2}$). H19 expression had no correlation with either outcome. Using the KM plotter tool (Gyorffy

et al. 2012) and restricting the samples to grade 3 serous tumours confirmed that high *Igf2* expression correlates with decreased progression free survival (p=0.03) and decreased overall survival (p=0.01). Based on this, we would predict that our tumours would have strong *IGF2* expression. This would be expected to be higher in tumour 277B, due to its consistent hyper-methylation of CTCF binding sites in the ICR. However, for tumours 1354A and 971A where the methylation states are close to normal, we would not expect to see particularly high *IGF2* expression. This may vary if sites five and six more heavily impact on expression than the earlier sites. It is not predictable how the methylation patterns observed in 620A could affect expression.

Clinical outcomes for patients with differential CTCF site methylation

We were interested to see if changes in methylation correlated with clinical outcomes for the patients involved in this study, to determine if there were any factors that could be impacted by the methylation findings (Table 4). The late stage tumours (971A, 620A) have different methylation profiles, while the early stage tumours (1354A, 277B) also lacked any similarity in methylation patterns. This suggests that there is no "early" or "late" stage methylation pattern in the *H19* ICR, however increasing the number of samples would be necessary to confirm this.

Table 4: Tumour characteristics and clinical outcomes in serous ovarian cancer patients.

Patient Sample	Age at diagnosis (years)	Diagnosis	Stage	Grade	Treatment	Survival (months)
1354A	67	Serous carcinoma	IA	3	N/A	9.8 (alive)
277B	58	Serous carcinoma	IC	3	Carboplatin/Paclitaxel	54.3 (alive)
971A	46	Serous papillary carcinoma	IIIC	3	Carboplatin/Paclitaxel	42.4 (deceased – ovarian cancer)
620A	78	Serous papillary carcinoma	IIIC	3	Carboplatin	13.9 (deceased –ovarian cancer)

Although the tumours we received were taken before any chemotherapy, most of the patients were later treated using a combination of drugs, commonly including carboplatin or paclitaxel. Paclitaxel targets tubulin, preventing disassembly of the mitotic spindle assembly and therefore disrupting cell division (Jordan & Wilson 2004). The resultant prolonged mitotic checkpoints trigger apoptosis. Carboplatin is a platinum based drug believed to act through crosslinking with DNA, resulting in apoptosis, similar to its predecessor cisplatin (Wang & Lippard 2005). It has previously been suggested that DNA methylation affects chemoresistance in ovarian cancer epithelial cells treated with cisplatin by conferring platinum resistance (Li *et al.* 2009b). Paclitaxel's mode of action is not directly affected by DNA methylation, and therefore is often used in patients with chemoresistance to carboplatin. Future work may interrogate methylated regions, such as the binding sites in the *H19* ICR to test for potential resistance to platinum based therapies.

Conclusion

In this research we have shown that methylation of multiple CpGs within the CTCF binding sites is generally stable, with the exception of CpGs at positions six and nine within the binding motif. Future work should investigate the importance of each CpG position within the binding site to CTCF binding affinity or occupancy. The average methylation across each binding site was found to be highly variable between tumours, with no consistent patterns of hypo- or hyper-methylation across samples. There is no evidence in our samples of a methylation pattern unique to early or late serous tumours, however far more samples would need to be tested to confirm this. It would be worth investigating whether the methylation patterns of some of these sites could predictive potential for resistance to platinum based

therapies such as carboplatin. A first step for this will be to investigate methylation status in a larger range of samples, both pre- and post-chemotherapy to observe changes in methylation status and patient outcome after treatment.

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References

Chen, CL, Ip, SM, Cheng, D, Wong, LC & Ngan, HYS 2000, 'Loss of imprinting of the IGF-II and H19 genes in epithelial ovarian cancer', *Clinical Cancer Research*, vol. 6, no. 2, pp. 474-479.

Cheng, YW, Idrees, K, Shattock, R, Khan, SA, Zeng, ZS, Brennan, CW, Paty, P*, et al.* 2010, 'Loss of imprinting and marked gene elevation are 2 forms of aberrant IGF2 expression in colorectal cancer', *International Journal of Cancer*, vol. 127, no. 3, pp. 568-577.

Cui, H, Niemitz, EL, Ravenel, JD, Onyango, P, Brandenburg, SA, Lobanenkov, VV & Feinberg, AP 2001, 'Loss of imprinting of insulin-like growth factor-II in Wilms' tumor commonly involves altered methylation but not mutations of CTCF or its binding site', *Cancer Res*, vol. 61, no. 13, Jul 1, pp. 4947-4950.

Essien, K, Vigneau, S, Apreleva, S, Singh, LN, Bartolomei, MS & Hannenhalli, S 2009, 'CTCF binding site classes exhibit distinct evolutionary, genomic, epigenomic and transcriptomic features', *Genome Biology*, vol. 10, no. 11.

Gyorffy, B, Lanczky, A & Szallasi, Z 2012, 'Implementing an online tool for genome-wide validation of survival-associated biomarkers in ovarian-cancer using microarray data from 1287 patients', *Endocr Relat Cancer*, vol. 19, no. 2, Apr, pp. 197-208.

Hao, Y, Crenshaw, T, Moulton, T, Newcomb, E & Tycko, B 1993, 'Tumor-suppressor activity of H19 RNA', *Nature*, vol. 365, no. 6448, pp. 764-767.

Hiura, H, Okae, H, Kobayash, H, Miyauchi, N, Sato, F, Sato, A, Suzuki, F*, et al.* 2012, 'High-throughput detection of aberrant imprint methylation in the ovarian cancer by the bisulphite PCR-Luminex method', *Bmc Medical Genomics*, vol. 5, Mar.

Jordan, MA & Wilson, L 2004, 'Microtubules as a target for anticancer drugs', *Nat Rev Cancer*, vol. 4, no. 4, 04//print, pp. 253-265.

Katainen, R, Dave, K, Pitkanen, E, Palin, K, Kivioja, T, Valimaki, N, Gylfe, AE*, et al.* 2015, 'CTCF/cohesin-binding sites are frequently mutated in cancer', *Nat Genet*, vol. 47, no. 7, 07//print, pp. 818-821.

Kim, HT, Choi, BH, Niikawa, N, Lee, TS & Chang, SI 1998, 'Frequent loss of imprinting of the H19 and IGF-II genes in ovarian tumors', *American Journal of Medical Genetics*, vol. 80, no. 4, pp. 391-395.

Krueger, F & Andrews, SR 2011, 'Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications', *Bioinformatics*, vol. 27, no. 11, Jun 1, pp. 1571-1572.

Li, H, Handsaker, B, Wysoker, A, Fennell, T, Ruan, J, Homer, N, Marth, G*, et al.* 2009a, 'The Sequence Alignment/Map format and SAMtools', *Bioinformatics*, vol. 25, no. 16, Aug 15, pp. 2078-2079.

Li, J, Abushahin, N, Pang, SJ, Xiang, L, Chambers, SK, Fadare, O, Kong, BH*, et al.* 2011, 'Tubal origin of 'ovarian' low-grade serous carcinoma', *Modern Pathology*, vol. 24, no. 11, Nov, pp. 1488-1499.

Li, J, Fadare, O, Xiang, L, Kong, BH & Zheng, WX 2012, 'Ovarian serous carcinoma: recent concepts on its origin and carcinogenesis', *Journal of Hematology & Oncology*, vol. 5, no. 8.

Li, LC & Dahiya, R 2002, 'MethPrimer: designing primers for methylation PCRs', *Bioinformatics*, vol. 18, no. 11, Nov, pp. 1427-1431.

Li, M, Balch, C, Montgomery, JS, Jeong, M, Chung, JH, Yan, P, Huang, TH*, et al.* 2009b, 'Integrated analysis of DNA methylation and gene expression reveals specific signaling pathways associated with platinum resistance in ovarian cancer', *BMC Med Genomics*, vol. 2, Jun 08, p. 34.

Ling, JQ, Li, T, Hu, JF, Vu, TH, Chen, HL, Qiu, XW, Cherry, AM*, et al.* 2006, 'CTCF Mediates Interchromosomal Colocalization Between Igf2/H19 and Wsb1/Nf1', *Science*, vol. 312, no. 5771, pp. 269-272.

Lövkvist, C, Dodd, IB, Sneppen, K & Haerter, JO 2016, 'DNA methylation in human epigenomes depends on local topology of CpG sites', *Nucleic Acids Research*, February 29, 2016.

Lu, L, Katsaros, D, Wiley, A, de la Longrais, IAR, Puopolo, M, Schwartz, P & Yu, H 2006, 'Promoter-specific transcription of insulin-like growth factor-II in epithelial ovarian cancer', *Gynecologic Oncology*, vol. 103, no. 3, pp. 990-995.

Mallona, I, Díez-Villanueva, A & Peinado, MA 2014, 'Methylation plotter: a web tool for dynamic visualization of DNA methylation data', *Source Code for Biology and Medicine*, vol. 9, no. 1, 2014//, p. 11.

Murphy, SK, Huang, ZQ, Wen, YQ, Spillman, MA, Whitaker, RS, Simel, LR, Nichols, TD*, et al.* 2006, 'Frequent IGF2/H19 domain epigenetic alterations and elevated IGF2 expression in epithelial ovarian cancer', *Molecular Cancer Research*, vol. 4, no. 4, pp. 283-292.

Plasschaert, RN, Vigneau, S, Tempera, I, Gupta, R, Maksimoska, J, Everett, L, Davuluri, R*, et al.* 2014, 'CTCF binding site sequence differences are associated with unique regulatory and functional trends during embryonic stem cell differentiation', *Nucleic Acids Res*, vol. 42, no. 2, Jan, pp. 774-789.

Ryan, D, Pederson, B, Triche Jr, T & Gruning, B 2016, 'PileOMeth 0.1.13', Zenodo, <http://doi.org/10.5281/zenodo.53211>.

Stadler, MB, Murr, R, Burger, L, Ivanek, R, Lienert, F, Scholer, A, van Nimwegen, E*, et al.* 2011, 'DNA-binding factors shape the mouse methylome at distal regulatory regions', *Nature*, vol. 480, no. 7378, Dec 14, pp. 490-495.

Takai, D, Gonzales, FA, Tsai, YC, Thayer, MJ & Jones, PA 2001, 'Large scale mapping of methylcytosines in CTCF-binding sites in the human H19 promoter and aberrant hypomethylation in human bladder cancer', *Human Molecular Genetics*, vol. 10, no. 23, November 1, 2001, pp. 2619-2626.

Tan, TZ, Yang, H, Ye, J, Low, J, Choolani, M, Tan, DS, Thiery, JP*, et al.* 2015, 'CSIOVDB: a microarray gene expression database of epithelial ovarian cancer subtype', *Oncotarget*, vol. 6, no. 41, Dec 22, pp. 43843-43852.

Vu, TH, Nguyen, AH & Hoffman, AR 2010a, 'Loss of IGF2 imprinting is associated with abrogation of long-range intrachromosomal interactions in human cancer cells', *Hum Mol Genet*, vol. 19, no. 5, Mar 1, pp. 901-919.

Wang, D & Lippard, SJ 2005, 'Cellular processing of platinum anticancer drugs', *Nat Rev Drug Discov*, vol. 4, no. 4, 04//print, pp. 307-320.

Wang, H, Maurano, MT, Qu, H, Varley, KE, Gertz, J, Pauli, F, Lee, K*, et al.* 2012a, 'Widespread plasticity in CTCF occupancy linked to DNA methylation', *Genome Res*, vol. 22, no. 9, Sep, pp. 1680-1688.

Figures for Manuscript: Differential methylation of CTCF sites in ovarian cancer

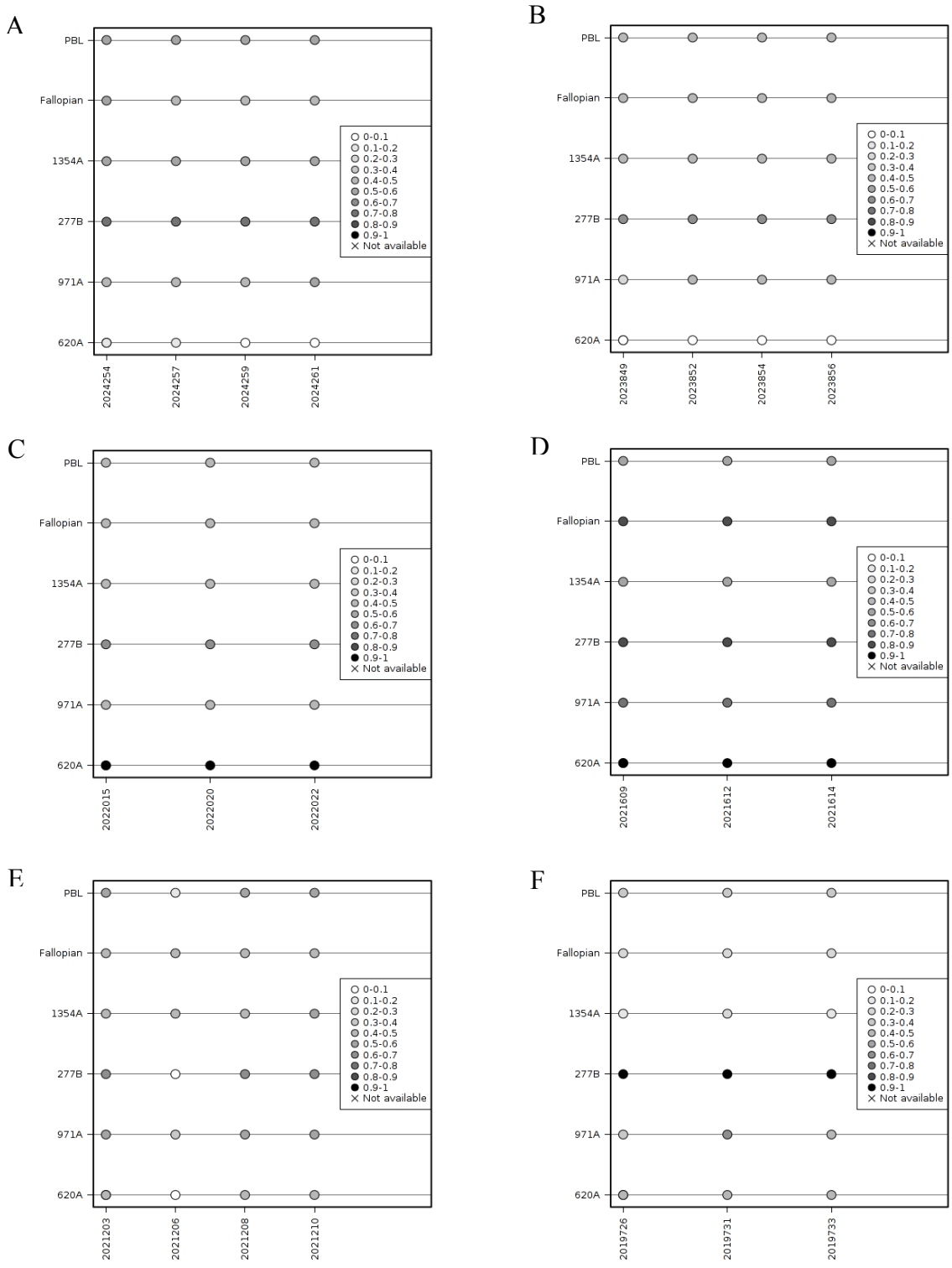

Figure 1: Methylation levels of individual CpG sites with the CTCF binding sites. **A**: CTCF binding site 1. **B**: CTCF binding site 2. **C**: CTCF binding site 4. **D**: CTCF binding site 5. **E:** CTCF binding site 6. **F:** CTCF binding site 7. Position on chromosome 11 is shown on the x axes.

