The Role of Imprinting in Embryonic Development and Tumorigenesis.

by

Teresa M. Holm

M.Sc. (Hons), Biochemistry, 1997; B.Sc., Biology, 1995
University of Auckland, New Zealand

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Signature of Author: _____
Department of Biology
November 07, 2005

Certified by: _____
Rudolf Jaenisch
Professor of Biology
Thesis Supervisor

Accepted by: _____
Stephen P. Bell
Professor of Biology
Co-Chair, Department Graduate Committee

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Abstract

Imprinting is a mammalian adaptation that results in the mono-allelic expression of a subset of genes depending on their parental origin. It is believed that DNA methylation marks are responsible for maintaining imprinted gene expression patterns. The 'parental conflict' hypothesis was proposed to explain the evolution of imprinting and is based on the assumption that mammals arose from an ancestor that was polyandrous (multiple fathers within one litter). According to this hypothesis, conflict between the male and female over the allocation of maternal resources to the offspring led to the evolution of imprinting. Consistent with this, many imprinted genes are involved in embryonic or placental growth by regulating mitogenic pathways or the cell cycle. Loss of imprinting (LOI) has been found at specific loci in cancers, raising the possibility that altered expression of imprinted genes may also contribute to tumorigenesis. To investigate the effect of global LOI on embryonic development and cancer formation, imprint free (IF) embryonic stem (ES) cells were generated using conditional inactivation/reactivation of the DNA methyltransferase *Dnmt1*. Tetraploid complementation and chimera experiments revealed that IF-embryos fail to develop beyond E11.5 and display an overgrowth phenotype. As developmental comparisons, parthenogenetic and androgenetic (AT) embryos were derived and found to develop to E9.5 and E7.5, respectively. Removing the imprinted methyl marks from AT-ES cells rescued embryonic development to E9.5-10.5 and restored pluripotency. However, IFAT embryos were not developmentally equivalent to biparentally-derived IF-embryos, suggesting that mechanisms other than DNA methylation may be involved in maintaining parent-specific gene expression patterns. To study the effect of LOI on cell growth, murine embryonic fibroblasts (MEFs) were derived from E13.5 chimeric IF-embryos and analyzed *in vitro*. IF-MEFs grew faster, were resistant to the cytostatic effects of TGFβ, and formed tumors in SCID mice. In addition, IF-MEFs were immortal and when exposed to H-Ras became fully transformed. Western blot analysis of IF-MEFs revealed abnormally low levels of $p19^{Arf}$ and p53, two critical regulators of growth arrest and potent tumor suppressors. Somatic contribution of IF-ES cells in chimeric adults led to highly penetrant tumor formation by 12 months of age, causing multiple cancer types derived from the IF cells. Taken together, these data are consistent with global LOI having a causal role in tumorigenesis by affecting the regulation of the p53-$p19^{Arf}$ pathway and predisposing IF cells to transformation.

Thesis Supervisor: Rudolf Jaenisch
Title: Professor of Biology

Dedication

I would like to dedicate this thesis to Lola Marjorie Mitchell. Who taught me my most important lesson yet, that 'faith in the future is the substance of things hoped for and the evidence of things not seen'.

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Chapter 1:

Introduction

1.1 Epigenetic regulation of gene expression during development and disease.

Epigenetics is used to describe the study of stable alterations in gene expression potential that arise during development and cellular proliferation (reviewed by Jaenisch and Bird, 2002). These epigenetic processes are essential for embryogenesis, cellular differentiation and tissue maintenance in mammals. In addition, epigenetic modifications provide safeguards to prevent viral genomes commandeering cellular processes for their own replication. Two epigenetic mechanisms that fulfill this criterion are DNA methylation and genomic imprinting.

1.1.1 DNA methylation

Methylation is a common form of DNA modification in animals, occurring at position five of cytosine residues (reviewed in Bird and Wolffe 1999) and (Bestor 2000)). The mammalian genome contains approximately 3 x 10^7 of 5-methylcytosine (m^5C) residues most of this is within 5′- m^5CpG-3′ dinucleotides, which is roughly equivalent to 1% of the genome (Walsh and Bestor 1999). Like histone modifications cytosine methylation raises the coding capacity of the genome and participates in the partitioning of genomes into active and inactive functional compartments. Some of the constituents of the inactive compartment associated with DNA methylation include imprinted genes, the

inactive female X chromosome, and parasitic DNA elements. It was proposed in 1975 that DNA methylation might be responsible for the stable maintenance of a particular gene expression pattern through mitotic cell division (Holliday and Pugh 1975; Riggs 1975). Since then, ample evidence has been obtained to support this concept, and DNA methylation is now recognized to be a significant contributor to the stability of gene expression states (Bird 2002). Specifically, DNA methylation establishes a silent chromatin state by collaborating with proteins that modify nucleosomes.

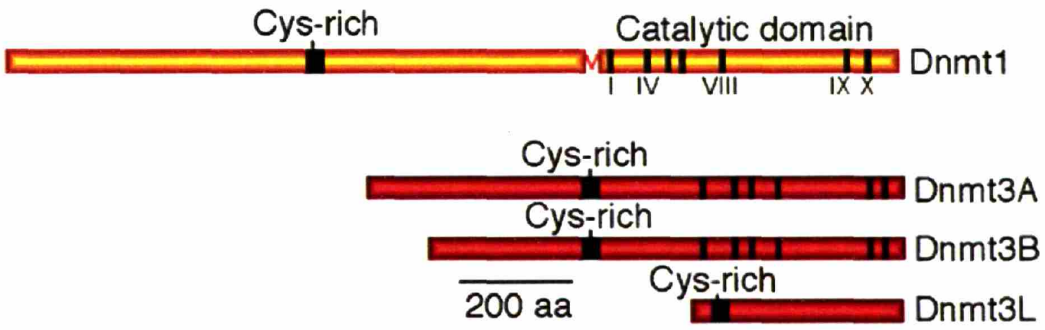
1.1.1.1 The DNA methyltransferases of mammals

Cellular DNA methylation patterns seem to be established by a complex interplay of at least three independent DNA methyltansferases: Dnmt1, Dnmt3A, Dnmt3B and Dnmt3L **(Fig 1.1)**. Genetic analysis of these various Dnmts has established that DNA methylation is essential for vertebrate development (Bestor 2000). Loss of methylation causes apoptosis in embryos and in fibroblasts but not in ES cells or in cancer cells (Jaenisch and Bird 2003).

1.1.1.1.1 Catalytic mechanism of DNA (cytosine-5)-methyltransferases

The 5 carbon position of cytosine is relatively unreactive, and its methylation in neutral aqueous solution has been called a 'chemically improbable reaction (Chen, MacMillan et al. 1991). Co-crystal structures of DNA-DNA methyltransferases showed that the target cytosine is everted from the DNA helix and inserted deep into the active site of the enzyme (Klimasauskas, Kumar et al. 1994).