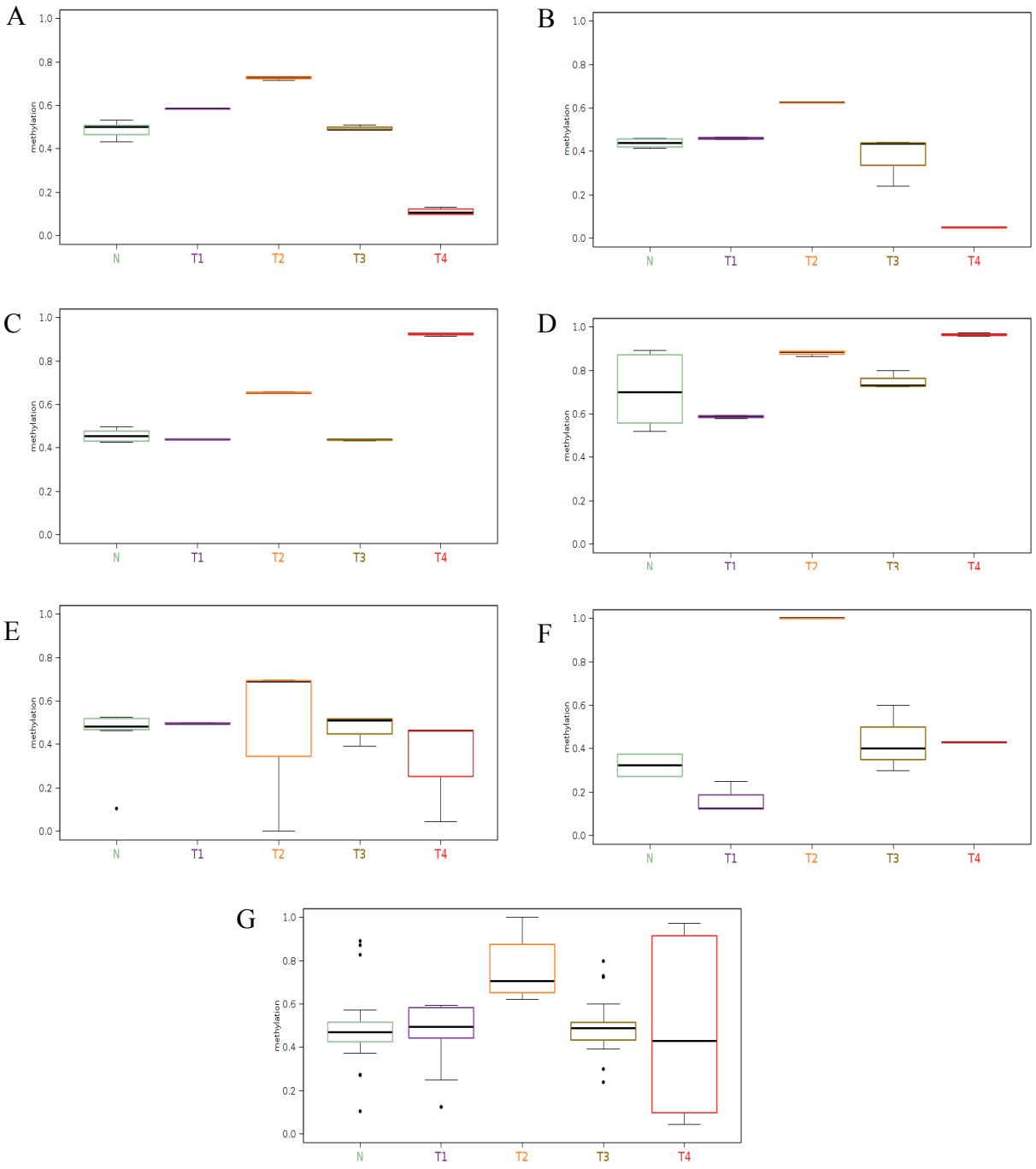

Figure 2: Distribution of methylation values of CpGs within the CTCF binding sites. Single dots are used to represent data points considered outliers, the upper and lower bounds of the box represent the third and first quintiles respectively and the error bars describe the maximum and minimum. **A**: CTCF binding site 1. **B**: CTCF binding site 2. **C**: CTCF binding site 4. **D**: CTCF binding site 5. **E:** CTCF binding site 6. **F:** CTCF binding site 7. **G:** The average methylation of all binding sites within the ICR. 'N' is the combination of values from normal fallopian tube and peripheral blood lymphocytes. 'T1' is 1354A (Stage 1A), 'T2' is 277B (Stage 1C), 'T3' is 971A (Stage 3C) and 'T4' is 620A (Stage 3C).

Chapter 4 – CTCF site methylation resistant to 5-aza-2-deoxycytidine (5aza) treatment in vitro

This chapter consists of an unpublished manuscript.

Chapter Overview

This manuscript is formatted for submission to the International Journal of Oncology. It describes our research into the efficacy of 5-aza-2-deoxycytidine (5aza) in removing DNA methylation in the imprinted *IGF2/H19* ICR in ovarian cancer cell lines. This region has a strong biological imperative to maintain methylation at "normal" levels in order to sustain imprinted regulation.

Previous work has investigated the methylation status of a few of the CTCF binding sites within the *IGF2/H19* ICR in several different cancer contexts, however the region is commonly referred to as hyper- or hypo-methylated without clear distinction of the seven different sites. Our work investigated the methylation status of each of the seven CTCF binding sites, excluding site 3 due to technical issues.

Our work determined the methylation status of each binding site in normal tissues, represented by peripheral blood lymphocytes and normal fallopian tissue, as well as in two ovarian cancer cell lines OVCAR3 and OVCAR5. We found that after treatment with 5aza, the methylation was strongly reduced, but not at all sites and not in both cell lines. This shows that 5aza's effects on this region are inconsistent and unreliable.

We then investigated the possible impact of methylation changes in long-range interactions at this locus that are reliant on CTCF protein binding. However due to unstable gene copies within the cell lines in preliminary counting, and therefore difficulty assessing interaction frequency using DNA FISH, our results were inconclusive. A molecular approach such as a quantitative 3C experiment may be better suited.

Finally, we investigated the expression of key genes *IGF2, H19, WSB1* and *ACTB* using qPCR. Both cell lines were found to have low expression of *IGF2* and *H19* compared to normal tissues, and are likely a poor approximate for primary disease. However, treatment with 5aza in OVCAR3 resulted in increased expression of *IGF2* and *H19*.

This manuscript is a unique study into the localised effects of 5aza treatment on DNA methylation of multiple sites within the ICR of the imprinted *IGF2/H19* region.

NOTE: After completion of this research, it was noted that both cell lines need to be genotyped to detect potential contamination of other cell types. This will be completed before submitting the paper for publication. Neither cell line has been noted previously as contaminated on the Database of Cross-Contaminated or Misidentifed Cell Lines (International Cell Line Authentication Committee).

Statement of Authorship

Title of paper	**CTCF site methylation resistant to 5-aza-2-deoxycytidine (5aza) treatment in vitro**
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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Nicole Williams
Contribution to the paper	Designed project and experiments Performed all experiments Collected all data Performed analysis of results Wrote manuscript
	02.12.16

Name of Co-Author	Reuben Jacob
Contribution to the paper	Designed project Cell culture & 5aza treatment
Signature	**Date** 29/11/2016

Name of Co-Author	Stephen Pederson
Contribution to the paper	Statistical modelling of bisulphite and qPCR data
Signature	**Date** 28/11/2016

Name of Co-Author	James Breen
Contribution to the paper	Bioinformatic processing of sequencing data
Signature	**Date** 28/11/2016.

Name of Co-Author	Noor Lokman
Contribution to the paper	Provided essential materials, patient information Contributed to interpretation of results
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Name of Co-Author	Carmela Ricciardelli
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Name of Co-Author	Frank Grützner
Contribution to the paper	Design of project and experiments Funding of project Editing of manuscript
Signature	2/12/16

Manuscript Title: CTCF site methylation resistant to 5-aza-2-deoxycytidine (5aza) treatment in vitro

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Abbreviations

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Running title
Williams et al: CTCF site methylation resistant to 5-aza-2-deoxycytidine (5aza) treatment in vitro

Abstract

Hypermethylation of tumour suppressor promotor regions occurs in many cancers, leading to the development of demethylating drugs such as 5-aza-2-deoxycytidine (5aza). However, studies to date have focussed on re-activation of hypermethylated tumour suppressors, but little is known about the mechanisms and localised effects of 5aza treatment in other regions of the genome. In this study, we treated OVCAR3 and OVCAR5 cells with 5aza and examined methylation of several CCCTC-binding factor (CTCF) binding sites within the imprinted *insulin-like growth factor II (IGF2)/H19* region which is frequently deregulated in cancer and subject to complex epigenetic regulation. We also investigated CTCF dependent long-range chromatin interaction and expression of the key genes *IGF2* and *H19*. Interestingly, our research shows that 5aza treatment does not affect methylation evenly across a ~5kb stretch of this imprinted region. The cell lines featured frequent amplification of the *IGF2* and *β-actin* regions, and while some interchromosomal interaction was observed it could not be quantified. Both cell lines showed an increase in expression of *IGF2* and *H19* after 5aza treatment. The results show that 5aza treatment does not work uniformly, and that different cancer may react differently to the demethylation agent.

Introduction

Hyper-methylation of tumour-suppressor gene promoters is a well-established characteristic of many cancers (Kulis & Esteller 2010; Merlo *et al.* 1995). The use of demethylating agents such as 5aza was an exciting development in cancer treatment, given the reversible nature of DNA methylation (Leone *et al.* 2002). 5aza works as the residue is incorporated into replicating DNA and binds methyltransferases, reducing the enzymes available to methylate surrounding DNA (Christman 2002).

One of the key regions deregulated in several cancers is the imprinted *IGF2/H19* region. The paternally expressed *IGF2* is a potent mitogenic growth factor heavily involved in foetal growth, while the maternally expressed *H19* is thought to have growth inhibitory effects (Hao *et al.* 1993). The regulation of this locus relies on differential DNA methylation of seven CTCF binding sites within the imprinting control region (ICR). CTCF is unable to bind the methylated sites, resulting in intra-chromosomal loops bringing downstream enhancers to the *IGF2* promotors (Li *et al.* 2008). Conversely, if CTCF is able to bind, this looping structure is blocked, the enhancers act on the *H19* promoter, and CTCF mediates long range inter-chromosomal interactions, such as with the *WD repeat and SOCS box-containing 1/Neurofibromatosis 1 (WSB1/NF1)* region (Ling *et al.* 2006). CTCF binding is

not always methylation sensitive as it is in the ICR (Wang *et al.* 2012a), and it is thought that CTCF binding can lead to a decrease in methylation in nearby sequences (Stadler *et al.* 2011).

OVCAR3 and OVCAR5 are ovarian cancer cell lines established from the ascites of patients with adenocarcinoma of the ovary (Hamilton *et al.* 1983; Langdon & Lawrie 2001). Previous studies in OVCAR3 observed hypo-methylation of CTCF binding site 6 in the ICR of *IGF2/H19* (Dammann *et al.* 2010). This study also showed that in the MCF10 mammary epithelial cell line, CTCF site 6 was non-responsive to 5aza treatment (Dammann *et al.* 2010). However, little else is known about global methylation patterns in these cell lines compared to normal tissues. When OVCAR3 is treated with 5aza, many genes were found to be upregulated, including *H19* (Menendez *et al.* 2007). We aimed to do a comprehensive analysis of all seven CTCF binding sites, in both OVCAR5 and OVCAR3, to determine if there are similar patterns in the two cell lines, and if the effects of 5aza treatment are uniform across all sites within the ICR.

Materials and Methods

Sample preparation

The cell lines OVCAR3 and OVCAR5 were cultured in Gibco RPMI 1640 medium (ThermoFisher, Waltham, MA) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin. Cells were grown in 75cm^3 flasks at 37^{o}C in a 5% CO_2 atmosphere until ~80% confluence before being harvested or seeded into fresh flasks. Cells were treated with 5aza (Sigma-Aldrich, Sydney, Australia), at 10µM concentration for 72 hours. The treated cells were harvested using trypsin (0.05% trypsin-EDTA) at the end of the 72 hour period to prevent recovery. Fallopian tube tissue was taken during debulking surgery at the Royal Adelaide Hospital (RAH) and stored at -80^{o}C before DNA extraction. All patient samples were taken under approval of the Royal Adelaide Hospital Human Research Ethics Committee (RAH Protocol No. 140101). All DNA was extracted using a standard phenol-chloroform protocol and stored at -20^{o}C for later use. RNA was extracted using TRIzol (ThermoFisher Scientific, Waltham, MA US) as per the manufacturer's instructions.

Bisulphite treatment and PCR

Genomic DNA was treated and cleaned using the EpiTect Bisulphite Kit (Qiagen, Melbourne, VIC) as per the manufacturer's instructions. Primers were designed using the MethPrimer program (Li & Dahiya 2002). PCRs were set up as 20µl reactions using 0.1µM

primers (Table 1), 10µl Kapa Uracil+ enzyme and 2X Ready Mix (Geneworks, Adelaide, SA) and ~2µl template DNA using a BioRad thermocycler with the program 95°C for 4 minutes, 95°C for 45 seconds, 60°C for 1.5 minutes, 72°C for 2 minutes for 5 cycles, then 95°C for 45 seconds, 60°C for 1.5 minutes, 72°C for 1.5 minutes for 25 cycles and 72°C for 4 minutes. PCR reactions were cleaned using an Ampure Bead clean up and were sequenced using an Illumina MiSeq Reagent Nano Kit v2 at 2x 150bp paired end reads on the Illumina MiSeq platform (ACRF, Adelaide, SA).

Bioinformatics and statistics

The DNA sequences were merged, and mapped against the GRCh37/hg19 assembly using Bismark (Krueger & Andrews 2011). Methylation base calls were performed using the PileOMeth tool (Ryan *et al.* 2016) and sorted using SAMtools (Li *et al.* 2009a). The sorted sequences were then fitted to a logistic regression model as follows:

$$logit\pi_{ijk}=y_{ijk}=\mu+\alpha_i+\beta_j+\gamma_k+\delta_{ij}+\lambda_{jk}+\zeta_{ik}$$

Where *yijk* is the logit transformed proportion of methylated bases in sample *i,* at position *j,* with 5aza staus *k*. The factors considered are μ, the overall average of the pooled peripheral blood lymphocytes and fallopian samples at the site of the first CpG motif, αi the change in average value between samples, βj as a measure of positional effects between CpGs within one binding site, γk representing the effects of 5aza, δij measuring any site-specific effects between samples, λjk to determine site-specific effects due to 5aza treatment and ζik to capture cell-line specifc effects due to 5aza treatment that have not been covered previously. Due to the complexity of the model, p-values were adjusted using Bonferroni's method to reduce false-positive results. The methylation data was then graphed using Methylation plotter (Mallona, Díez-Villanueva & Peinado 2014).

qPCR

RNA was converted using the BioRad iScript cDNA synthesis kit as per the manufacturer's instructions. The 20µl reaction was diluted to 100µl using DEPC treated sterile water, and 5µl was used per well. KiCqStart SYBR green primers for *ACTB, H19* and *WSB1* were purchased from Sigma Aldrich (Castle Hill, NSW) and Power SYBR Green Master Mix (Life Technologies) were used as per the manufacturer's instructions. Primers for *IGF2* (F: CCCCTCCGACCGTGCT; R: TGGACTGCTTCCAGGTGTCAT, provided by Assoc. Prof Briony Forbes (Flinders University, Adelaide). Each sample was tested in technical triplicate and efficiency determined by the standard curve method. MicroAmp Fast

Optical 96-well Reaction qPCR plates (Applied BioSystems) were read by the StepOne Plus Real-time PCR system (Applied BioSystems) using a standard protocol of 95°C for 10 minutes, followed by cycles of 95°C for 15 seconds and 60°C for 1 minute for 40 cycles. The melt curve protocol was 95°C for 15 seconds, 60°C for 1 minute, increasing in 0.3°C increments, then 95°C for 15 seconds. Results were analysed using the StepOne Software v2.3 (Applied BioSystems).

The qPCR results were analysed using the ΔΔCt method, with allowances for slightly different amplification efficiencies between primer sets. The results were fitted to the following model:

$$y_{ijk}=\mu+\alpha_i+\beta_j+(\alpha\beta)_{ij}+\varepsilon_{ijk}$$

where y_{ijk} represents the scaled Ct values for gene/primers (i), with control/treatment (j) and replicates (k) considered. Other terms include μ as the average Ct values of the housekeeper β-actin, αi as the change in Ct values due to each primer, βj representing change due to 5aza treatment, $(\alpha\beta)ij$ as the change in Ct value for gene i after treatment with 5aza which is not considered by the additive combination of αi and βj. This term effectively represents the ΔΔCt value. Finally εijk represents the general error term such that $\varepsilon \sim (0,\sigma)$. P-values were adjusted using Bonferroni's method.

BAC identification and isolation

BACs covering the *ACTB, IGF2/H19* and *WSB1/NF1* regions in humans were found for the GRCh37/h19 assembly using the UCSC genome browser (http://genome.ucsc.edu/cgi-bin/hgGateway). BACs were ordered from the Children's Hospital Oakland Research Institute (Oakland, CA). Clones were grown overnight at 37°C before being pelleted and extracted using a standard phenol-chloroform protocol. The regions of interests were then confirmed through PCR and sequencing (Table 2).

DNA FISH

Cells were trypsinised (0.05% trypsin-EDTA), treated with 0.075M KCl and fixed using a 3:1 methanol acetic acid solution. Fixed cells were dropped onto cleaned slides and slowly dried in humid conditions. Slides were pre-treated with pepsin, fixed with 1% formaldehyde and dehydrated in an ethanol series (70%, 90%, 100%). They were denatured

in 70% formamide/2X SSC at 70°C for 90 secs, dehydrated again using an ethanol series (70%, 90%, 100%) before hybridising with labelled BAC probes.

BAC DNA was fluorescently labelled with Vysis SpectrumOrange or SpectrumGreen dUTPs (Abbott Molecular, Abbott Park, IL) using Exo-Klenow (New England Biolabs, Ipswich, MA) and 9-mer random primers (Geneworks, Adelaide, SA). Labelled BACs were ethanol precipitated with Hybloc Competitor DNA (Applied Genetics Laboratories Inc., Melbourne, FL) and salmon sperm before being dissolved in 5µl deionised formamide and 5µl hybridisation mix (10% dextran sulphate in 2X SSC). The probe was then denatured at 80°C for 10 mins and re-annealed at 37°C for 30 mins before hybridising overnight on the pre-treated slides.

Hybridised slides were thoroughly washed three times with 2X SSC and 50% formamide for 5 minutes at 42°C, 2X SSC for 5 minutes at 42°C, 0.1X SSC for 5 mins at 60°C and finally 2X SSC for 5 minutes at 42°C. The slides were airdried and mounted with coverslips using Vectashield mounting medium (Vector, Burlingame, CA). Nuclei were visualised using AxioVision 4 software on an AxioImager 2.1 epifluorescence microscope (Zeiss, Oberkochen, BW) using filters for GFP green, DS Red and DAPI. All images were taken using the 100x lens.

Results

Differential CTCF site methylation in peripheral blood lymphocytes and fallopian tube

First we investigated the methylation status of each of our CTCF binding sites using targeted amplicon bisulphite sequencing. Although all 7 binding sites were targeted, binding site 3 was not covered by the designed amplicon and site 7 was excluded for statistical analysis due to low read depth (Appendix A). Peripheral blood lymphoctyes (PBL) and fallopian tube tissue served as normal tissues for comparison. As expected for an imprinted region in normal tissues, both samples displayed ~50% methylation at sites 1, 2, 4 and 6. However the fallopian tissue showed higher methylation (~86%) at site 5, and both fallopian tube and PBL had lower than expected methylation at site 7, at 27%-37% respectively (Figure 2).

Ovarian cancer cell lines show specific changes in CTCF site methylation

Each of the sites successfully amplified were found to contain 3 or 4 CpG residues (Figure 1). These were defined by their position in the binding motif. We investigated

whether there were differences between individual residues within each binding site. Interestingly, there was little variation between CpGs within a binding site in a single sample, and positional differences were only noted in residues at position 6 and 9 in binding sites 5 and 6 (Table 3). In order to investigate the difference between the samples, we determined the average of the methylation at each site (Figure 2). OVCAR3 and OVCAR5 both showed similar methylation levels to PBLs and fallopian tube at CTCF sites 1, 2 and 4 (Table 4). Interestingly, we discovered that both cell lines had significantly lower methylation at site 5. We also observed that CTCF binding site 6 showed a difference between the cell lines, where OVCAR3 was hypomethylated and OVCAR5 was hypermethylated compared to Fallopian tube.

Specific CTCF binding sites resist demethylation after 5aza treatment

We then investigated whether all CTCF sites respond equally to demethylation by 5aza, by comparing the loss of methylation at each site. Interestingly, when the two cell lines were treated with 5aza, we observed distinct effects across the CTCF sites. Both OVCAR3 and OVCAR5 had significantly reduced methylation at CTCF binding sites 1 and 6 (Table 4). At CTCF site 1, OVCAR3 methylation was reduced by ~21% (~47% down to 26%) while OVCAR5 had a smaller reduction of ~13% (~54% down to 41%) (Figure 2). At site 6, the two cell lines were affected more consistently, with methylation reducing 16% in OVCAR3 (~41% down to ~28%) and 14% in OVCAR5 (~66% down to ~52%). However, at sites 2, 4, and 5, methylation was reduced only in OVCAR3 by 13%, 27% and 26% respectively.

Amplification of IGF2 and ACTB loci and possible disruption of interaction in OVCAR cell lines

Changes in CTCF site methylation is thought to affect CTCF binding and hence long-range interaction frequency. To investigate interaction, we used BAC clones of the *IGF2, ACTB* and *WSB1* regions in human and performed DNA FISH to investigate the *IGF2/H19-WSB1* interaction *in situ.* Due to the variable methylation levels in the cell lines before 5aza treatment, we wanted to determine if there was an effect on the long-range interactions of the *IGF2* region. We observed highly variable numbers of signals, particularly of *ACTB* and *IGF2* in the cell lines (Figure S1). We quantified the number of signals for each probe in each cell line in preliminary counting. This suggested far more than two copies of these regions are present (Table 5), particularly in OVCAR5 where we observed on average ~4 copies of *ACTB*, 3 copies of *WSB1* and ~4 copies of *IGF2*. However up to 11 copies of *IGF2* were observed in a single OVCAR5 cell (Appendix A). Similarly, OVCAR3 had on average

~4 copies of *ACTB*, however, *IGF2* and *WSB1* were less variable and close to the expected 2 copies. Only 2 interactions were observed, one control interaction between *ACTB* and *WSB*1, and one between *IGF2* and *WSB1,* both in OVCAR5.

Expression of IGF2, H19 and WSB1

We investigated the expression of these key genes using qPCR, comparing both cell lines to a normal fallopian sample (Agilent Technologies, Santa Clara, CA). Only 5aza treated OVCAR3 was included, due to sample quality issues in 5aza treated OVCAR5. Before 5aza treatment, both OVCAR3 and OVCAR5 had significantly lower expression of *IGF2* compared to fallopian tissue (Table 6). There was no difference in expression of *WSB1*, however OVCAR3 had lower expression of *H19* than either OVCAR5 or fallopian tissue. After treatment with 5aza, expression of both *IGF2* and *H19* increased in OVCAR3 compared to the level pre-treatment. The increase was far more pronounced in *H19* compared to *IGF2,* while there was no effect on the expression of *WSB1* (Table 7).

Discussion

Positional methylation differences at individual CpG residues within CTCF binding sites at the IGF2/H19 locus

Most CpG residues in the CTCF binding sites surveyed showed methylation levels consistent with other CpG residues nearby. However, at CTCF sites 5 and 6, both OVCAR3 and OVCAR5 had residues at positions 6 and 9 that differed significantly from other residues (Table 3). Interestingly, position 6 also differed between PBL and fallopian tube at CTCF site 6. It is unclear how these positional differences may affect CTCF binding, if at all, or how such pronounced methylation differences occur in a small region that is presumably controlled by the same methyltransferases, where nearby CpGs would have the same methylation states (Lövkvist *et al.* 2016).

Differential methylation at CTCF sites in ovarian cancer cell lines

OVCAR3 and OVCAR5 generally displayed similar methylation levels to normal tissues at CTCF sites 1, 2 and 4, however differed significantly at sites 5 and 6 (Table 4). Both cell lines were significantly hypomethylated at CTCF site 5, although this may be due to the higher than expected levels observed in the fallopian tissue. However, OVCAR3 was hypomethylated at site 6, where OVCAR5 was hypermethylated, relative to normal tissues. This variability is similar to what we have previously observed in primary serous ovarian tumours (Williams, Thesis Chapter 3 2016). Interestingly, the two normal samples, PBL and

fallopian tissue, did not match up at sites 5 and 6 as they did at sites 1, 2 and 4. Especially at site 5, the fallopian tissue had distinctly higher methylation than PBLs, although as this was primary tissue taken from a single patient there may be some variability in that individual. Including more samples in future study will help to shed light on whether this variability is due to sample numbers or actual differential methylation in fallopian tube compared to PBLs.

Demethylation of CTCF binding sites by 5aza is non-uniform in ovarian cancer cell lines

5aza treatment was found to have variable effects on the two cell lines. While 5aza was able to significantly reduce methylation in both cell lines at CTCF sites 1 and 6, all other sites were only impacted in the OVCAR3 samples (Table 4). It is possible that this difference between the cell lines may be due to differences in growth, as 5aza requires replicating DNA to work (Christman 2002). However, we would expect this to result in different effects across the entirety of the ICR, rather than only sites in the middle. As all sites in this region are ~5kb apart at most, there should be little difference in the replication timing across the sites. Therefore, to the best of our knowledge these results are the first that may indicate that some methylation dependent sites are more resistant to demethylation than others within the same regulatory region.

Amplification of IGF2 and ACTB loci and possible disruption of interaction in OVCAR cell lines

We counted the number of signals we observed in the two cell lines and compared to the copies observed in normal lymphocytes (Table 5). The variability seen, particularly in OVCAR5, may be due to replication of the normal two copies in some cells, however it is unlikely that this is the case in all cells. Furthermore, we identified some cells with very high signal counts (Appendix A). As listed in canSAR 3.0 (Tym *et al.* 2016), OVCAR5 has 3 copies of *IGF2* and *H19,* and 2 copies of *WSB1*. OVCAR3 is listed as having 2 copies of *IGF2* and *H19*, and 4 copies of *WSB1*. Future work should genotype these cell lines to check for contamination or genetic alterations with another cell type, or whether some other change has occurred. The variability in the number of signals we observed in DNA FISH experiments on OVCAR3 and OVCAR5 made it statistically difficult to differentiate between background interactions and true interaction frequencies. We suggest a more molecular approach such as a quantitative form of 3C (Hagege *et al.* 2007a) would be more suitable to assess the interaction frequency.

Expression of IGF2, H19 and WSB1 before and after 5aza treatment

The low expression of *IGF2* and *H19* we observed in both OVCAR3 and OVCAR5 (Table 6) suggests these cell lines are a poor approximate for primary tissues such as fallopian tube, or disease where overexpression of *IGF2* is commonly observed (Huang & Murphy 2013). However, we are still able to observe changes after 5aza treatment. 5aza treatment increased expression of both *IGF2* and *H19* in OVCAR3 cells, however there was no change in *WSB1* expression (Table 7). This correlates with other studies that show increased *H19* expression in OVCAR3 after 5aza treatment (Menendez *et al.* 2007). Although we see increased expression of the growth inhibitors like *H19,* 5aza treatment also increased expression of the growth factor *IGF2.* This shows that treatment with non-targeted drugs such as 5aza can have undesirable knock-on effects on other genes. Interestingly, the extra copies of *IGF2* we observed did not appear to affect expression, as it remained significantly below what was observed in normal fallopian tissue. We suggest there may be silencing mechanisms, other than normal genomic imprinting, that are involved to mitigate effects of the increased copies.

Conclusion

Our research has highlighted the variable demethylation effects of 5aza treatment on the *IGF2/H19* ICR in the OVCAR3 and OVCAR5 cell lines. 5aza treatment was far more effective in OVCAR3 cells than in OVCAR5, where some binding sites were not affected. Furthermore, the amount of decrease in methylation varied at each CTCF binding site. There may be some evidence to suggest certain sites are resistant to demethylation by 5aza. We identified amplification in copies of *IGF2* and *β-actin* using DNA FISH and although attempts to quantify the interaction frequency in the cell lines were frustrated by the variability in signal number, there may be an indication that interactions may be negatively affected by the amplification of the loci. Finally, 5aza treatment was observed to increase expression of both *IGF2* and *H19.* We propose further research is necessary into the effects of 5aza on imprinted expression, particularly where duplications may have occurred.

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References

Christman, JK 2002, '5-Azacytidine and 5-aza-2'-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy', *Oncogene*, vol. 21, no. 35, Aug 12, pp. 5483-5495.

Dammann, RH, Kirsch, S, Schagdarsurengin, U, Dansranjavin, T, Gradhand, E, Schmitt, WD & Hauptmann, S 2010, 'Frequent aberrant methylation of the imprinted IGF2/H19 locus and LINE1 hypomethylation in ovarian carcinoma', *International Journal of Oncology*, vol. 36, no. 1, pp. 171-179.

Hagege, H, Klous, P, Braem, C, Splinter, E, Dekker, J, Cathala, G, de Laat, W*, et al.* 2007a, 'Quantitative analysis of chromosome conformation capture assays (3C-qPCR)', *Nat Protoc*, vol. 2, no. 7, pp. 1722-1733.

Hamilton, TC, Young, RC, McKoy, WM, Grotzinger, KR, Green, JA, Chu, EW, Whang-Peng, J*, et al.* 1983, 'Characterization of a human ovarian carcinoma cell line (NIH:OVCAR-3) with androgen and estrogen receptors', *Cancer Res*, vol. 43, no. 11, Nov, pp. 5379-5389.

Hao, Y, Crenshaw, T, Moulton, T, Newcomb, E & Tycko, B 1993, 'Tumor-suppressor activity of H19 RNA', *Nature*, vol. 365, no. 6448, pp. 764-767.

Huang, Z & Murphy, SK 2013, 'Increased Intragenic IGF2 Methylation is Associated with Repression of Insulator Activity and Elevated Expression in Serous Ovarian Carcinoma', *Front Oncol*, vol. 3, p. 131.

Kent, WJ, Sugnet, CW, Furey, TS, Roskin, KM, Pringle, TH, Zahler, AM, Haussler*, et al.* 2002, 'The Human Genome Browser at UCSC', *Genome Research*, vol. 12, no. 6, June 1, 2002, pp. 996-1006.

Krueger, F & Andrews, SR 2011, 'Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications', *Bioinformatics*, vol. 27, no. 11, Jun 1, pp. 1571-1572.

Kulis, M & Esteller, M 2010, 'DNA methylation and cancer', *Adv Genet*, vol. 70, pp. 27-56.

Langdon, SP & Lawrie, SS 2001, 'Establishment of ovarian cancer cell lines', *Methods Mol Med*, vol. 39, pp. 155-159.

Leone, G, Teofili, L, Voso, MT & Lubbert, M 2002, 'DNA methylation and demethylating drugs in myelodysplastic syndromes and secondary leukemias', *Haematologica*, vol. 87, no. 12, Dec, pp. 1324-1341.

Li, H, Handsaker, B, Wysoker, A, Fennell, T, Ruan, J, Homer, N, Marth, G*, et al.* 2009a, 'The Sequence Alignment/Map format and SAMtools', *Bioinformatics*, vol. 25, no. 16, Aug 15, pp. 2078-2079.

Li, LC & Dahiya, R 2002, 'MethPrimer: designing primers for methylation PCRs', *Bioinformatics*, vol. 18, no. 11, Nov, pp. 1427-1431.

Li, T, Hu, J-F, Qiu, X, Ling, J, Chen, H, Wang, S, Hou, A*, et al.* 2008, 'CTCF regulates allelic expression of Igf2 by orchestrating a promoter-polycomb repressive complex 2 intrachromosomal loop', *Molecular and Cellular Biology*, vol. 28, no. 20, pp. 6473-6482.

Ling, JQ, Li, T, Hu, JF, Vu, TH, Chen, HL, Qiu, XW, Cherry, AM*, et al.* 2006, 'CTCF Mediates Interchromosomal Colocalization Between Igf2/H19 and Wsb1/Nf1', *Science*, vol. 312, no. 5771, pp. 269-272.

Lövkvist, C, Dodd, IB, Sneppen, K & Haerter, JO 2016, 'DNA methylation in human epigenomes depends on local topology of CpG sites', *Nucleic Acids Research*, February 29, 2016.

Mallona, I, Díez-Villanueva, A & Peinado, MA 2014, 'Methylation plotter: a web tool for dynamic visualization of DNA methylation data', *Source Code for Biology and Medicine*, vol. 9, no. 1, 2014//, p. 11.

Menendez, L, Walker, D, Matyunina, LV, Dickerson, EB, Bowen, NJ, Polavarapu, N, Benigno, BB*, et al.* 2007, 'Identification of candidate methylation-responsive genes in ovarian cancer', *Mol Cancer*, vol. 6, Jan 25, p. 10.

Merlo, A, Herman, JG, Mao, L, Lee, DJ, Gabrielson, E, Burger, PC, Baylin, SB*, et al.* 1995, '5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers', *Nat Med*, vol. 1, no. 7, Jul, pp. 686-692.

Ryan, D, Pederson, B, Triche Jr, T & Gruning, B 2016, 'PileOMeth 0.1.13', Zenodo, <http://doi.org/10.5281/zenodo.53211>.

Schmidt, D, Schwalie, PC, Wilson, MD, Ballester, B, Goncalves, A, Kutter, C, Brown, GD*, et al.* 2012, 'Waves of retrotransposon expansion remodel genome organization and CTCF binding in multiple mammalian lineages', *Cell*, vol. 148, no. 1-2, Jan 20, pp. 335-348.

Stadler, MB, Murr, R, Burger, L, Ivanek, R, Lienert, F, Scholer, A, van Nimwegen, E*, et al.* 2011, 'DNA-binding factors shape the mouse methylome at distal regulatory regions', *Nature*, vol. 480, no. 7378, Dec 14, pp. 490-495.

Tym, JE, Mitsopoulos, C, Coker, EA, Razaz, P, Schierz, AC, Antolin, AA & Al-Lazikani, B 2016, 'canSAR: an updated cancer research and drug discovery knowledgebase', *Nucleic Acids Research*, vol. 44, no. D1, January 4, 2016, pp. D938-D943.

Wang, H, Maurano, MT, Qu, H, Varley, KE, Gertz, J, Pauli, F, Lee, K*, et al.* 2012a, 'Widespread plasticity in CTCF occupancy linked to DNA methylation', *Genome Res*, vol. 22, no. 9, Sep, pp. 1680-1688.