Figure 1.1 DNA Methyltransferases in mammals



Sequence relationships among mammalian DNA methyltransferases. Catalytic motifs are designated with roman numerals. Motifs are absent from Dnmt3L, whereas the cysteine-rich regions and other sequences show strong similarities with Dnmt3A and Dnmt3B. At right is a ClustalW representation of sequence similarities within the region spanning catalytic motifs I to VIII. The corresponding region of Dnmt3L was identified by alignment with Dnmt3A and Dnmt3B. Adapted from Bourc'his et al 2001.

DNA methyl transferases add a methyl group to the 6 position, thereby pushing electrons to the 5 position, which then attack the methyl group of S-adenosyl-L-methionine resulting in 5-methylcytosine **(Fig 1.2)** (Bestor 2000).

1.1.1.1.2 DNA methyltransferase-1 (Dnmt1)

Dnmt1 was the first methyltransferase to be discovered (Bestor, Laudano et al. 1988). Pioneering work has established that Dnmt1 has a 10-40 fold preference for methylating hemimethylated DNA (Yoder, Soman et al. 1997). Dnmt1 is the most abundant methyltransferase in somatic cells and localizes to replication foci in S phase where it interacts with the proliferating cell nuclear antigen (PCNA) (Iida, Suetake et al. 2002). Dnmt1 is often referred to as the maintenance methyltransferase because it is believed to be the enzyme responsible for copying methylation patterns after DNA replication. Homologues of *Dnmt1* have been found in nearly all eukaryotes whose DNA bears m^5CpG but not in those that lack it, indicating that methyltransferases have an important and conserved function in DNA methylation (Bestor 2000).

Targeted mutations of the *Dnmt1* gene result in a recessive lethal phenotype and produce a number of unique phenotypes in mice (Li, Bestor et al. 1992). Firstly, the *Dnmt1* mutation produces a lethal differentiation phenotype in which homozygous mutant embryonic stem (ES) cells grow normally with a severely demethylated genome but undergo cell-autonomous apoptosis when induced to differentiate (Li, Bestor et al. 1992). Secondly, embryos homozygous for mutations at *Dnmt1* show mis-expression of imprinted genes (Li, Beard et al. 1993; Li, Beard et al. 1993).

Figure 1.2 Cytosine Methylation in Vertebrates.

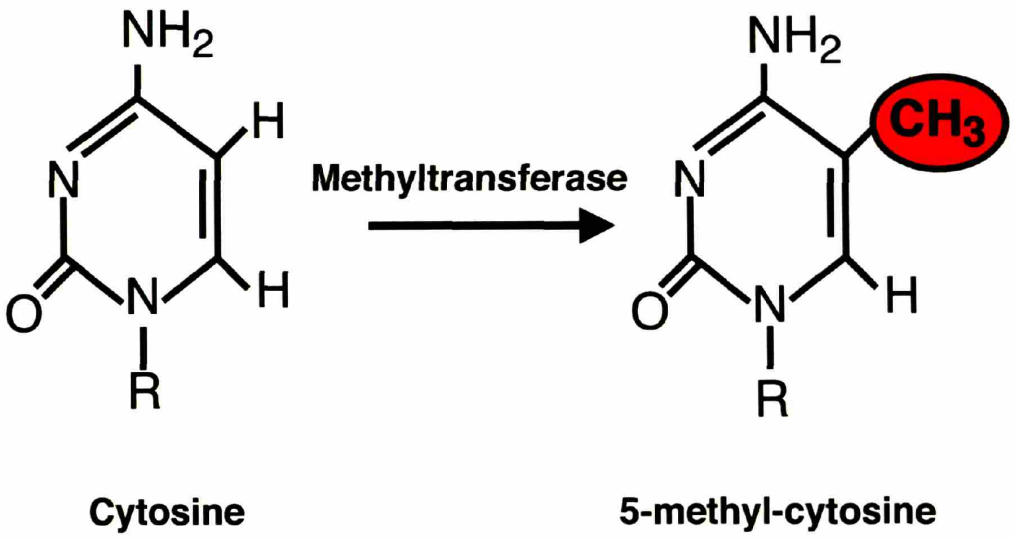


Figure 1.2 Cytosine Methylation in Vertebrates
Addition of a methyl group to cytosine by methyltransferases results in 5-mehtyl-cytosine

Finally, homozygous *Dnmt1*-null embryos display transient ectopic expression of all copies of *Xist* and faults in X-inactivation (Beard, Li et al. 1995). ES cells in a demethylated state for extended lengths of time have an increased frequency of chromosomal deletions and rearrangements, most likely due to an increased rate of homologous recombination among demethylated and unmasked repeat sequences (Chen, Pettersson et al. 1998). The finding that a small amount of m^5C persist in the genome of *Dnmt1*-null ES cells and that these cells partially retain the capacity to methylate newly integrated retroviral DNA, is consistent with the existence of one or more additional DNA methyltransferases (Lei, Oh et al. 1996).

Interestingly, Dnmt1 is localized exclusively to the nuclei of somatic cells and is cytoplasmic only in the oocyte and preimplantation embryo. Expression of *Dnmt1* is controlled by multiple promoters (including female- and male-specific) and results in transcripts with alternative 5' exons **(Fig 1.3)**. Usage of the oocyte specific promoter leads to translation of a protein that is shorter than the full-length (somatic) form by 118 N-terminal amino acids (Mertineit, Yoder et al. 1998). This truncated oocyte specific form of Dnmt1 (Dnmt1o) is enzymatically active and accumulates to very high levels in the oocyte; it is nuclear only at the earliest stages of oocyte growth and becomes localized to the cytoplasmic shell of the oocyte prior to ovulation (Carlson, Page et al. 1992). After fertilization the Dnmt1o protein is cytoplasmic in pre-implantation embryos but enters, and then exits, the nucleus at the 8-cell stage (Mertineit, Yoder et al. 1998) (Carlson, Page et al. 1992).