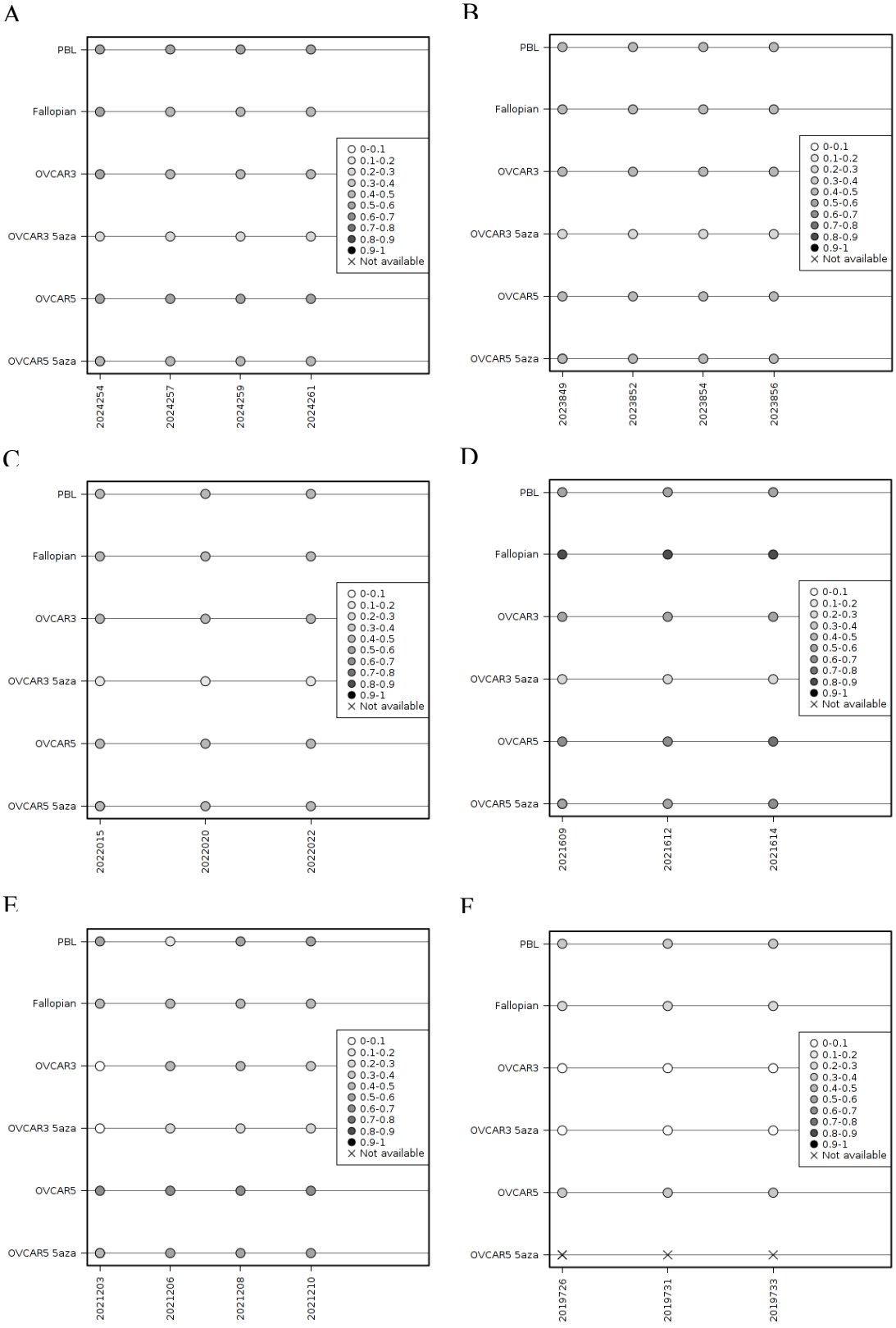
Figure legends for Manuscript: CTCF site methylation resistant to 5-aza-2-deoxycytidine (5aza) treatment in vitro

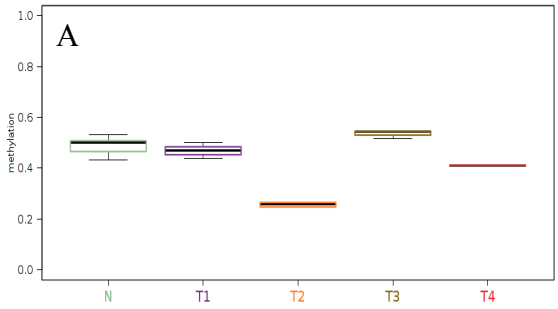
Figure 1: Methylation levels of individual CpG sites with the CTCF binding sites. **A**: CTCF binding site 1. **B**: CTCF binding site 2. **C**: CTCF binding site 4. **D**: CTCF binding site 5. **E:** CTCF binding site 6. **F:** CTCF binding site 7. Position on chromosome 11 is shown on the x axes.

Figure 2: Distribution of methylation values of CpGs within the CTCF binding sites. Single dots are used to represent data points considered outliers, the upper and lower bounds of the box represent the third and first quintiles respectively and the error bars describe the maximum and minimum. **A**: CTCF binding site 1. **B**: CTCF binding site 2. **C**: CTCF binding site 4. **D**: CTCF binding site 5. **E:** CTCF binding site 6. **F:** CTCF binding site 7. 'N' is the combination of values from normal fallopian tube and peripheral blood lymphocytes. 'T1' is OVCAR3, 'T2' is OVCAR3 treated with 5aza, 'T3' is OVCAR5 and 'T4' is OVCAR5 treated with 5aza.

Figure S1: DNA FISH dot assay of *Igf2/H19-Wsb1* interaction using fluorescently labelled BAC probes. *ActB-Wsb1* probe pair is used to estimate the random frequency of two probes interacting. All probes under normal conditions (lymphocytes) show two copies of each region.

Figures for Manuscript: CTCF site methylation resistant to 5-aza-2-deoxycytidine (5aza) treatment in vitro

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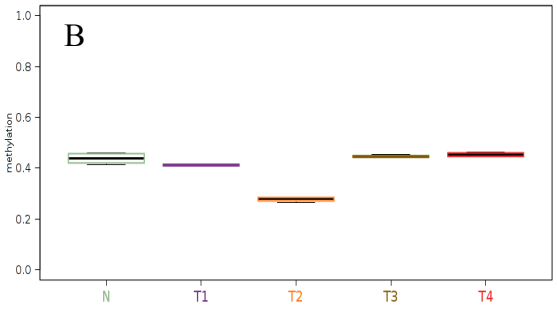
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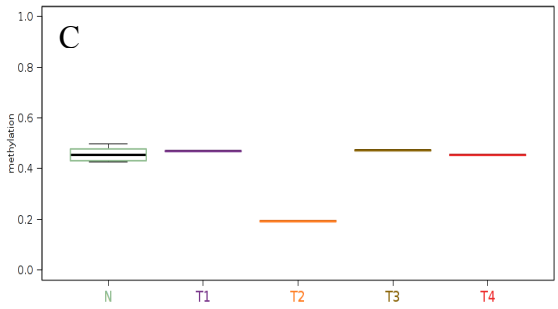
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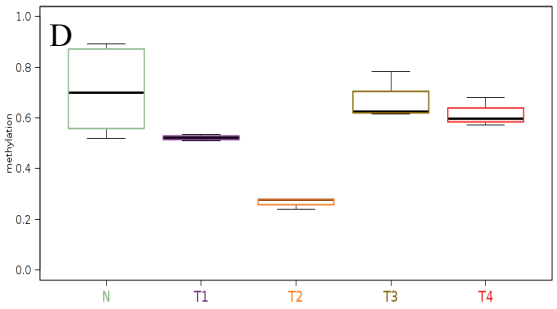
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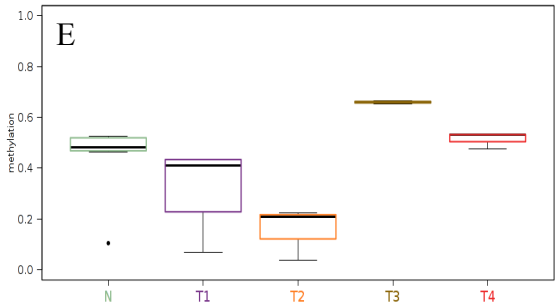
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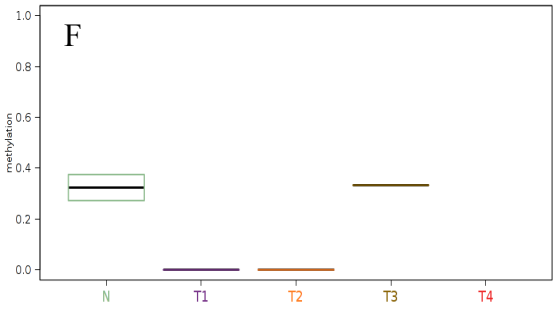
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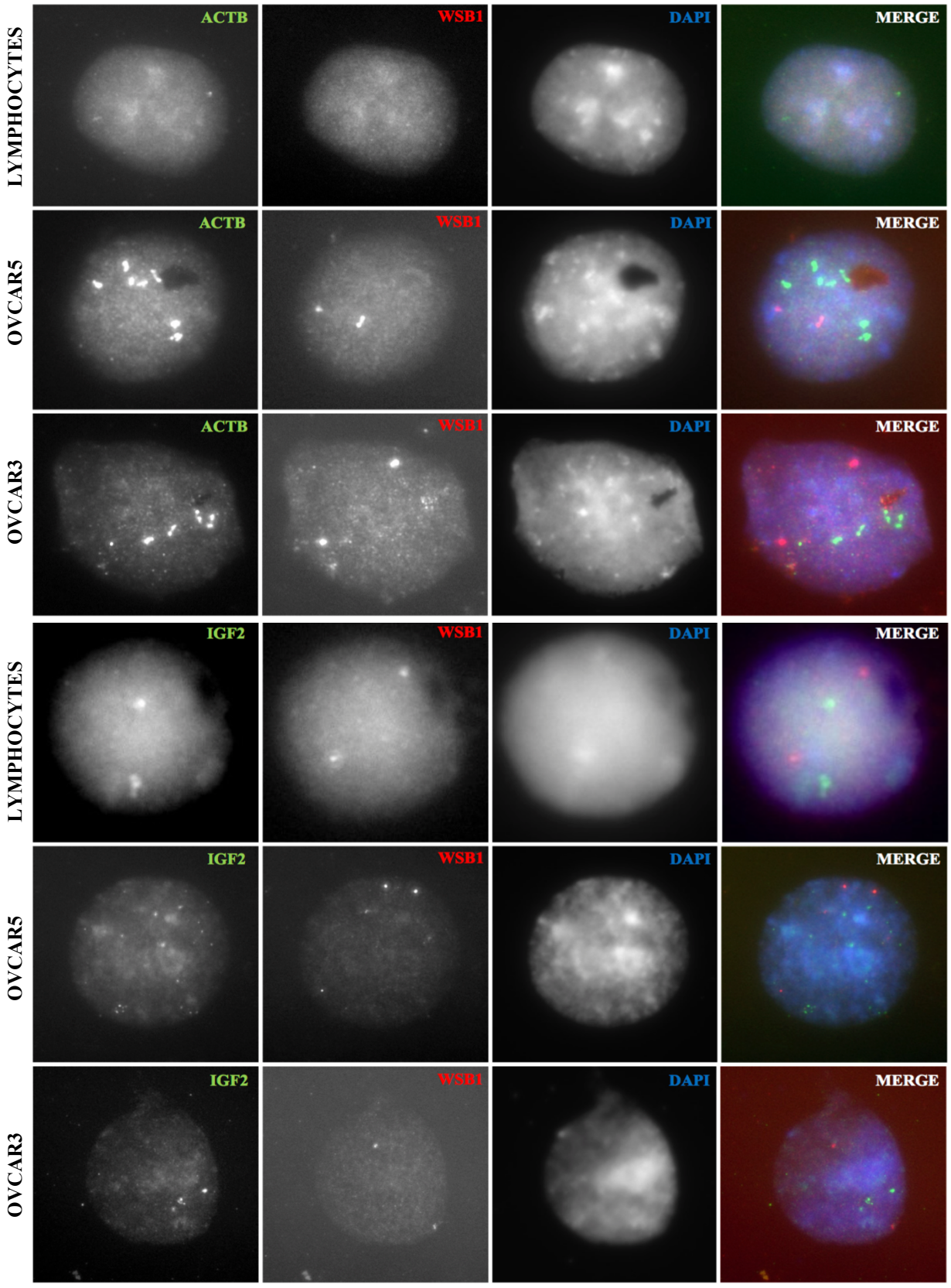
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WSB1
DAPI
MERGE
OVCAR5
ACTB
WSB1
DAPI
MERGE
OVCAR3
ACTB
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DAPI
MERGE
LYMPHOCYTES
IGF2
WSB1
DAPI
MERGE
OVCAR5
IGF2
WSB1
DAPI
MERGE
OVCAR3
IGF2
WSB1
DAPI
MERGE

Tables for Manuscript: CTCF site methylation resistant to 5-aza-2-deoxycytidine (5aza) treatment in vitro

Table 1: Details of primers and product sizes for each CTCF site for bisulphite treated gDNA.

Target	Forward primer (5'-3')	Reverse Primer (5'-3')	Product size (bp)
CTCF 1	TTTGTTGATTTTATTAAGGGAGGTT	TTCTATAAATAAACCCCAACCAAAC	147
CTCF 2	ATGTGTATTTTTGGAGGTTTTTTTT	AACTCCCATAAATATTCTATCCCTCA	223
CTCF 3	GTTTAAGTTTTTTTTGGATGGGG	CAAACCATAACACTAAAACCCTCA	190
CTCF 4	ATGAATATTTTTGGAGGTTTTTTTT	ATAAATATCCTATCCCTAATAACCCC	216
CTCF 5	ATGTGTATTTTTGGAGGTTTTTTTT	ACTCCCATAAATATCCTATACCTCAC	222
CTCF 6	TATGGGTATTTTTGGAGGTTTTTT	ACTCCCATAAATATCCTATTCCCA	224
CTCF 7	TTTTATTAAAGGTTAAGGTGGTGAT	CAAAACAAAATCCCCACAAC	254

Table 2: BAC clones used in DNA FISH experiments and region specific PCR primers.

BAC name	Target gene/region	Forward primer 5'-3'	Reverse primer 5'-3'
RP11-93G19	*ActB*	CAT GTA CGT TGC TAT CCA GGC	CTC CTT AAT GTC ACG CAC GAT
RP11-542J6	*Igf2/H19*	TAC CGC CCAT CTC CCT TCT CA	GGT GAG GGT CGT GCC AAT TA
RP11-458L21	*Wsb1/Nf1*	TGA ACC ATG CTG TCT GCT GT	TGA CAC CGA GCA CAG ACT TC

Table 3: Methylation differences for individual CpG residues between 5aza treated and non-treated OVCAR3, OVCAR5 and normal samples.

Sample	CTCF site	Estimate	Std. Error	z value	Pr(>|z|)	adjP	
Normal	5[#]	1.47	0.03	56.70	0	0	***
OVCAR3:pos6	5[#]	-0.40	0.05	-8.94	4.09E-19	9.40E-18	***
OVCAR5:pos6	5[#]	-0.91	0.05	-20.03	3.18E-89	7.30E-88	***
OVCAR3:pos9	5[#]	-0.61	0.05	-13.20	8.53E-40	1.96E-38	***
OVCAR5:pos9	5[#]	-1.17	0.05	-25.05	1.83E-138	4.22E-137	***
Normal	6[#]	-0.16	0.02	-9.95	2.51E-23	8.52E-22	***
OVCAR3:pos6	6[#]	0.21	0.07	3.14	0.001675	0.05694	
OVCAR5:pos6	6[#]	0.10	0.07	1.36	0.1745	1	
PBL:pos6	6[#]	-2.20	0.04	-55.91	0	0	***
OVCAR3:pos9	6[#]	-2.09	0.10	-20.30	1.29E-91	4.39E-90	***
OVCAR5:pos9	6[#]	-0.03	0.07	-0.38	0.7054	1	
PBL:pos9	6[#]	-0.01	0.03	-0.17	0.8644	1	

[#]Normal samples were not pooled and treated separately due to discrepancies between peripheral blood lymphocytes and fallopian tube tissue– cell lines compared only to fallopian tube. Estimate, Std Error and z value columns presented at 2dp. Bonferroni's method was used to obtain adjusted p-values. Site 7 is excluded due to insufficient read depth to perform analysis (Appendix A). Significant results, shown by asterisk, were determined as those with estimates ±0.3 different to normal samples.

Table 4: Average methylation differences for across CTCF binding sites between 5aza treated and non-treated OVCAR3, OVCAR5 and normal samples.

Sample	CTCF site	Estimate	Std. Error	z value	Pr(>|z|)	adjP	
Normal	1	0.05	0.10	0.48	0.6336	1	
OVCAR3	1	-0.19	0.17	-1.07	0.2826	1	
OVCAR5	1	0.35	0.18	1.99	0.04697	0.3758	
5azaTRUE	1	-0.85	0.17	-5.06	4.10E-07	3.28E-06	***
Normal	2	-0.21	0.01	-20.76	1.05E-95	3.14E-94	
OVCAR3	2	-0.14	0.02	-7.50	6.56E-14	1.97E-12	
OVCAR5	2	-0.03	0.02	-1.55	0.1208	1	
5azaTRUE	2	-0.04	0.01	-3.67	0.0002433	0.0073	
OVCAR3 5azaTRUE	2	-0.68	0.02	-37.69	0	0	***
Normal	4	-0.23	0.01	-25.95	1.78E-148	1.96E-147	
OVCAR3	4	0.04	0.01	2.46	0.01404	0.1544	
OVCAR5	4	0.09	0.01	6.40	1.61E-10	1.77E-09	
5azaTRUE	4	-0.08	0.03	-2.64	0.008283	0.09112	
OVCAR3 5azaTRUE	4	-1.15	0.04	-30.73	2.44E-207	2.68E-206	***
Normal	5[#]	1.47	0.03	56.70	0	0	***
OVCAR3	5[#]	-1.40	0.03	-43.48	0	0	***
OVCAR5	5[#]	-0.39	0.03	-12.25	1.65E-34	3.81E-33	***
5azaTRUE	5[#]	-0.24	0.02	-13.04	6.83E-39	1.57E-37	
OVCAR3 5azaTRUE	5[#]	-0.70	0.03	-27.35	1.23E-164	2.84E-163	***
Normal	6[#]	-0.16	0.02	-9.95	2.51E-23	8.52E-22	***
OVCAR3	6[#]	-0.27	0.05	-5.37	7.76E-08	2.64E-06	***
OVCAR5	6[#]	0.79	0.05	14.62	1.96E-48	6.67E-47	***
5azaTRUE	6[#]	-0.58	0.05	-11.69	1.41E-31	4.78E-30	***
OVCAR3 5azaTRUE	6[#]	-0.39	0.07	-5.52	3.48E-08	1.18E-06	***

[#]Normal samples were not pooled and treated separately due to discrepancies between peripheral blood lymphocytes and fallopian tube tissue– cell lines compared only to fallopian tube. Estimate, Std Error and z value columns presented at 2dp. Bonferroni's method was used to obtain adjusted p-values. Site 7 is excluded due to insufficient read depth to perform analysis (Appendix A). Significant results, shown by asterisk, were determined as those with estimates ±0.3 different to normal samples.

Table 5: Summary of number of signals and SD observed for each probe in the three cell types used.

	ACTB	WSB1	IGF2	N
Lymphocytes	2.05 ± 0.69	2.1 ± 0.45	2.1 ± 0.31	20
OVCAR3	4.3 ± 0.86	2.15 ± 0.67	2.55 ± 0.83	20
OVCAR5	4.6 ± 1.19	3.0 ± 1.17	4.1 ± 2.57	20

Table 6: Comparison of *IGF2, H19* and *WSB1* expression in OVCAR3 and OVCAR5 to fallopian tissue. Bonferroni's method is used to obtain adjusted p-value.

Gene	Cell Line	logFC	Std. Error	t	p	adjP	
H19	OVCAR3	-3.646	1.222	-2.985	0.006106	0.03663	*
H19	OVCAR5	-1.731	1.246	-1.39	0.1764	1	
Igf2	OVCAR3	-8.637	1.287	-6.71	4.045e-07	2.427e-06	***
Igf2	OVCAR5	-7.67	1.313	-5.842	3.7e-06	2.22e-05	***
Wsb1	OVCAR3	1.635	1.091	1.499	0.146	0.876	
Wsb1	OVCAR5	1.368	1.112	1.23	0.2297	1	

Table 7: Change in *IGF2, H19* and *WSB1* expression in OVCAR3 cells treated with 5aza. Bonferroni's method is used to obtain adjusted p-value.

Gene	logFC	Std.Err	t	p	adjP
H19	7.29	1.166	6.253	2.117e-05	6.35e-05
Igf2	5.207	1.205	4.32	0.0007063	0.002119
Wsb1	1.192	0.7894	1.51	0.1533	0.46

Chapter 5 - Conclusions

This chapter consists of a conventional thesis conclusion.

Chapter Overview

This chapter summarises my research and highlights key findings. It also discusses the significance of the work in the field and identifies future research directions.

Conclusions, Significance and Future Directions

Higher order chromatin organisation has been studied for decades, however is an area of epigenetics where the complexity and extent of its role in regulation is only now starting to be recognised. However little is understood the evolutionary trajectory and potential role in speciation and human disease. Our research looked to improve our understanding of long-range chromatin interactions by investigating established interactions involving the well-known *IGF2/H19* region in both evolutionary and disease contexts.

Epigenetic changes affecting gene expression, genomic imprinting or hybrid phenotypes have long fascinated scientists. Cattle hybrids offer an excellent system to investigate this as hybrid over-growth phenotypes are implicated to result from aberrant imprinting regulation. This work has contributed new information on the variability of *IGF2/H19* expression and regulation in cattle hybrids. First, we established that the *IGF2/H19-WSB1* interaction that was originally observed in mice is conserved in bovine species and if any differences occur between different breeds. Using cattle embryonic fibroblasts of both *B. p. indicus* and *B. p. taurus* purebreds and reciprocal hybrids, we found that although these long-range interactions still occur in these hybrid animals, there is no difference in the frequency of the interactions, which appear to be individually variable. Interestingly this did not appear to affect the expression of these genes, where we identified higher expression of *H19* in *B. p. indicus* purebreds compared to *B. p. taurus* purebreds or hybrids, and higher expression of *IGF2* in purebreds compared to hybrids. These results suggest that in this system, mechanisms other than long range interactions are modulating the expression of these genes, or at least the effect of these interactions are minor compared to other factors. Furthermore, we observed no difference in *IGF2* or *H19* expression between the hybrids at this embryonic stage. As there is an established birthweight difference between reciprocal hybrids in this bovine system, we expected some early molecular signatures of this growth difference. However, there appears to be no change at this developmental stage, suggesting that the growth difference becomes apparent later. As *IGF2* strongly influences foetal growth and *H19* is believed to be involved in myogenesis, it is possible that the impact of these genes will be observed at different stages. Future research in this area should investigate the methylation patterns of CTCF binding sites and the sequence of the binding sites themselves to identify any other changes that might indicate differences in the embryonic stage. It would also be interesting to confirm the presence of other important features used in *IGF2/H19* regulation in the bovine system, such as the enhancer elements downstream of *H19,* which may explain the differences in expression we observed. It would be ideal to extend these

experiments into a range of embryonic tissues, to overcome some of the limitations of using cultured cell lines.

The *IGF2/H19* region is frequently affected in cancer, but few studies have investigated in detail how the CTCF sites of the ICR are affected. To investigate this DNA methylation, long-range interactions and gene expression in this region was analysed in ovarian cancer. We examined both primary tumours and cell lines, each of which had unique advantages. The OVCAR3 and OVCAR5 cell lines were able to be treated in culture with demethylating drugs, and enabled us to perform DNA FISH to investigate interaction frequency. The use of primary tumours allowed us to investigate DNA methylation patterns in pre-treatment disease with cells unaffected by culture conditions.

With access to primary ovarian serous tumours, and by doing targeted amplicon bisulphite sequencing, we were able to investigate the pattern of methylation in the *H19* ICR. Previous research had selected a few binding sites within the ICR to sample, however we aimed to observed all seven binding sites. Of the sites we successfully analysed, we showed that methylation patterns did not correlate with early or late tumour stage, suggesting that methylation changes to the ICR are individually variable. Overall, our results show that the patterns of hypo- or hypermethylation observed in ovarian cancers are more complex, and there is likely to be high variability even within small regions such as the ICR. Others have suggested similar patterns, with opposite methylation patterns between the *IGF2* DMR and the sixth CTCF binding site (Dammann *et al.* 2010), however we have shown this occurs within a much smaller span of DNA than previously thought. Future research would ideally expand the scope of this experiment, looking at far more tumours from a larger range of stages, and whether the methylation patterns changed between pre-treatment and post-chemotherapy. It would also be worthwhile to investigate whether any of these methylation patterns could be used to predict resistance to platinum-based chemotherapy, where the efficacy may be affected by DNA methylation.

We also investigated the efficacy of 5aza in removing methylation in the *H19* ICR in the OVCAR3 and OVCAR5 ovarian cancer cell lines, and the impact of this on gene expression and long range interactions. We successfully bisulphite sequenced five of the CTCF binding sites in the ICR, and showed that 5aza treatment strongly reduced methylation, albeit only at some sites and in some cases, differently between the two cell lines. This work highlights the inconsistent effects of 5aza treatment, even within a small region of DNA. There is also

the possibility that some of these binding sites are more resistant to demethylation than others, something that could be investigated in future work. We were unable to estimate the interaction frequency in these cells using DNA FISH, due to the variability in the number of signals we observed within the cell populations. This variability makes it difficult to identify specific interactions rather than coincidentally overlapping signals. There may be some evidence that interactions are decreased or negatively affected by the increase in copies of *IGF2*. We believe that a quantitative 3C approach may be better suited to estimate interaction frequency in this case. We also investigated expression differences between the two cell lines and fallopian tube. Interestingly, the cell lines had equivalent *β-Actin* and *WSB1* expression to fallopian tube, however both cell lines had significantly lower *IGF2* and *H19* expression. Treatment of OVCAR3 with 5aza increased expression of *H19*, supporting the idea that 5aza can be used to reverse promoter hyper-methylation of tumour suppressors, however there was also an increase in *IGF2* expression. As *IGF2* overexpression correlates with poor prognosis in ovarian cancer, it is an unfortunate side-effect that use of 5aza appears to increase its expression. Further research could look into the effects of 5aza in a larger range cell lines, focussing on the CTCF sites we identified as being differently impacted.

Overall this project has furthered our understanding of the dynamics of the factors involved in long-range interactions. We describe evidence that interactions at *IGF2/H19* are not affected by hybridisation in bovine offspring, and how interactions may not reflect expression changes in *IGF2* and *H19* as predicted. We also show that in ovarian cancer tumours there is differential methylation of individual CTCF binding sites, rather than consistent changes across the *IGF2/H19* ICR. Furthermore, we show that these sites respond differently to 5aza treatment in ovarian cancer cell lines, suggesting some sites could be resistant to demethylating treatment.

Appendix A

Identification of CTCF binding sites in the human *H19* ICR

Using the UCSC genome browser (Kent *et al.* 2002) and the GRCh37/*hg19* assembly, we selected a 15kb region of DNA upstream of and including the *H19* gene. Using MethPrimer (Li & Dahiya 2002), we predicted CpG islands to determine regions of potential methylation differentiation. Using the general consensus motif for CTCF binding sites (Schmidt *et al.* 2012), we identified seven CTCF binding sites, all of which occurred within regions deemed CpG islands. Table S1 outlines the location and sequence of each of the binding sites identified.

Table S1: Chromosomal position and motif of CTCF sites in the *H19* ICR in humans.

Target site	Position	Motif
CTCF1	chr11:2,024,249-2,024,262	CCGCGCGGCGGCAG
CTCF2	chr11:2,023,844-2,023,857	CCGCGCGGCGGCAG
CTCF3	chr11:2,023,443-2,023,456	CCGCGCGGCGGCAG
CTCF4	chr11:2,022,010-2,022,023	CCGCGTGGCGGCAG
CTCF5	chr11:2,021,604-2,021,617	CTGCGCGGCGGCAG
CTCF6	chr11:2,020,198-2,020,211	CCGCGCGGCGGCAG
CTCF7	chr11:2,019,721-2,019,734	CCGCGAGCCGTAAG

Read depth of bisulphite sequencing correctly aligned to each CTCF binding site

Due to the reduced sequence complexity of bisuphite treated DNA, and the repetitive nature of the ICR, we expected that there may be some overlap in which CTCF sites would be covered by any given amplicon. As can be seen in Figure S1, the majority of reads mapped correctly to the targeted site. CTCF site 7 had considerably lower read depth than the other sites (Table S2), which had to be taken into account in the statistical modelling to prevent false positive results. Later research should try to improve the coverage of this site, as well as CTCF site 3, where the amplicon narrowly missed the target site. The estimates of methylation levels at each CTCF site are only calculated from the reads that correctly mapped to that site, ignored misaligned sequences.

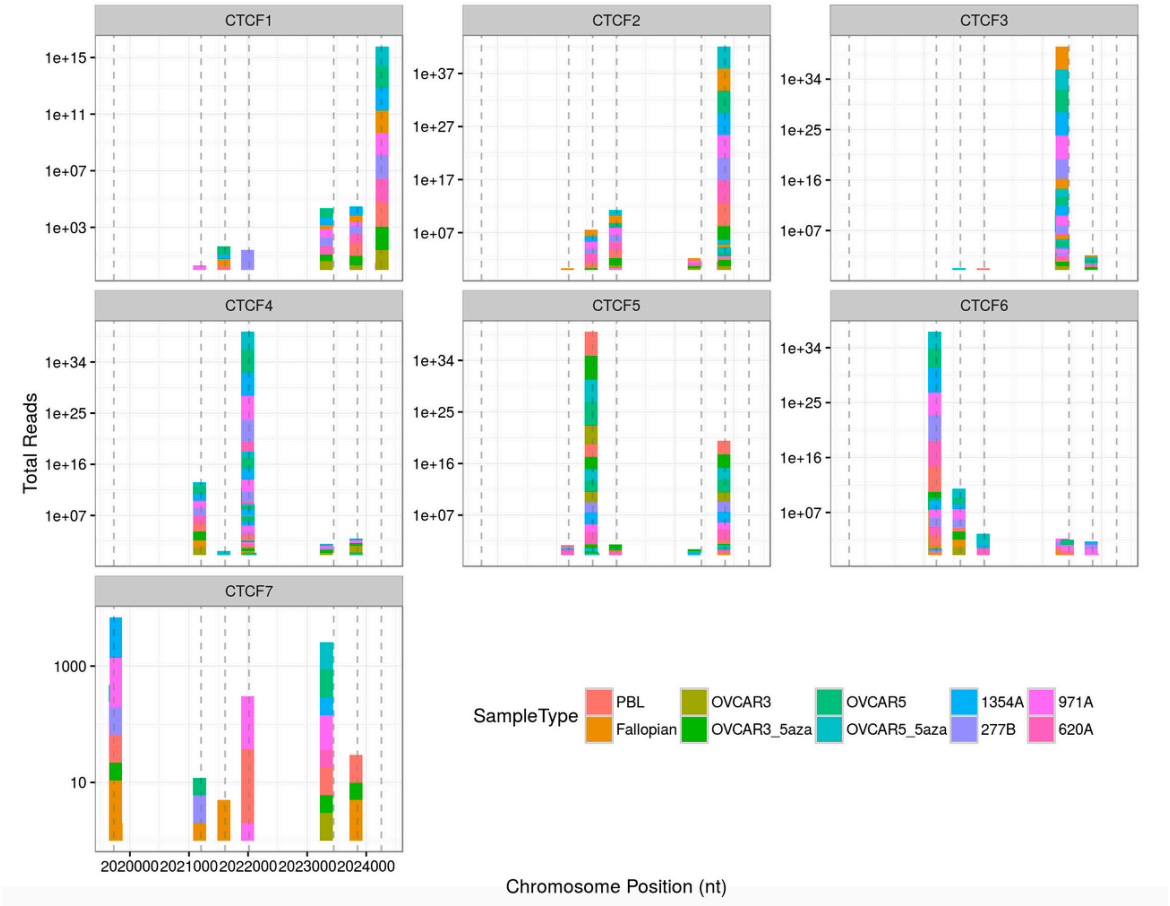

Figure S1: Alignment of reads to each CTCF site by sample. Figure produced by Stephen Pederson.

Table S2: Raw read counts for CTCF site 7.

Sample	Group	Position	Unmethylated	Methylated	Percent methylation
PBL	Normal	P2	5	3	38%
PBL	Normal	P4	5	3	38%
PBL	Normal	P9	5	3	38%
Fallopian	Normal	P2	19	7	27%
Fallopian	Normal	P4	18	7	28%
Fallopian	Normal	P9	18	7	28%
OVCAR3	Cell-Line	P2	4	0	0%
OVCAR3	Cell-Line	P4	4	0	0%
OVCAR3	Cell-Line	P9	4	0	0%
OVCAR3_5aza	Cell-Line	P2	3	0	0%
OVCAR3_5aza	Cell-Line	P4	3	0	0%
OVCAR3_5aza	Cell-Line	P9	3	0	0%
OVCAR5	Cell-Line	P2	4	2	33%
OVCAR5	Cell-Line	P4	4	2	33%
OVCAR5	Cell-Line	P9	4	2	33%
1354A	Tumour	P2	7	1	12%
1354A	Tumour	P4	6	2	25%
1354A	Tumour	P9	7	1	12%
277B	Tumour	P2	0	5	100%
277B	Tumour	P4	0	5	100%
277B	Tumour	P9	0	5	100%
971A	Tumour	P2	6	7	54%
971A	Tumour	P4	4	9	69%
971A	Tumour	P9	7	6	46%
620A	Tumour	P2	5	3	38%
620A	Tumour	P4	5	3	38%
620A	Tumour	P9	5	3	38%

Expression of *IGF2, H19, WSB1* and *ACTB* in primary ovarian serous tumours

Due to technical issues, the results of the qPCR experiment were not included in the manuscript of chapter 3 of this thesis, however the details have been included here. Each of the tumours had RNA extracted and tested for concentration and quality using a spectrometer and gel electrophoresis. The RNA was used in cDNA synthesis using the BioRad iScript synthesis kit. These samples were included on qPCR plates together with normal ovary and fallopian RNA from Agilent, as well as ovarian cancer cell line samples referred to in chapter 4 of this thesis. Unexpectedly, there was amplification in the –RT controls for nearly all tumour samples (numerical IDs) with every primer set except *H19*, where the values were borderline (Figure S2). This could be due to gDNA contamination, as it is unlikely that the primers were unsuitable as evidenced by the other successful reactions. However, there was extensive troubleshooting of other primer sets against the same target transcripts in these samples, as many returned multiple melt curves suggesting more than one target was being detected. This experiment will need to be repeated before the results can be included in the paper manuscript, however due to time constraints, was not possible to do before submitting the thesis.