Figure 1.3 Sex-specific exons and mRNAs from the *Dnmt1* gene

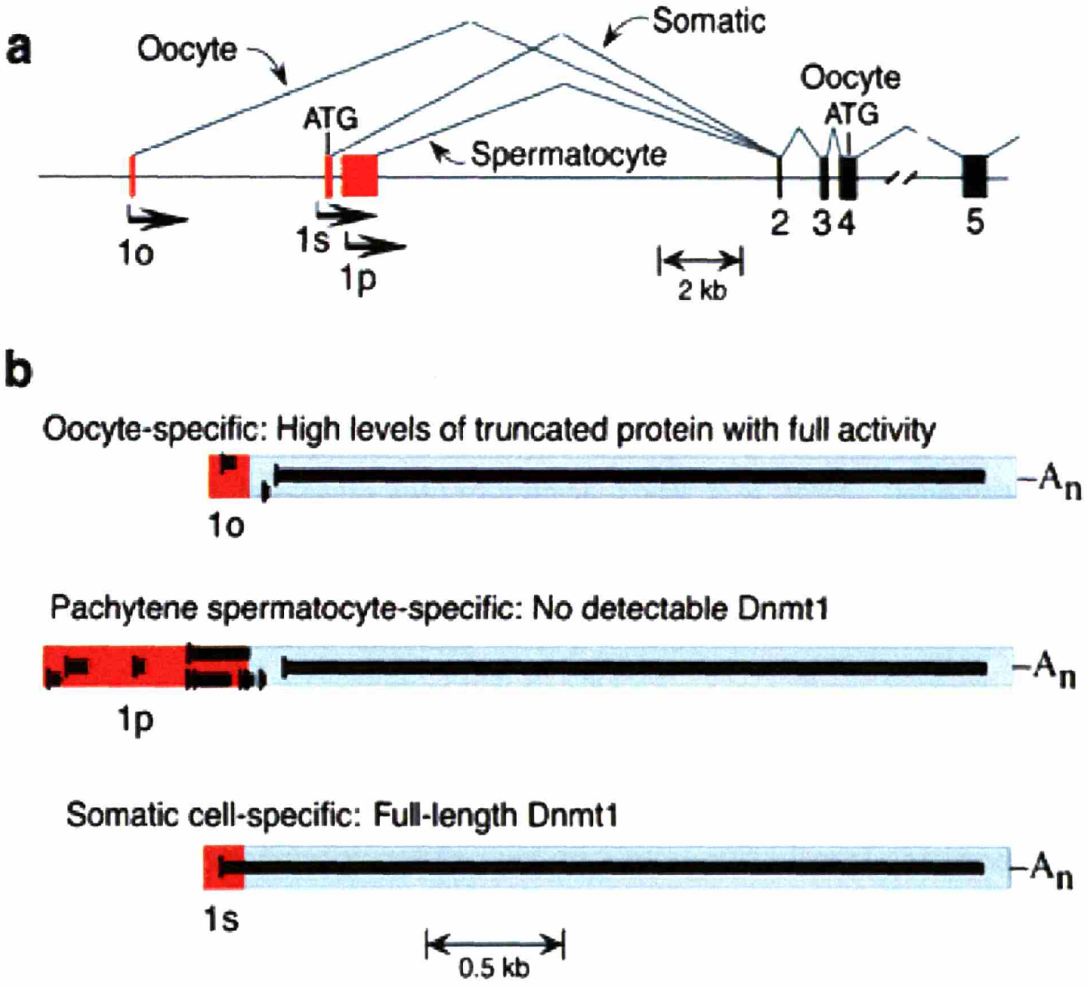


Figure 1.3 Sex-specific exons and mRNAs from the Dnmt1 gene.
(a) 5' region of Dnmt1 on proximal mouse chromosome 9. Exon 1o is oocyte-specific, exon 1s is specific to somatic cells of both sexes and exon 1p is restricted to pachytene spermatocytes (30). The ATG codon in exon 1s is used for initiation of translation in somatic cells; a truncated form arises from use of the ATG codon in exon 4 in oocytes (30). (b) mRNA products of sex-specific exons. Effect of the alternative promoter use and splicing on organization of mature Dnmt1 mRNAs are indicated. Heavy horizontal bars indicate open reading frames; short vertical bars indicate ATG initiation codons. Adapted from Bestor et al 2000.

Dnmt1 does not become fully nuclear again until after implantation, following its replacement by the full-length somatic form, which is transcribed from the somatic cell promoter. The biological function of the elaborate nuclear-cytoplasmic trafficking of Dnmt1 during oogenesis and early development is currently unknown, although targeted mutation of *Dnmt1o* results in a loss of maternal imprints (Howell, Bestor et al. 2001).

Promoter 1s, located 7 kb upstream of exon 1o, is active in somatic cells and functions as the housekeeping promoter (Yoder, Soman et al. 1997). Exon 1s contains the ATG codon that initiates full-length Dnmt1 (Mertineit, Yoder et al. 1998). Promoter 1s is activated shortly after implantation and by embryonic day (E)7, all detectable Dnmt1 protein is the 1s full-length form (Mertineit, Yoder et al. 1998). Although 1s promoter is active in all dividing cells, it does become downregulated under conditions of growth arrest (Mertineit, Yoder et al. 1998).

Promoter 1p, lying downstream of exon 1s, is active only in the pachytene spermatocyte (Mertineit, Yoder et al. 1998). Exon 1p contains multiple short open reading frames that are predicted to interfere with translation of the Dnmt1 open reading frame. In keeping with this expectation, the pachytene spermatocyte does not contain detectable amounts of the Dnmt1 protein and the abundant mRNA that contains exon 1p is not associated with polyribosomes (Trasler, Alcivar et al. 1992; Mertineit, Yoder et al. 1998).

The relationship between Dnmt1 protein and mRNA levels in germ cells is unusual. The protein is present at very high levels in mature oocytes and preimplantation embryos, but mRNA levels are low at these stages. Conversely, *Dnmt1* mRNA levels in

the pachytene spermatocyte are high, but protein levels are low. An analysis of mRNA levels therefore gives a large underestimate of protein levels in oocytes and early embryos, and a significant overestimate in pachytene spermatocytes (Mertineit, Yoder et al. 1998).

1.1.1.1.3 Dnmt3a, 3b and 3L

More recently, the *Dnmt3* family of methyltransferase genes from mouse and human have been characterized (Okano, Bell et al. 1999). These genes are highly conserved and orthologues have been identified in zebrafish, *Arabidopsis thaliana* and maize (Bestor 2000).

In mice, *Dnmt3a* and *Dnmt3b* are highly expressed in the developing implantation embryo (Okano, Bell et al. 1999). Functional studies in transgenic flies have shown that Dnmt3a can carryout *de novo* DNA methylation (Lyko, Ramsahoye et al. 1999). Surprisingly, *Dnmt3a* knockout mice are born live but become runted and die around four weeks of age (Okano, Bell et al. 1999). These observations, coupled with *in vitro* data indicating that Dnmt3A and Dnmt3B enzymes have an equal preference for hemi- and unmethylated DNA substrates have led to them being termed the '*de novo* DNA methyltransferases' (Okano, Xie et al. 1998)

In humans, mutations in *DNMT3B* are responsible for intercranial facial (ICF) syndrome (Wijmenga, van den Heuvel et al. 1998) and *DNMT3B* currently remains the only DNA methyltransferase to be mutated in a human disease. The ICF syndrome results in immunodeficiency and mild facial anomalies, in addition numerous cytogenetic abnormalities that largely affect the pericentric regions of chromosome 1, 9 and 16 have

been observed. These pericentric regions contain a type of repetitive DNA termed 'classical satellite', or 'satellites 2 and 3', and are normally heavily methylated. In ICF patients, the classical satellite regions are nearly completely unmethylated, as are CpG islands on the inactive X chromosome (Jeanpierre, Turleau et al. 1993; Kondo, Bobek et al. 2000). Thus, DNMT3B appears to be specialized for the methylation of only a particular compartment of the genome. To date, none of the ICF patients have been found to be homozygous for null alleles of *DNMT3B* (Xu, Bestor et al. 1999). This suggests that complete loss of DNMT3B activity might be lethal and in support of this, *Dnmt3b* knockout mice are non-viable and show numerous developmental defects and growth impairment after E9.5 (Okano, Bell et al. 1999).

Dnmt3L by itself has no catalytic DNA methyltransferase activity. However, it does co-localize with Dnmt3a and Dnmt3b within the cell, and is essential for establishing methylation imprints in both the female and male germline (Bourc'his, Xu et al. 2001; Hata, Okano et al. 2002).

1.1.1.2 Mammalian DNA methylation patterns

The pattern of DNA methylation during mammalian embryogenesis is dynamic, beginning with a wave of demethylation during the cleavage stages, followed by genome-wide *de novo* methylation after implantation (Jaenisch 1997). Demethylation is an active process that strips the male genome of methylation within hours of fertilization (Mayer, Niveleau et al. 2000; Oswald, Engemann et al. 2000). In contrast the maternal genome is passively demethylated during subsequent cleavage divisions (Li 2002). The extent of methylation in the genome of the gastrulating embryo is high due to *de novo* methylation

but it tends to decrease in specific tissues during differentiation (Ehrlich, Gama-Sosa et al. 1982). *De novo* methylation occurs rarely during normal postgastrulation development but it is observed frequently during the establishment of cell lines and in some cancers (Jones, Wolkowicz et al. 1990; Kawai, Hirose et al. 1994). **(Fig 1.4)**

One of the most striking features of vertebrate DNA methylation patterns is the presence of CpG islands. These GC-rich regions of DNA possess high densities of CpGs that are usually positioned at the 5' ends of genes. Initial computational studies have estimated that the human genome contains a predicted 29,000 CpG islands, most of which remain unmethylated throughout embryonic development and in adults (Robertson and Wolffe 2000; Egger, Liang et al. 2004). However, a small proportion of CpG islands become methylated during development and as a consequence the associated promoter is stably silenced. This process of developmentally programmed methylation is also involved in genomic imprinting (see section 1.1.2 below) and X chromosome inactivation (Jaenisch and Bird 2003). While the *de novo* methylation events that occur in germ cells or during early embryogenesis occur rapidly, the rate of accumulation of methylated CpGs in somatic cells appears to be very slow. For example, the *de novo* methylation of a provirus in murine erythroleukemia cells takes many weeks to complete (Bird and Wolffe 1999). Similarly, the recovery of global DNA methylation levels following chronic treatment of mouse cells with the DNA methylation inhibitor 5-azacytidine requires months to complete (Egger, Liang et al. 2004).

Figure 1.4 Temporal changes in DNA methylation levels during development

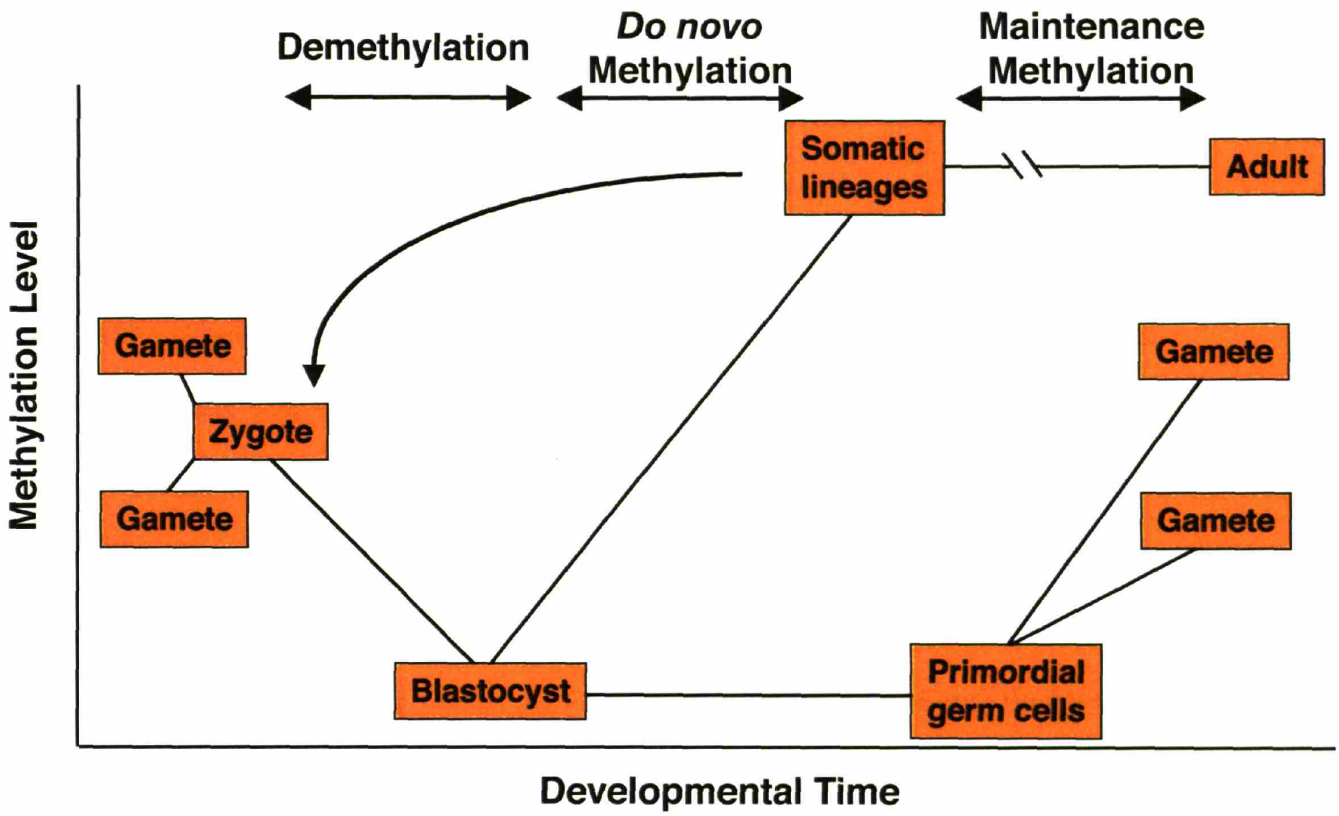


Figure 1.4 Temporal changes in DNA methylation levels during development.
Shows the changes in the global levels of genomic methylation relative to the different stages of embryonic development and the somatic and germ cell lineages. Adapted from Jaenisch 1997.