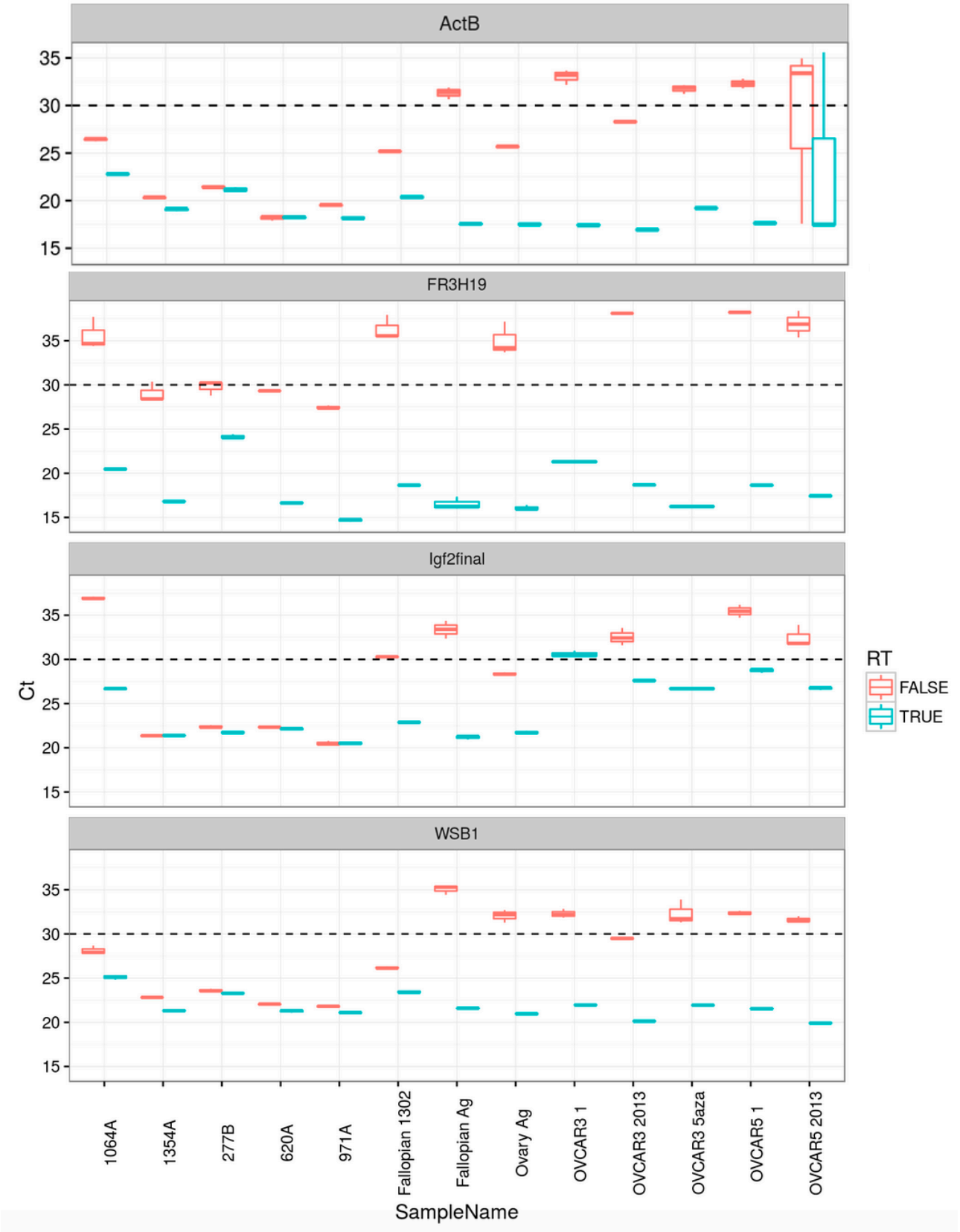

Figure S2: Results of qPCR experiments using multiple primer sets, *ACTB, H19, IGF2* and *WSB1* on tumour material. Figure produced by Stephen Pederson.

Estimating interaction frequency in OVCAR3 and OVCAR5 using DNA FISH

As described in Chapter 4, we experienced difficulties assessing the frequency of interaction between the *IGF2/H19* and *WSB1/NF1* regions due to variability in the number of signals we observed. This technique relies on a control interaction frequency, in this case between *ACTB* and *WSB1*, to estimate the random chance that two signals will overlap when the regions are not know to interact otherwise. However, as we observed very high signal counts, particularly for *ACTB* and *IGF2* in OVCAR5, we were unable to tell background, random interactions from true interactions. We have suggested a molecular technique such as 3C or the associated chromosome trap (ACT) technique may be better suited than FISH to determine the interaction frequency.

Table S3: Recorded interactions and signal numbers for *ACTB* and *WSB1* from DNA FISH experiments.

Samples	Cell count	Interaction (1=yes, 0=no)	No of ACTB signals	No of WSB1 signals
Lymphocytes	1	0	2	2
	2	0	2	0
	3	0	1	2
	4	0	2	1
	5	0	2	0
	6	0	1	1
	7	0	3	2
	8	0	2	2
	9	0	2	2
	10	0	2	2
	11	0	1	2
	12	0	2	0
	13	0	2	0
	14	0	3	0
	15	0	2	4
	16	0	2	2
	17	0	4	2
	18	0	2	2
	19	0	2	2
	20	0	2	1
OVCAR3	1	0	7	3
	2	0	4	2
	3	0	6	2
	4	0	4	2
	5	0	4	3
	6	0	4	2
	7	0	4	2
	8	0	4	2
	9	0	3	2
	10	0	4	2
	11	0	4	2

	12	0	4	3
	13	0	4	2
	14	0	4	2
	15	0	4	2
	16	0	4	2
	17	0	4	3
	18	0	5	2
	19	0	5	2
	20	0	4	2
OVCAR5	1	0	4	2
	2	0	4	3
	3	0	4	4
	4	0	4	2
	5	0	7	2
	6	0	4	2
	7	0	4	3
	8	0	7	2
	9	0	3	2
	10	0	3	3
	11	0	4	2
	12	0	4	2
	13	0	6	2
	14	0	5	2
	15	0	4	3
	16	0	6	3
	17	0	4	3
	18	0	5	5
	19	1	6	2
	20	0	4	4

Table S4: Recorded interactions and signal numbers for *IGF2* and *WSB1* from DNA FISH experiments.

Samples	Cell count	Interaction (1=yes, 0=no)	No of IGF2 signals	No of WSB1 signals
Lymphocytes	1	0	2	2
	2	0	2	2
	3	0	2	2
	4	0	2	2
	5	0	2	3
	6	0	2	2
	7	0	2	2
	8	0	2	2
	9	0	2	2
	10	0	2	2
	11	0	2	2
	12	0	2	2
	13	0	3	3
	14	0	2	2
	15	0	2	2
	16	0	2	2
	17	0	2	1
	18	0	2	2
	19	0	3	3
	20	0	2	2
OVCAR3	1	0	3	2
	2	0	2	2
	3	0	2	2
	4	0	2	2
	5	0	2	2
	6	0	4	3
	7	0	2	4
	8	0	4	2
	9	0	4	2
	10	0	4	2
	11	0	3	2
	12	0	2	2
	13	0	2	1
	14	0	2	2
	15	0	2	2
	16	0	2	2
	17	0	2	2
	18	0	2	3
	19	0	3	1
	20	0	2	3
OVCAR5	1	0	10	4

2	0	5	3
3	0	2	2
4	0	2	2
5	0	2	2
6	1	5	3
7	0	2	2
8	0	11	2
9	0	2	6
10	0	2	3
11	0	4	3
12	0	3	2
13	0	3	2
14	0	3	4
15	0	4	3
16	0	4	4
17	0	3	5
18	0	5	2
19	0	3	2
20	0	7	4

Full Reference List

Anand-Ivell, R, Hiendleder, S, Viñoles, C, Martin, GB, Fitzsimmons, C, Eurich, A, Hafen, B*, et al.* 2011, 'INSL3 in the Ruminant: A Powerful Indicator of Gender- and Genetic-Specific Feto-Maternal Dialogue', *PLoS ONE*, vol. 6, no. 5, p. e19821.

Apostolou, E, Ferrari, F, Walsh, RM, Bar-Nur, O, Stadtfeld, M, Cheloufi, S, Stuart, HT*, et al.* 2013, 'Genome-wide chromatin interactions of the Nanog locus in pluripotency, differentiation, and reprogramming', *Cell Stem Cell*, vol. 12, no. 6, Jun 6, pp. 699-712.

Auersperg, N 2013, 'The origin of ovarian cancers--hypotheses and controversies', *Front Biosci (Schol Ed)*, vol. 5, pp. 709-719.

Australian Institute of Health & Welfare 2010, 'Ovarian cancer in Australia: an overview, 2010', *Cancer Series*, no. 52.

Bartolomei, MS & Ferguson-Smith, AC 2011, 'Mammalian Genomic Imprinting', *Cold Spring Harbor Perspectives in Biology*, vol. 3, no. 7, July 1, 2011.

Bast, RC, Jr., Hennessy, B & Mills, GB 2009, 'The biology of ovarian cancer: new opportunities for translation', *Nat Rev Cancer*, vol. 9, no. 6, Jun, pp. 415-428.

Bebbere, D, Bauersachs, S, Fürst, RW, Reichenbach, H-D, Reichenbach, M, Medugorac, I, Ulbrich, SE*, et al.* 2013, 'Tissue-Specific and Minor Inter-Individual Variation in Imprinting of <italic>IGF2R</italic> Is a Common Feature of <italic>Bos taurus</italic> Concepti and Not Correlated with Fetal Weight', *PLoS ONE*, vol. 8, no. 4, p. e59564.

Bell, AC, West, AG & Felsenfeld, G 1999, 'The Protein CTCF Is Required for the Enhancer Blocking Activity of Vertebrate Insulators', *Cell*, vol. 98, no. 3, 8/6/, pp. 387-396.

Berry, NB & Bapat, SA 2008, 'Ovarian cancer plasticity and epigenomics in the acquisition of a stem-like phenotype', *J Ovarian Res*, vol. 1, p. 8.

Bickmore, WA 2013, 'The spatial organization of the human genome', *Annu Rev Genomics Hum Genet*, vol. 14, pp. 67-84.

Botta, M, Haider, S, Leung, IXY, Lio, P & Mozziconacci, J 2010, 'Intra- and inter-chromosomal interactions correlate with CTCF binding genome wide', *Mol Syst Biol*, vol. 6, no. 426.

Branco, MR & Pombo, A 2006, 'Intermingling of Chromosome Territories in Interphase Suggests Role in Translocations and Transcription-Dependent Associations', *PLoS Biol*, vol. 4, no. 5, p. e138.

Brown, KW, Villar, AJ, Bickmore, W, Clayton-Smith, J, Catchpoole, D, Maher, ER & Reik, W 1996, 'Imprinting mutation in the Beckwith-Wiedemann syndrome leads to biallelic IGF2 expression through an H19-independent pathway', *Hum Mol Genet*, vol. 5, no. 12, Dec, pp. 2027-2032.

Brown, MA, Tharel, LM, Brown, AH, Jr., Jackson, WG & Miesner, JR 1993, 'Genotype x environment interactions in preweaning traits of purebred and reciprocal cross Angus and Brahman calves on common bermudagrass and endophyte-infected tall fescue pastures', *J Anim Sci*, vol. 71, no. 2, Feb, pp. 326-333.

Buiting, K, Saitoh, S, Gross, S, Dittrich, B, Schwartz, S, Nicholls, RD & Horsthemke, B 1995, 'Inherited microdeletions in the Angelman and Prader-Willi syndromes define an imprinting centre on human chromosome 15', *Nat Genet*, vol. 9, no. 4, 04//print, pp. 395-400.

Chambers, EV, Bickmore, WA & Semple, CA 2013, 'Divergence of Mammalian Higher Order Chromatin Structure Is Associated with Developmental Loci', *PLoS Comput Biol*, vol. 9, no. 4, p. e1003017.

Chaumeil, J & Skok, JA 2012, 'The role of CTCF in regulating V(D)J recombination', *Curr Opin Immunol*, vol. 24, no. 2, Apr, pp. 153-159.

Chen, CL, Ip, SM, Cheng, D, Wong, LC & Ngan, HYS 2000, 'Loss of imprinting of the IGF-II and H19 genes in epithelial ovarian cancer', *Clinical Cancer Research*, vol. 6, no. 2, pp. 474-479.

Chen, Z, Hagen, DE, Elsik, CG, Ji, T, Morris, CJ, Moon, LE & Rivera, RM 2015, 'Characterization of global loss of imprinting in fetal overgrowth syndrome induced by assisted reproduction', *Proc Natl Acad Sci U S A*, vol. 112, no. 15, Apr 14, pp. 4618-4623.

Cheng, YW, Idrees, K, Shattock, R, Khan, SA, Zeng, ZS, Brennan, CW, Paty, P*, et al.* 2010, 'Loss of imprinting and marked gene elevation are 2 forms of aberrant IGF2 expression in colorectal cancer', *International Journal of Cancer*, vol. 127, no. 3, pp. 568-577.

Chess, A, Simon, I, Cedar, H & Axel, R 1994, 'Allelic inactivation regulates olfactory receptor gene expression', *Cell*, vol. 78, no. 5, Sep 9, pp. 823-834.

Christman, JK 2002, '5-Azacytidine and 5-aza-2'-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy', *Oncogene*, vol. 21, no. 35, Aug 12, pp. 5483-5495.

Constancia, M, Hemberger, M, Hughes, J, Dean, W, Ferguson-Smith, A, Fundele, R, Stewart, F*, et al.* 2002, 'Placental-specific IGF-II is a major modulator of placental and fetal growth', *Nature*, vol. 417, no. 6892, pp. 945-948.

Cremer, T & Cremer, M 2010, 'Chromosome territories', *Cold Spring Harb Perspect Biol*, vol. 2, no. 3, Mar, p. a003889.

Cui, H, Niemitz, EL, Ravenel, JD, Onyango, P, Brandenburg, SA, Lobanenkov, VV & Feinberg, AP 2001, 'Loss of imprinting of insulin-like growth factor-II in Wilms' tumor commonly involves altered methylation but not mutations of CTCF or its binding site', *Cancer Res*, vol. 61, no. 13, Jul 1, pp. 4947-4950.

Cui, HM, Onyango, P, Brandenburg, S, Wu, YQ, Hsieh, CL & Feinberg, AP 2002, 'Loss of imprinting in colorectal cancer linked to hypomethylation of H19 and IGF2', *Cancer Research*, vol. 62, no. 22, pp. 6442-6446.

Dammann, RH, Kirsch, S, Schagdarsurengin, U, Dansranjavin, T, Gradhand, E, Schmitt, WD & Hauptmann, S 2010, 'Frequent aberrant methylation of the imprinted IGF2/H19 locus and LINE1 hypomethylation in ovarian carcinoma', *International Journal of Oncology*, vol. 36, no. 1, pp. 171-179.

Dawson, WD 1965, 'Fertility and Size Inheritance in a Peromyscus Species Cross', *Evolution*, vol. 19, no. 1, pp. 44-55.

Deligianni, C & Spilianakis, CG 2012, 'Long-range genomic interactions epigenetically regulate the expression of a cytokine receptor', *EMBO Rep*, vol. 13, no. 9, pp. 819-826.

Dindot, SV, Farin, PW, Farin, CE, Romano, J, Walker, S, Long, C & Piedrahita, JA 2004, 'Epigenetic and Genomic Imprinting Analysis in Nuclear Transfer Derived Bos gaurus/Bos taurus Hybrid Fetuses', *Biology of Reproduction*, vol. 71, no. 2, August 1, 2004, pp. 470-478.

Dixon, JR, Selvaraj, S, Yue, F, Kim, A, Li, Y, Shen, Y, Hu, M*, et al.* 2012, 'Topological domains in mammalian genomes identified by analysis of chromatin interactions', *Nature*, vol. 485, no. 7398, pp. 376-380.