1.1.1.3 The function of DNA methylation

1.1.1.3.1 Genome defense and structural integrity

Much attention in the methylation field has focused on CpG islands, primarily because of the propensity of these sequences to become aberrantly hypermethylated in tumors, resulting in the transcriptional silencing of their associated gene (Baylin, Herman et al. 1998; Jones and Laird 1999). Most CpG-dinucleotides however, reside in the relatively CpG-poor bulk genomic DNA and are hypermethylated (Cooper and Krawczak 1989). A closer examination of the distribution of this fraction of CpG dinucleotides within the genome has revealed that most reside within parasitic DNA elements or retrotransposons, such as endogenous retroviruses, LINE elements and SINE elements, which are CpG-rich (Yoder, Walsh et al. 1997; Colot and Rossignol 1999). These parasitic elements account for almost 40% of the human genome and it is thought that DNA methylation may have arisen as a genome defense system to silence expression of these elements and limit their spread throughout the genome (Yoder, Walsh et al. 1997). Parasitic DNA elements represent a significant threat to the structural integrity of the genome because they can mediate recombination between non-allelic repeats, resulting in chromosomal rearrangements and translocations. In addition, active retrotransposons can integrate into, and disrupt the expression or function of, host genes (Kazazian and Moran 1998; Montagna, Santacatterina et al. 1999). For instance, many retrotransposons contain strong promoters that if integrated within a transcriptional unit, could result in internal initiation. If integration is in the sense orientation, then it can produce a truncated transcript. Alternatively, integration in the antisense orientation (relative to the normal

direction of transcription of the targeted gene) can result in inhibition of gene expression by transcriptional interference. Expression of genes encoded by retrotransposons, such as reverse transcriptase, are essential for their mobility and the DNA methylation of retrotransposon promoters has been shown to silence their transcription (Kochanek, Renz et al. 1995).

Evidence supporting the genome defense hypothesis comes from several sources. *Dnmt1* homozygous knockout ES cells, which retain only 30% normal methylation levels, have a ten-fold increase in the rate of mutations involving gene rearrangements (Chen, Pettersson et al. 1998). These cells also show a large increase in transcription from endogenous transposable elements (such as IAP elements) compared with wild-type ES cells. More recently it has been shown that mice derived from ES cells carrying the hypomorphic allele of *Dnmt1* (known as the ChiP allele) accumulate extensive chromosomal rearrangements and retroviral insertions that ultimately result in cancer of the thymus and premature death (Eden, Gaudet et al. 2003; Gaudet, Hodgson et al. 2003). Similarly, human tumor cells often display global genome hypomethylation of parasitic and repetitive DNAs, resulting in the re-expression of viral elements (Florl, Lower et al. 1999; Grassi, Girault et al. 1999). However conversely, human tumors often display a region-specific gain of DNA methylation at CpG islands that occurs in conjunction with the hypomethylation of repetitive DNA. The net outcome of these changes in the normal DNA methylation pattern is a global alteration of gene transcription. When oncogenes and tumor suppressors are affected in this way, the outcome is progression towards a cancer phenotype (discussed further in section 1.2).

1.1.1.3.2 Transcriptional repression

The connection between CpG methylation and transcriptional silencing has been recognized for the past twenty years (Jones, Veenstra et al. 1998; Nan, Ng et al. 1998). Although it was established that local cytosine methylation could directly interfere with the binding of transcriptional activators, such a mechanism could not easily account for more global silencing phenomena, such as X-chromosome inactivation.

The methyl-CpG binding proteins

Although DNA methylation is clearly repulsive to some DNA binding proteins, it is singularly attractive to others. Early studies detected methyl-CpG binding activities and implicated these as mediators of transcriptional repression (Hendrich and Bird 1998). Progress in understanding the mechanism of repression came with the characterization of MeCP2 and its relatives the methyl-CpG binding domain proteins MBD1-MBD4 (Lewis, Meehan et al. 1992) (Hendrich and Bird 1998). Biochemical and transient transfection studies identified a domain in each protein (except mammalian MBD3) that could target it specifically to methylated CpG sites *in vitro* and *in vivo* (Hendrich and Bird 1998) (Nan, Meehan et al. 1993).

Chromatin immunoprecipitation analyses have shown that MeCP2 and MBD2 associate with loci in a manner that is strictly dependent on DNA methylation (Billard, Magdinier et al. 2002; El-Osta, Kantharidis et al. 2002; Rietveld, Caldenhoven et al. 2002). Studies with crude nuclear extracts support the notion that methyl-CpG binding proteins, such as MeCp2 MBD2 and MBD1, function as transcriptional repressors

because in their absence, methylated reporter genes are expressed (Boyes and Bird 1991). A critical breakthrough in understanding how methylation-mediated repression worked came from the finding that MeCP2 interacts with a co-repressor complex containing histone deacetylases (HDACs) (Jones, Veenstra et al. 1998; Nan, Ng et al. 1998). MBD2 also associates with HDACs, extending a relationship between histone modification and DNA methylation that is likely to be better understood in the future (Ng, Zhang et al. 1999; Feng and Zhang 2001).

Mbd2-null mice are viable and fertile, but lack the methylated DNA binding complex MeCP1 (comprises MBD2 plus the NuRD chromatin remodeling complex) (Ng, Zhang et al. 1999; Zhang, Ng et al. 1999; Hendrich, Guy et al. 2001). Cells deficient in MBD2 exhibit significant de-repression of endogenous genes and are defective in the methylated-mediated repression of transiently transfected genes. For example, MBD2 normally binds to the *Interleukin-4* (*Il4*) gene and in $Mbd2^{-/-}$ mice, inappropriate expression of *Il4* is found in a subset of naïve T helper cells (Hutchins, Mullen et al. 2002).

The past decade has seen considerable progress in our understanding of how DNA methylation is translated into functional states in the genome. The MBD family of proteins has been identified that bind to methylated DNA and associate with enzymes that alter the fundamental properties of chromatin. At a superficial level one could attribute the functional properties of methylated DNA to these MBD associated proteins.

However, in contrast to the profound effects of loss of DNMTs, the consequences of losing methyl-CpG binding proteins are much less severe. This discrepancy between MBD and DNMT null animals may be caused by redundancy within the MBD family or

alternatively other proteins that recognize DNA methyl marks may be involved. Recent studies support the view that repression is multilayered and involves many collaborating processes. If so, a combination of mutations in several methyl-CpG binding proteins should produce a more severe phenotype (Wade 2001; Jaenisch and Bird 2003).

MeCP2 and Rett syndrome

In October 1999, it was announced that over 80% patients with the neurological disorder Rett syndrome had mutations in the *MeCP2* gene (Amir, Van den Veyver et al. 1999). Rett syndrome is an X-linked neurological disorder occurring in heterozygous females that is characterized by normal early development, followed by a period of regression. Patients lose speech and purposeful hand movements while acquiring a variety of other neurological symptoms. Stabilization generally occurs and most patients survive into adulthood (Rett 1966; Hagberg, Aicardi et al. 1983; Hagberg, Goutieres et al. 1985). The condition is almost always due to spontaneous mutations in *MeCP2* that are clustered in three regions: the methyl binding domain (MBD), the transcriptional repression domain, or the C terminus. Notably, most missense mutations are tightly concentrated in the MBD domain, leading to reduced binding of MeCP2 to methylated DNA (Ballestar, Yusufzai et al. 2000). This suggests that binding to methyl-CpG is essential for MeCP2 function.

Mice with null mutations in *MeCP2* show phenotypic similarities to the human condition that includes delayed onset of symptoms, small brain size, reduced dendritic arborization, tremors, inertia, abnormal gait and arrhythmic breathing. Specific inactivation of *MeCP2* in the brain leads to the same phenotype as global deletion,

emphasizing the neurological basis of Rett syndrome (Chen, Akbarian et al. 2001; Guy, Hendrich et al. 2001; Shahbazian, Young et al. 2002).

On the basis of the genetic and biochemical properties of MeCP2, it has been widely believed that the symptoms of Rett syndrome are due to a failure of effective silencing of methylated genes. However, microarray analysis of brain transcripts from wild-type and *MeCP2* mutant mice has detected few if any gene expression differences (Tudor, Akbarian et al. 2002). Thus, it is possible that MeCP2 has a function in the brain that is not related to the repression of methylated genes. Alternatively, the absence of one methyl-CpG binding protein may be so well compensated by other related proteins that only subtle effects on gene expression occur, which are below detectable levels using current technology.

1.1.2 Imprinting

The genetic non-equivalence of the mammalian maternal and paternal genomes was uncovered by elegant pronuclear transplantation studies in the 1980s (McGrath and Solter 1984; Surani, Barton et al. 1984). This surprising revelation forced classical Mendelian genetics to undergo a number of revisions and led to the discovery of imprinting, defined as the monoallelic expression of a gene in a parent-of-origin specific manner. To date autosomal imprinting has only been demonstrated in eutherian (placental) mammals. However, female marsupials exhibit a form of genomic imprinting as they preferentially inactivate the paternal X chromosome in all somatic cells (Cooper, Woolley et al. 1983). A similar mode of imprinted X inactivation occurs in the

extraembryonic tissues of eutherian females and may represent the ancestral form of X inactivation (Takagi and Sasaki 1975).

1.1.2.1 The function of imprinting in mammalian development

A question of central importance to the field is the functional significance of genomic imprinting in mammals. The highly restricted developmental potential of androgenotes with two paternal genomes and gynogenotes or parthenogenotes with two maternal genomes was interpreted to mean that genomic imprinting was critical to development of mammals. It should be noted that androgenotes and parthenogenotes do not fail to imprint correctly or completely but rather have a genome-wide imbalance in the dosage of imprinted genes. For example, the androgenotes will have double the dosage of paternally expressed genes and no expression of maternally expressed genes. The same condition holds for single or partial chromosomal uniparental disomies, which have been extensively studied in mice, and often lead to developmental anomalies (Reviewed in Surani 1994 and Latham, McGrath et al. 1995). This leaves open the possibility that imprinting is dispensable under conditions where the imprints on both parental genomes are erased (Jaenisch 1997). There is some indirect evidence for this, parthenogenetic embryos that are generated from nuclei of immature oocytes at a stage in development when imprints are not fully established develop to a later stage than parthenogenotes from more mature oocytes whose imprints are in place (Kono, Obata et al. 1996; Kono, Sotomaru et al. 2002).

Figure 1.5 Mouse chromosome localization of imprinted genes

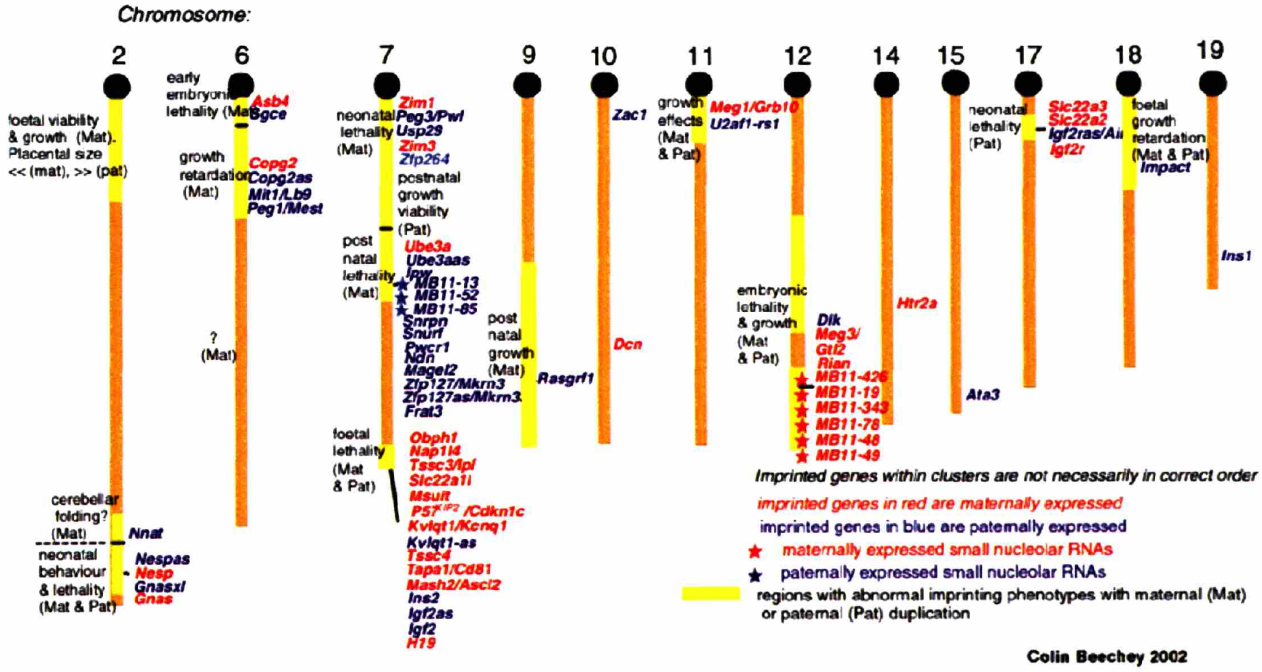


Figure1.5 Mouse chromosome localization of imprinted genes.
Shows the distribution of imprinted genes in clusters on chromosomes throughout the mouse genome. Imprinted genes that are maternally expressed are shown in red and imprinted genes shown in blue are paternally expressed.