Dunzinger, U, Nanda, I, Schmid, M, Haaf, T & Zechner, U 2005, 'Chicken orthologues of mammalian imprinted genes are clustered on macrochromosomes and replicate asynchronously', *Trends Genet*, vol. 21, no. 9, pp. 488-492.

Edwards, CA & Ferguson-Smith, AC 2007, 'Mechanisms regulating imprinted genes in clusters', *Curr Opin Cell Biol*, vol. 19, no. 3, 6//, pp. 281-289.

Essien, K, Vigneau, S, Apreleva, S, Singh, LN, Bartolomei, MS & Hannenhalli, S 2009, 'CTCF binding site classes exhibit distinct evolutionary, genomic, epigenomic and transcriptomic features', *Genome Biology*, vol. 10, no. 11.

Esteller, M 2002, 'CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future', *Oncogene*, vol. 21, no. 35, Aug 12, pp. 5427-5440.

Ezkurdia, I, Juan, D, Rodriguez, JM, Frankish, A, Diekhans, M, Harrow, J, Vazquez, J*, et al.* 2014, 'Multiple evidence strands suggest that there may be as few as 19,000 human protein-coding genes', *Hum Mol Genet*, vol. 23, no. 22, Nov 15, pp. 5866-5878.

Fang, P, Lev-Lehman, E, Tsai, T-F, Matsuura, T, Benton, CS, Sutcliffe, JS, Christian, SL*, et al.* 1999, 'The Spectrum of Mutations in UBE3ACausing Angelman Syndrome', *Human Molecular Genetics*, vol. 8, no. 1, January 1, 1999, pp. 129-135.

Feeley, KM & Wells, M 2001, 'Precursor lesions of ovarian epithelial malignancy', *Histopathology*, vol. 38, no. 2, pp. 87-95.

Filippova, GN, Cheng, MK, Moore, JM, Truong, J-P, Hu, YJ, Di Kim, N, Tsuchiya, KD*, et al.* 2005, 'Boundaries between Chromosomal Domains of X Inactivation and Escape Bind CTCF and Lack CpG Methylation during Early Development', *Developmental Cell*, vol. 8, no. 1, pp. 31-42.

Flesken-Nikitin, A, Hwang, C-I, Cheng, C-Y, Michurina, TV, Enikolopov, G & Nikitin, AY 2013, 'Ovarian surface epithelium at the junction area contains a cancer-prone stem cell niche', *Nature*, vol. 495, pp. 241-245.

Fuss, SH, Omura, M & Mombaerts, P 2007, 'Local and cis effects of the H element on expression of odorant receptor genes in mouse', *Cell*, vol. 130, no. 2, Jul 27, pp. 373-384.

Gebert, C, Wrenzycki, C, Herrmann, D, Gröger, D, Reinhardt, R, Hajkova, P, Lucas-Hahn, A*, et al.* 2006, 'The bovine IGF2 gene is differentially methylated in oocyte and sperm DNA', *Genomics*, vol. 88, no. 2, 8//, pp. 222-229.

Glusman, G, Yanai, I, Rubin, I & Lancet, D 2001, 'The complete human olfactory subgenome', *Genome Res*, vol. 11, no. 5, May, pp. 685-702.

Goodall, JJ & Schmutz, SM 2007, 'IGF2 gene characterization and association with rib eye area in beef cattle', *Animal Genetics*, vol. 38, no. 2, pp. 154-161.

Gribnau, J, Hochedlinger, K, Hata, K, Li, E & Jaenisch, R 2003, 'Asynchronous replication timing of imprinted loci is independent of DNA methylation, but consistent with differential subnuclear localization', *Genes Dev*, vol. 17, no. 6, Mar 15, pp. 759-773.

Guelen, L, Pagie, L, Brasset, E, Meuleman, W, Faza, MB, Talhout, W, Eussen, BH*, et al.* 2008, 'Domain organization of human chromosomes revealed by mapping of nuclear lamina interactions', *Nature*, vol. 453, no. 7197, 06/12/print, pp. 948-951.

Gyorffy, B, Lanczky, A & Szallasi, Z 2012, 'Implementing an online tool for genome-wide validation of survival-associated biomarkers in ovarian-cancer using microarray data from 1287 patients', *Endocr Relat Cancer*, vol. 19, no. 2, Apr, pp. 197-208.

Hagege, H, Klous, P, Braem, C, Splinter, E, Dekker, J, Cathala, G, de Laat, W*, et al.* 2007a, 'Quantitative analysis of chromosome conformation capture assays (3C-qPCR)', *Nat Protoc*, vol. 2, no. 7, pp. 1722-1733.

Hagege, H, Klous, P, Braem, C, Splinter, E, Dekker, J, Cathala, G, de Laat, W*, et al.* 2007b, 'Quantitative analysis of chromosome conformation capture assays (3C-qPCR)', *Nat. Protocols*, vol. 2, no. 7, pp. 1722-1733.

Haldane, JBS 1922, 'Sex ratio and unisexual sterility in hybrid animals', *Journal of Genetics*, vol. 12, no. 2, 1922//, pp. 101-109.

Hamilton, TC, Young, RC, McKoy, WM, Grotzinger, KR, Green, JA, Chu, EW, Whang-Peng, J*, et al.* 1983, 'Characterization of a human ovarian carcinoma cell line (NIH:OVCAR-3) with androgen and estrogen receptors', *Cancer Res*, vol. 43, no. 11, Nov, pp. 5379-5389.

Handoko, L, Xu, H, Li, G, Ngan, CY, Chew, E, Schnapp, M, Lee, CWH*, et al.* 2011, 'CTCF-mediated functional chromatin interactome in pluripotent cells', *Nature Genetics*, vol. 43, no. 7, pp. 630-638.

Hao, Y, Crenshaw, T, Moulton, T, Newcomb, E & Tycko, B 1993, 'Tumor-suppressor activity of H19 RNA', *Nature*, vol. 365, no. 6448, pp. 764-767.

Hark, AT, Schoenherr, CJ, Katz, DJ, Ingram, RS, Levorse, JM & Tilghman, SM 2000, 'CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus', *Nature*, vol. 405, no. 6785, May 25, pp. 486-489.

Hatada, I, Ohashi, H, Fukushima, Y, Kaneko, Y, Inoue, M, Komoto, Y, Okada, A*, et al.* 1996, 'An imprinted gene p57KIP2 is mutated in Beckwith-Wiedemann syndrome', *Nat Genet*, vol. 14, no. 2, Oct, pp. 171-173.

Hedborg, F, Holmgren, L, Sandstedt, B & Ohlsson, R 1994, 'The cell type-specific IGF2 expression during early human development correlates to the pattern of overgrowth and neoplasia in the Beckwith-Wiedemann syndrome', *Am J Pathol*, vol. 145, no. 4, pp. 802-817.

Hiendleder, S, Lewalski, H & Janke, A 2008, 'Complete mitochondrial genomes of Bos taurus and Bos indicus provide new insights into intra-species variation, taxonomy and domestication', *Cytogenet Genome Res*, vol. 120, no. 1-2, pp. 150-156.

Hiura, H, Okae, H, Kobayash, H, Miyauchi, N, Sato, F, Sato, A, Suzuki, F*, et al.* 2012, 'High-throughput detection of aberrant imprint methylation in the ovarian cancer by the bisulphite PCR-Luminex method', *Bmc Medical Genomics*, vol. 5, Mar.

Hou, C, Dale, R & Dean, A 2010, 'Cell type specificity of chromatin organization mediated by CTCF and cohesin', *Proc Natl Acad Sci U S A*, vol. 107, no. 8, Feb 23, pp. 3651-3656.

Hu, L, McArthur, C & Jaffe, RB 2010, 'Ovarian cancer stem-like side-population cells are tumourigenic and chemoresistant', *British Journal of Cancer*, vol. 102, no. 8, pp. 1276-1283.

Huang, Z & Murphy, SK 2013, 'Increased Intragenic IGF2 Methylation is Associated with Repression of Insulator Activity and Elevated Expression in Serous Ovarian Carcinoma', *Front Oncol*, vol. 3, p. 131.

Hubertus, J, Lacher, M, Rottenkolber, M, Muller-Hocker, J, Berger, M, Stehr, M, von Schweinitz, D*, et al.* 2011, 'Altered expression of imprinted genes in Wilms tumors', *Oncol Rep*, vol. 25, no. 3, Mar, pp. 817-823.

Issa, JPJ, Vertino, PM, Boehm, CD, Newsham, IF & Baylin, SB 1996, 'Switch from monoallelic to biallelic human IGF2 promoter methylation during aging and carcinogenesis', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 21, pp. 11757-11762.

Jiang, H, Lin, X, Liu, Y, Gong, W, Ma, X, Yu, Y, Xie, Y*, et al.* 2012, 'Transformation of epithelial ovarian cancer stemlike cells into mesenchymal lineage via EMT results in cellular heterogeneity and supports tumor engraftment', *Molecular Medicine*, vol. 18, pp. 1197-1208.

Jin, F, Li, Y, Dixon, JR, Selvaraj, S, Ye, Z, Lee, AY, Yen, CA*, et al.* 2013, 'A high-resolution map of the three-dimensional chromatin interactome in human cells', *Nature*, vol. 503, no. 7475, Nov 14, pp. 290-294.

Jordan, MA & Wilson, L 2004, 'Microtubules as a target for anticancer drugs', *Nat Rev Cancer*, vol. 4, no. 4, 04//print, pp. 253-265.

Kalhor, R, Tjong, H, Jayathilaka, N, Alber, F & Chen, L 2012, 'Genome architectures revealed by tethered chromosome conformation capture and population-based modeling', *Nat Biotechnol*, vol. 30, no. 1, Jan, pp. 90-98.

Kanduri, C, Pant, V, Loukinov, D, Pugacheva, E, Qi, CF, Wolffe, A, Ohlsson, R*, et al.* 2000, 'Functional association of CTCF with the insulator upstream of the H19 gene is parent of origin-specific and methylation-sensitive', *Current Biology*, vol. 10, no. 14, pp. 853-856.

Karst, AM & Drapkin, R 2010, 'Ovarian cancer pathogenesis: a model in evolution', *J Oncol*, vol. 2010, p. 932371.

Katainen, R, Dave, K, Pitkanen, E, Palin, K, Kivioja, T, Valimaki, N, Gylfe, AE*, et al.* 2015, 'CTCF/cohesin-binding sites are frequently mutated in cancer', *Nat Genet*, vol. 47, no. 7, 07//print, pp. 818-821.

Kemp, Christopher J, Moore, James M, Moser, R, Bernard, B, Teater, M, Smith, Leslie E, Rabaia, Natalia A*, et al.* 2014, 'CTCF Haploinsufficiency Destabilizes DNA Methylation and Predisposes to Cancer', *Cell Reports*, no. 0.

Kent, WJ, Sugnet, CW, Furey, TS, Roskin, KM, Pringle, TH, Zahler, AM, Haussler*, et al.* 2002, 'The Human Genome Browser at UCSC', *Genome Research*, vol. 12, no. 6, June 1, 2002, pp. 996-1006.

Kim, HT, Choi, BH, Niikawa, N, Lee, TS & Chang, SI 1998, 'Frequent loss of imprinting of the H19 and IGF-II genes in ovarian tumors', *American Journal of Medical Genetics*, vol. 80, no. 4, pp. 391-395.

Kim, TH, Abdullaev, ZK, Smith, AD, Ching, KA, Loukinov, DI, Green, RD, Zhang, MQ*, et al.* 2007, 'Analysis of the vertebrate insulator protein CTCF-binding sites in the human genome', *Cell*, vol. 128, no. 6, pp. 1231-1245.

Kishino, T, Lalande, M & Wagstaff, J 1997, 'UBE3A/E6-AP mutations cause Angelman syndrome', *Nat Genet*, vol. 15, no. 1, 01//print, pp. 70-73.

Kitsberg, D, Selig, S, Brandeis, M, Simon, I, Keshet, I, Driscoll, DJ, Nicholls, RD*, et al.* 1993, 'Allele-specific replication timing of imprinted gene regions', *Nature*, vol. 364, no. 6436, pp. 459-463.

Krueger, F & Andrews, SR 2011, 'Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications', *Bioinformatics*, vol. 27, no. 11, Jun 1, pp. 1571-1572.

Kulis, M & Esteller, M 2010, 'DNA methylation and cancer', *Adv Genet*, vol. 70, pp. 27-56.

Kurukuti, S, Tiwari, VK, Tavoosidana, G, Pugacheva, E, Murrell, A, Zhao, Z, Lobanenkov, V*, et al.* 2006, 'CTCF binding at the H19 imprinting control region mediates maternally inherited higher-order chromatin conformation to restrict enhancer access to Igf2', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 28, Jul 11, pp. 10684-10689.

Langdon, SP & Lawrie, SS 2001, 'Establishment of ovarian cancer cell lines', *Methods Mol Med*, vol. 39, pp. 155-159.

Leone, G, Teofili, L, Voso, MT & Lubbert, M 2002, 'DNA methylation and demethylating drugs in myelodysplastic syndromes and secondary leukemias', *Haematologica*, vol. 87, no. 12, Dec, pp. 1324-1341.

Li, H, Handsaker, B, Wysoker, A, Fennell, T, Ruan, J, Homer, N, Marth, G*, et al.* 2009a, 'The Sequence Alignment/Map format and SAMtools', *Bioinformatics*, vol. 25, no. 16, Aug 15, pp. 2078-2079.

Li, J, Abushahin, N, Pang, SJ, Xiang, L, Chambers, SK, Fadare, O, Kong, BH*, et al.* 2011, 'Tubal origin of 'ovarian' low-grade serous carcinoma', *Modern Pathology*, vol. 24, no. 11, Nov, pp. 1488-1499.

Li, J, Fadare, O, Xiang, L, Kong, BH & Zheng, WX 2012, 'Ovarian serous carcinoma: recent concepts on its origin and carcinogenesis', *Journal of Hematology & Oncology*, vol. 5, no. 8.

Li, LC & Dahiya, R 2002, 'MethPrimer: designing primers for methylation PCRs', *Bioinformatics*, vol. 18, no. 11, Nov, pp. 1427-1431.

Li, M, Balch, C, Montgomery, JS, Jeong, M, Chung, JH, Yan, P, Huang, TH*, et al.* 2009b, 'Integrated analysis of DNA methylation and gene expression reveals specific signaling pathways associated with platinum resistance in ovarian cancer', *BMC Med Genomics*, vol. 2, Jun 08, p. 34.

Li, T, Hu, J-F, Qiu, X, Ling, J, Chen, H, Wang, S, Hou, A*, et al.* 2008, 'CTCF regulates allelic expression of Igf2 by orchestrating a promoter-polycomb repressive complex 2 intrachromosomal loop', *Molecular and Cellular Biology*, vol. 28, no. 20, pp. 6473-6482.

Ling, J & Hoffman, AR 2011, 'Associated chromosome trap for identifying long-range DNA interactions', *Journal of visualized experiments : JoVE*, no. 50.

Ling, JQ & Hoffman, AR 2007, 'Epigenetics of long-range chromatin interactions', *Pediatric Research*, vol. 61, no. 5, pp. 11R-16R.

Ling, JQ, Li, T, Hu, JF, Vu, TH, Chen, HL, Qiu, XW, Cherry, AM*, et al.* 2006, 'CTCF Mediates Interchromosomal Colocalization Between Igf2/H19 and Wsb1/Nf1', *Science*, vol. 312, no. 5771, pp. 269-272.

Lomvardas, S, Barnea, G, Pisapia, DJ, Mendelsohn, M, Kirkland, J & Axel, R 2006, 'Interchromosomal interactions and olfactory receptor choice', *Cell*, vol. 126, no. 2, Jul 28, pp. 403-413.

Long, JE & Cai, X 2007, 'Igf-2r expression regulated by epigenetic modification and the locus of gene imprinting disrupted in cloned cattle', *Gene*, vol. 388, no. 1-2, Feb 15, pp. 125-134.

Lopez-Serra, P & Esteller, M 2012, 'DNA methylation-associated silencing of tumor-suppressor microRNAs in cancer', *Oncogene*, vol. 31, no. 13, Mar 29, pp. 1609-1622.

Lövkvist, C, Dodd, IB, Sneppen, K & Haerter, JO 2016, 'DNA methylation in human epigenomes depends on local topology of CpG sites', *Nucleic Acids Research*, February 29, 2016.

Lu, L, Katsaros, D, Wiley, A, de la Longrais, IAR, Puopolo, M, Schwartz, P & Yu, H 2006, 'Promoter-specific transcription of insulin-like growth factor-II in epithelial ovarian cancer', *Gynecologic Oncology*, vol. 103, no. 3, pp. 990-995.

MacHugh, DE, Shriver, MD, Loftus, RT, Cunningham, P & Bradley, DG 1997, 'Microsatellite DNA variation and the evolution, domestication and phylogeography of taurine and zebu cattle (Bos taurus and Bos indicus)', *Genetics*, vol. 146, no. 3, Jul, pp. 1071-1086.