In addition, adult mice have been cloned from migrating primordial germ cells at different stages of migration demonstrating that partial erasure of the methylation associated with imprinting is competent to support full development (Yamazaki, Low et al. 2005). To determine the overall function of imprinting, studies of individual imprinted genes have been made (**Figure 1.5; Appendix 1**). Of these genes, which now numbers over 30, a trend has emerged where many of these genes fall into one of two categories. Particularly striking is the number of imprinted genes whose protein directly acts within the fetal growth pathway mediated by insulin like growth factor-2 (Igf2).

This includes *Igf2* itself and three genes that modulate its activity, the *mannose-6-phsophate/Igf2 receptor* (*Igf2r*), *Grb10* and *H19*. Targeted mutations in each of these genes confirm that these genes affect the rate of fetal growth (DeChiara, Efstratiadis et al. 1990; Filson, Louvi et al. 1993; Lau, Stewart et al. 1994; Leighton, Ingram et al. 1995). In addition, a group of genes including $p57^{kip2}$, *Gnas, Rasgrf1* and *Mash2* are involved in regulating fetal overgrowth and/or cell cycle (Tilghman 1999). Unlike egg laying animals, mammalian embryos develop in an almost parasitic-like manner and nutrients that flow from the mother's placenta to the developing embryo must be regulated. So it stands to reason that a new mechanism most be imposed to regulate embryonic growth as failure to do so could be lethal to both mother and offspring.

Imprinting genes have recently been identified in a new role that is emerging in the development of the brain. The first indications that this was likely came from human behavior studies on children with Prader-Willi (PWS) and Angelman (AS) syndromes. Both diseases are often caused by large megabase deletions at 15q11-q13, but in the case of PWS, the deletion occurs on the paternal chromosome, whereas in AS, the affected

chromosome is always of maternal origin (Lalande 1996; Nicholls, Saitoh et al. 1998). Children with PWS are hypotonic, fail to suckle and develop obesity and mental retardation with time. In addition they have short stature and small hands and feet. AS patients are ataxic, hyperactive and much more severely retarded. Targeted deletions of the syntenic chromosomal region in the mouse appear to recapitulate the phenotypic characteristics of both diseases (Jiang, Armstrong et al. 1998; Yang, Adamson et al. 1998). These data suggest that PWS is caused by the loss of expression of multiple paternal specific genes in this region, whereas AS may solely be due to the loss-of-function of a single gene, *Ube3a*. *Ube3a* is imprinted exclusively in the brain and encodes a ubiquitin ligase (Rougeulle, Glatt et al. 1997; Vu and Hoffman 1997).

Knowledge regarding the function of imprinting has been expanded through chimera studies of androgenetic and pathenogenetic embryos. Overall, researchers observed that androgenetic chimeras are oversized and exhibit striking muscle hypertrophy. In contrast gynogenetic chimeras are growth retarded, consistent with a role for imprinted genes in fetal growth. When cellular distribution studies were under-taken, differences in the number and tissue contribution of uniparental cells in the brain were found. Androgenetic cells inhibited overall brain size and contributed primarily to the hypothalamus but not to the cortex. Gynogenetic cells appeared to enhance forebrain growth and were more likely to contribute to the cortex, striatum and hippocampus (Mann, Gadi et al. 1990; Allen, Logan et al. 1995; Keverne, Fundele et al. 1996). Although these data come about as a result of imbalanced imprinting, where genes are either over or under expressed, it is possible to hypothesize from this work that

imprinting may have contributed over time to the rapid expansion of the cortex relative to other parts of the brain.

Recent evidence has suggested that the role of imprinting in regulating embryonic growth may extend into the next generation by influencing the maternal behavior. Disruption of the imprinted gene *Peg1*, which is expressed broadly in the embryo but restricted to the brain in adults, was shown to cause a general retardation of embryonic growth. Surprisingly, the mutation in adult females is associated with poor maternal care, including impaired placentophagia. Interestingly *Peg1* expression is high in the adult hypothalamus, an organ that has been implicated in maternal behavior (Lefebvre, Viville et al. 1998).

1.1.2.2 The evolution of genomic imprinting

Haig and colleagues suggested a theory for the evolution of imprinting by drawing a connection between the growth effects displayed by mutations in some imprinted genes and the behavioral effects in others. They proposed that imprinting will arise in polyandrous animals as result of a conflict between males and females over the allocation of maternal resources to offspring. Fathers will favor strategies for extracting the maximal amount of resources for their offspring at the expense of the mother and her future offspring by other sires. The mother counters this by using imprinting to allocate resources equally among all her litter. This struggle results in a comprised growth rate, leading to the suggestion that imprinting is a genetic 'tug-of-war' (Moore and Haig 1991).

According to Haig's model, paternally expressed genes should promote growth and maternally expressed genes should negatively affect growth during the period where the mother is solely responsible for the embryo's nutritional resources. This prediction holds true for a number of imprinted genes **(Appendix I),** of which none had been identified at the time of Haig's proposal. Some of the most striking examples include the paternally expressed *Igf2* gene, a potent growth factor, that has a severe growth reduction phenotype when disrupted in mice (DeChiara, Efstratiadis et al. 1990), and its maternally expressed receptor *Igf2r*, in which mutations lead to oversized and nonviable embryos (Filson, Louvi et al. 1993; Lau, Stewart et al. 1994; Wang, Fung et al. 1994). The Igf2r protein is not involved in signal transduction but acts to sequester excess Igf2 and transport it to lysosomes for destruction. The most convincing piece of data with respect to the parental conflict model comes from the double mutant, which is normal-sized and viable (Filson, Louvi et al. 1993). In addition to many imprinted genes being directly involved in fetal growth, almost all are also imprinted in the placenta, a critical organ for allocating maternal resources throughout embryogenesis (Tilghman 1999).