Mahy, NL, Perry, PE & Bickmore, WA 2002, 'Gene density and transcription influence the localization of chromatin outside of chromosome territories detectable by FISH', *J Cell Biol*, vol. 159, no. 5, Dec 9, pp. 753-763.

Mallona, I, Díez-Villanueva, A & Peinado, MA 2014, 'Methylation plotter: a web tool for dynamic visualization of DNA methylation data', *Source Code for Biology and Medicine*, vol. 9, no. 1, 2014//, p. 11.

Malnic, B, Godfrey, PA & Buck, LB 2004, 'The human olfactory receptor gene family', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 8, February 24, 2004, pp. 2584-2589.

Malnic, B, Hirono, J, Sato, T & Buck, LB 1999, 'Combinatorial receptor codes for odors', *Cell*, vol. 96, no. 5, Mar 5, pp. 713-723.

Martin, D, Pantoja, C, Miñán, AF, Valdes-Quezada, C, Moltó, E, Matesanz, F, Bogdanović, O*, et al.* 2011, 'Genome-wide CTCF distribution in vertebrates defines equivalent sites that aid the identification of disease-associated genes', *Nat Struct Mol Biol*, vol. 18, no. 6, 06//print, pp. 708-714.

Menendez, L, Walker, D, Matyunina, LV, Dickerson, EB, Bowen, NJ, Polavarapu, N, Benigno, BB*, et al.* 2007, 'Identification of candidate methylation-responsive genes in ovarian cancer', *Mol Cancer*, vol. 6, Jan 25, p. 10.

Merlo, A, Herman, JG, Mao, L, Lee, DJ, Gabrielson, E, Burger, PC, Baylin, SB*, et al.* 1995, '5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers', *Nat Med*, vol. 1, no. 7, Jul, pp. 686-692.

Mittal, KR, Zeleniuchjacquotte, A, Cooper, JL & Demopoulos, RI 1993, 'Contralateral ovary in unilateral ovarian-carcinoma - A search for preneoplastic lesions', *International Journal of Gynecological Pathology*, vol. 12, no. 1, pp. 59-63.

Moon, H, Filippova, G, Loukinov, D, Pugacheva, E, Chen, Q, Smith, ST, Munhall, A*, et al.* 2005, 'CTCF is conserved from Drosophila to humans and confers enhancer blocking of the Fab-8 insulator', *EMBO Rep*, vol. 6, no. 2, Feb, pp. 165-170.

Moore, T & Haig, D 1991, 'Genomic imprinting in mammalian development: a parental tug-of-war', *Trends Genet*, vol. 7, no. 2, Feb, pp. 45-49.

Murphy, SK, Huang, Z, Wen, Y, Simel, LR, Price, TM & Berchuck, A 2009, 'Intragenic methylation at a novel CTCF binding site is associated with elevated IGF2 expression in serous epithelial ovarian cancer', *Gynecologic Oncology*, vol. 112, no. 2, pp. S138-S138.

Murphy, SK, Huang, ZQ, Wen, YQ, Spillman, MA, Whitaker, RS, Simel, LR, Nichols, TD*, et al.* 2006, 'Frequent IGF2/H19 domain epigenetic alterations and elevated IGF2 expression in epithelial ovarian cancer', *Molecular Cancer Research*, vol. 4, no. 4, pp. 283-292.

Murrell, A, Heeson, S & Reik, W 2004, 'Interaction between differentially methylated regions partitions the imprinted genes Igf2 and H19 into parent-specific chromatin loops', *Nat Genet*, vol. 36, no. 8, Aug, pp. 889-893.

Nakahashi, H, Kwon, KR, Resch, W, Vian, L, Dose, M, Stavreva, D, Hakim, O*, et al.* 2013, 'A genome-wide map of CTCF multivalency redefines the CTCF code', *Cell Rep*, vol. 3, no. 5, May 30, pp. 1678-1689.

Nijman, IJ, Bradley, DG, Hanotte, O, Otsen, M & Lenstra, JA 1999, 'Satellite DNA polymorphisms and AFLP correlate with Bos indicus-taurus hybridization', *Animal Genetics*, vol. 30, no. 4, pp. 265-273.

Nowack, MK, Shirzadi, R, Dissmeyer, N, Dolf, A, Endl, E, Grini, PE & Schnittger, A 2007, 'Bypassing genomic imprinting allows seed development', *Nature*, vol. 447, no. 7142, May 17, pp. 312-315.

Ohlsson, R, Renkawitz, R & Lobanenkov, V 2001, 'CTCF is a uniquely versatile transcription regulator linked to epigenetics and disease', *Trends Genet*, vol. 17, no. 9, Sep, pp. 520-527.

Ohta, T, Gray, TA, Rogan, PK, Buiting, K, Gabriel, JM, Saitoh, S, Muralidhar, B*, et al.* 1999, 'Imprinting-Mutation Mechanisms in Prader-Willi Syndrome', *The American Journal of Human Genetics*, vol. 64, no. 2, pp. 397-413.

Parelho, V, Hadjur, S, Spivakov, M, Leleu, M, Sauer, S, Gregson, HC, Jarmuz, A*, et al.* 2008, 'Cohesins functionally associate with CTCF on mammalian chromosome arms', *Cell*, vol. 132, no. 3, Feb 8, pp. 422-433.

Phillips, JE & Corces, VG 2009, 'CTCF: master weaver of the genome', *Cell*, vol. 137, no. 7, Jun 26, pp. 1194-1211.

Plasschaert, RN, Vigneau, S, Tempera, I, Gupta, R, Maksimoska, J, Everett, L, Davuluri, R*, et al.* 2014, 'CTCF binding site sequence differences are associated with unique regulatory and functional trends during embryonic stem cell differentiation', *Nucleic Acids Res*, vol. 42, no. 2, Jan, pp. 774-789.

Poole, RL, Leith, DJ, Docherty, LE, Shmela, ME, Gicquel, C, Splitt, M, Temple, IK*, et al.* 2012, 'Beckwith-Wiedemann syndrome caused by maternally inherited mutation of an OCT-binding motif in the IGF2/H19-imprinting control region, ICR1', *Eur J Hum Genet*, vol. 20, no. 2, 02//print, pp. 240-243.

Quitschke, WW, Taheny, MJ, Fochtmann, LJ & Vostrov, AA 2000, 'Differential effect of zinc finger deletions on the binding of CTCF to the promoter of the amyloid precursor protein gene', *Nucleic Acids Research*, vol. 28, no. 17, September 1, 2000, pp. 3370-3378.

Reddy, KL, Zullo, JM, Bertolino, E & Singh, H 2008, 'Transcriptional repression mediated by repositioning of genes to the nuclear lamina', *Nature*, vol. 452, no. 7184, 03/13/print, pp. 243-247.

Reed, ML & Leff, SE 1994, 'Maternal imprinting of human SNRPN, a gene deleted in Prader-Willi syndrome', *Nat Genet*, vol. 6, no. 2, Feb, pp. 163-167.

Renfree, MB, Hore, TA, Shaw, G, Marshall Graves, JA & Pask, AJ 2009, 'Evolution of Genomic Imprinting: Insights from Marsupials and Monotremes', *Annual Review of Genomics and Human Genetics*, vol. 10, no. 1, pp. 241-262.

Ryan, D, Pederson, B, Triche Jr, T & Gruning, B 2016, 'PileOMeth 0.1.13', Zenodo, <http://doi.org/10.5281/zenodo.53211>.

Ryba, T, Hiratani, I, Lu, J, Itoh, M, Kulik, M, Zhang, J, Schulz, TC*, et al.* 2010, 'Evolutionarily conserved replication timing profiles predict long-range chromatin interactions and distinguish closely related cell types', *Genome Res*, vol. 20, no. 6, Jun, pp. 761-770.

Schmidt, D, Schwalie, PC, Wilson, MD, Ballester, B, Goncalves, A, Kutter, C, Brown, GD*, et al.* 2012, 'Waves of retrotransposon expansion remodel genome organization and CTCF binding in multiple mammalian lineages', *Cell*, vol. 148, no. 1-2, Jan 20, pp. 335-348.

Schütt, S, Florl, AR, Shi, W, Hemberger, M, Orth, A, Otto, S, Schulz, WA*, et al.* 2003, 'DNA Methylation in Placentas of Interspecies Mouse Hybrids', *Genetics*, vol. 165, no. 1, p. 223.

Spilianakis, CG, Lalioti, MD, Town, T, Lee, GR & Flavell, RA 2005, 'Interchromosomal associations between alternatively expressed loci', *Nature*, vol. 435, no. 7042, 06/02/print, pp. 637-645.

Stadler, MB, Murr, R, Burger, L, Ivanek, R, Lienert, F, Scholer, A, van Nimwegen, E*, et al.* 2011, 'DNA-binding factors shape the mouse methylome at distal regulatory regions', *Nature*, vol. 480, no. 7378, Dec 14, pp. 490-495.

Stringer, JM, Suzuki, S, Pask, AJ, Shaw, G & Renfree, MB 2012, 'Promoter-specific expression and imprint status of marsupial IGF2', *PLoS ONE*, vol. 7, no. 7, p. e41690.

Szabo, PE, Tang, SHE, Silva, FJ, Tsark, WMK & Mann, JR 2004, 'Role of CTCF binding sites in the Igf2/H19 imprinting control region', *Molecular and Cellular Biology*, vol. 24, no. 11, pp. 4791-4800.

Takai, D, Gonzales, FA, Tsai, YC, Thayer, MJ & Jones, PA 2001, 'Large scale mapping of methylcytosines in CTCF-binding sites in the human H19 promoter and aberrant hypomethylation in human bladder cancer', *Human Molecular Genetics*, vol. 10, no. 23, November 1, 2001, pp. 2619-2626.

Tan, TZ, Yang, H, Ye, J, Low, J, Choolani, M, Tan, DS, Thiery, JP*, et al.* 2015, 'CSIOVDB: a microarray gene expression database of epithelial ovarian cancer subtype', *Oncotarget*, vol. 6, no. 41, Dec 22, pp. 43843-43852.

Tennant, MK, Thrasher, JB, Twomey, PA, Drivdahl, RH, Birnbaum, RS & Plymate, SR 1996, 'Protein and messenger ribonucleic acid (mRNA) for the type 1 insulin-like growth factor (IGF) receptor is decreased and IGF-II mRNA is increased in human prostate carcinoma compared to benign prostate epithelium', *Journal of Clinical Endocrinology & Metabolism*, vol. 81, no. 10, pp. 3774-3782.

Tomao, F, Papa, A, Rossi, L, Strudel, M, Vici, P, Lo Russo, G & Tomao, S 2013, 'Emerging role of cancer stem cells in the biology and treatment of ovarian cancer: basic knowledge and therapeutic possibilities for an innovative approach', *Journal of Experimental & Clinical Cancer Research*, vol. 32, no. 1, p. 48.

Tycko, B & Morison, IM 2002, 'Physiological functions of imprinted genes', *J Cell Physiol*, vol. 192, no. 3, Sep, pp. 245-258.

Tym, JE, Mitsopoulos, C, Coker, EA, Razaz, P, Schierz, AC, Antolin, AA & Al-Lazikani, B 2016, 'canSAR: an updated cancer research and drug discovery knowledgebase', *Nucleic Acids Research*, vol. 44, no. D1, January 4, 2016, pp. D938-D943.

Ulaner, GA, Vul, TH, Li, T, Hu, JF, Yao, XM, Yang, YW, Gorlick, R*, et al.* 2003, 'Loss of imprinting of IGF2 and H19 in osteosarcoma is accompanied by reciprocal methylation changes of a CTCF-binding site', *Human Molecular Genetics*, vol. 12, no. 5, pp. 535-549.

Vandijk, MA, Vanschaik, FMA, Bootsma, HJ, Holthuizen, P & Sussenbach, JS 1991, 'Initial characterisation of the 4 promoters of the human insulin-like growth factor-II gene', *Molecular and Cellular Endocrinology*, vol. 81, no. 1-3, pp. 81-94.

Vrana, PB, Fossella, JA, Matteson, P, del Rio, T, O'Neill, MJ & Tilghman, SM 2000, 'Genetic and epigenetic incompatibilities underlie hybrid dysgenesis in Peromyscus', *Nat Genet*, vol. 25, no. 1, May, pp. 120-124.

Vrana, PB, Guan, XJ, Ingram, RS & Tilghman, SM 1998, 'Genomic imprinting is disrupted in interspecific Peromyscus hybrids', *Nat Genet*, vol. 20, no. 4, Dec, pp. 362-365.

Vu, TH & Hoffman, AR 1994, 'Promoter-specific imprinting of the human insulin-like growth factor-II gene', *Nature*, vol. 371, no. 6499, pp. 714-717.

Vu, TH, Nguyen, AH & Hoffman, AR 2010a, 'Loss of IGF2 imprinting is associated with abrogation of long-range intrachromosomal interactions in human cancer cells', *Hum Mol Genet*, vol. 19, no. 5, Mar 1, pp. 901-919.

Vu, TH, Nguyen, AH & Hoffman, AR 2010b, 'Loss of IGF2 imprinting is associated with abrogation of long-range intrachromosomal interactions in human cancer cells', *Human Molecular Genetics*, vol. 19, no. 5, March 1, 2010, pp. 901-919.

Wang, D & Lippard, SJ 2005, 'Cellular processing of platinum anticancer drugs', *Nat Rev Drug Discov*, vol. 4, no. 4, 04//print, pp. 307-320.

Wang, H, Maurano, MT, Qu, H, Varley, KE, Gertz, J, Pauli, F, Lee, K*, et al.* 2012a, 'Widespread plasticity in CTCF occupancy linked to DNA methylation', *Genome Res*, vol. 22, no. 9, Sep, pp. 1680-1688.

Wang, H, Maurano, MT, Qu, H, Varley, KE, Gertz, J, Pauli, F, Lee, K*, et al.* 2012b, 'Widespread plasticity in CTCF occupancy linked to DNA methylation', *Genome Research*, vol. 22, no. 9, September 1, 2012, pp. 1680-1688.

Wang, J, Valo, Z, Smith, D & Singer-Sam, J 2007, 'Monoallelic Expression of Multiple Genes in the CNS', *PLoS ONE*, vol. 2, no. 12, p. e1293.

Weksberg, R, Shuman, C & Beckwith, JB 2010, 'Beckwith-Wiedemann syndrome', *Eur J Hum Genet*, vol. 18, no. 1, Jan, pp. 8-14.

Wright, ML 2014, 'Investigating the evolution of replication timing and monoallelic expression in mammals and birds', PhD thesis, University of Adelaide.

Wright, ML, Casey, A, Necsulea, A, Julien, P, Kaessmann, H & Grutzner, F in prep., 'Imprinted orthologs in platypus display an amount of monoallelic expression, which is not exclusive to one allele.', *Unpublished*.

Wright, ML & Grutzner, F in prep., 'Asynchronous replication of imprinted-orthologs in platypus, and the conservation of replication timing initiation in imprinted orthologs across amniote evolution.', *Unpublished*.

Xiang, R, Ghanipoor-Samami, M, Johns, WH, Eindorf, T, Rutley, DL, Kruk, ZA, Fitzsimmons, CJ*, et al.* 2013, 'Maternal and Paternal Genomes Differentially Affect Myofibre Characteristics and Muscle Weights of Bovine Fetuses at Midgestation', *PLoS ONE*, vol. 8, no. 1, p. e53402.

Xiao, T, Wallace, J & Felsenfeld, G 2011, 'Specific sites in the C terminus of CTCF interact with the SA2 subunit of the cohesin complex and are required for cohesin-dependent insulation activity', *Mol Cell Biol*, vol. 31, no. 11, Jun, pp. 2174-2183.

Zakharova, IS, Shevchenko, AI & Zakian, SM 2009, 'Monoallelic gene expression in mammals', *Chromosoma*, vol. 118, no. 3, Jun, pp. 279-290.

Zeitz, MJ, Ay, F, Heidmann, JD, Lerner, PL, Noble, WS, Steelman, BN & Hoffman, AR 2013, 'Genomic Interaction Profiles in Breast Cancer Reveal Altered Chromatin Architecture', *PLoS ONE*, vol. 8, no. 9, p. e73974.

Zhang, H, Niu, BB, Hu, JF, Ge, SF, Wang, HB, Li, T, Ling, JQ*, et al.* 2011, 'Interruption of intrachromosomal looping by CCCTC binding factor decoy proteins abrogates genomic imprinting of human insulin-like growth factor II', *Journal of Cell Biology*, vol. 193, no. 3, May, pp. 475-487.

Zhang, S, Kubota, C, Yang, L, Zhang, Y, Page, R, O'Neill, M, Yang, X*, et al.* 2004, 'Genomic imprinting of H19 in naturally reproduced and cloned cattle', *Biol Reprod*, vol. 71, no. 5, Nov, pp. 1540-1544.

Zhao, Z, Tavoosidana, G, Sjolinder, M, Gondor, A, Mariano, P, Wang, S, Kanduri, C*, et al.* 2006, 'Circular chromosome conformation capture (4C) uncovers extensive networks of epigenetically regulated intra- and interchromosomal interactions', *Nature Genetics*, vol. 38, no. 11, Nov, pp. 1341-1347.