However there are a number of imprinted genes that do not fit into the parental conflict model quite so neatly. For example, *Peg1* mutations affect both embryonic growth and adult maternal behavior (rearing, nesting, and feeding behaviors). Although this could be viewed as the father ensuring the expression of a gene in his daughters that is beneficial to his grandchildren (Lefebvre, Viville et al. 1998), it is not immediately apparent why the mother would choose to silence this gene, as they share a common genetic interest in the offspring. *Snrpn*, which encodes a paternal specific protein that functions in RNA processing and is located within the deletion found in PWS, represents

another example. *Snrpn* is widely expressed in the embryo but contrary to what is expected from the parental conflict model, targeted mutagenesis does not result in any deleterious phenotype. However, a deletion in the promoter of *Snrpn* affects the expression of a number of other paternally expressed imprinted genes located nearby. As a result, the phenotype of the *Snrpn* promoter-deleted mice resembles PWS, including a failure to suckle their young (Yang, Adamson et al. 1998). Thus, it is possible that although *Snrpn* itself is not necessary for embryonic development, its imprinted regulation may be required for the parental specific expression of neighboring genes (Tilghman 1999).

Because the Haig hypothesis is based on the polyandrous nature of the first mammals, it follows that imprinting should not be found in monogamous species, as both parents have a common interest in all offspring so therefore there is no conflict. To test the parental conflict model, imprinting was examined in the North American beach mouse (*Permyscus polionotus*), a rare example of a monogamous mammal. Imprinting was retained for the *Igf2*, *Igf2r* and *H19* loci just as in the *Mus musculus* (Vrana, Guan et al. 1998), thus one prediction of the parental conflict model was not met. However, this finding needs to be interpreted carefully, as even limited partner exchange is sufficient to induce parent offspring conflict. Indeed, partner exchange has been observed when *P. polionotus* females have been captured on their own and then presented with new males (Tilghman 1999). Interestingly, when these rodents are bred to the polyandrous *Peromyscus maniculatus*, the hybrid offspring demonstrate significant disruption in imprinting, suggesting that signals governing genomic imprinting are rapidly evolving

and that disruptions in the process may contribute to mammalian speciation (Vrana, Guan et al. 1998).

1.1.2.3 The establishment and erasure of the methylation mark in the germ line.

The study of the mechanism behind the establishment, erasure, and re-setting of imprinting is a rapidly expanding field. A number of studies have shown the importance of CpG methylation in maintaining the imprinted status of genes in the soma and it seems likely that this would also be the case in the gamete (Reik, Dean et al. 2001). Consistent with this, *cis* methylation marks are different in the egg and sperm and are maintained during early embryogenesis when global methylation is lost (Tilghman 1999). More definitive functional studies have confirmed the importance of these *cis*-acting elements for the maintenance of imprinting by generating transgenic mice or targeted mutations (Wutz, Smrzka et al. 1997; Thorvaldsen, Duran et al. 1998; Yang, Adamson et al. 1998). The differentially methylated regions (DMRs) between different imprinted genes show very little similarity, with the exception of the high density of CpG dinucleotides that are necessary to maintain the methylation imprint. The mechanisms responsible for initiating the methylation of DMRs are currently unknown.

Primordial germ cells (PGCs) are set aside very early during mouse embryonic development, and up until E10.5 they are identical in both males and females. After this stage of development, the methylation that maintains imprinting in PGCs is erased and biallelic expression of imprinted genes can be detected (Brandeis, Kafri et al. 1993; Tada, Tada et al. 1998; Davis, Yang et al. 2000; Ueda, Abe et al. 2000). How this occurs is as of yet unclear, imprint methylation marks could be lost passively through the inhibition

of Dnmt1 and successive rounds of replication or by an active process of demethylation. Indeed there is circumstantial evidence to suggest that both mechanisms might be employed (Kafri, Gao et al. 1993; Rougier, Bourc'his et al. 1998) but no DNA demethylase has been identified. A number of researchers have made use of germ cell lines in culture that have been derived from PGCs at different stages of embryonic development (known as embryonic germ (EG) cells). The study of EG cells overcomes the problem of examining an extremely small population of cells but results should be viewed with caution, as the process of culturing could induce epigenetic changes not present in PGCs. Despite this, Tada and colleagues showed that EG cell lines derived from E12.5 embryos contain an activity that could initiate demethylation of both imprinted and non-imprinted DNA in nuclei of somatic cell hybrids, this finding is consistent with the presence of either a demethylase or an inhibitor of Dnmt1 in EG cells (Tada, Tada et al. 1997).

After erasure, *de novo* methylation begins in both germ lines at the late fetal stages and continues after birth (Kafri, Ariel et al. 1992; Brandeis, Kafri et al. 1993). Oocytes are in meiotic arrest and methylation occurs during their growth (Obata, Kaneko-Ishino et al. 1998), whereas during spermatogenesis, methylation occurs before meiosis (Davis, Yang et al. 2000; Ueda, Abe et al. 2000). Nuclear transplant experiments indicate that this DNA methylation coincides roughly with the acquisition of functional imprints both for autosomal genes and for X chromosome imprinting (Obata, Kaneko-Ishino et al. 1998; Tada, Obata et al. 2000). Thus far, evidence suggests that the establishment of the methylation imprints is dependent on the expression of *Dnmt3L*, which by itself has no detectable DNA methyltransferase activity but cooperates with the

de novo methyltransferase DNMT3a to establish maternal imprints (Bourc'his, Xu et al. 2001; Hata, Okano et al. 2002).

1.1.2.4 Reading mechanisms for imprint gene silencing.

1.1.2.4.1 Short-range

Once imprints are introduced in the parental germlines, maintained in the early embryo and then fully matured during differentiation, they need to be 'read'. This is defined as a the conversion of methylation and chromatin codes into differential gene expression, which at this point is thought to only take place at the level of transcription (Jouvenot, Poirier et al. 1999). Generally, the 'reading' mechanisms of imprinted genes are complex and often involve the regulation of more than one gene that is clustered in a particular region. The reading mechanisms of DMRs described to date, fall into four different categories: promoter sequences, overlapping antisense transcripts, boundary elements and silencers. **(Fig 1.6)**

Promoter sequences.

Promoter regions are often rich in CpG regions, thus one of the commonest mechanisms for achieving transcriptional silencing of one allele is by promoter methylation. How a methylated promoter is transcriptional repressed is poorly understood, although it is clear that methyl-binding proteins (MBPs) are able to form complexes with histone deacetylases (Reik and Walter 2001). Thus, it is assumed that MBP-HDAC complexes lead to a closed chromatin conformation that prevents transcription factor access to the promoter (Bird and Wolffe 1999). Consistent with this,

differences in specific patterns of histone methylation and acetylation have been described between alleles of imprinted genes (Reik and Walter 2001).

Antisense Transcripts.

Regulation by antisense RNA is associated with a number of imprinted genes. Thus far, all antisense transcripts associated with imprinted loci are themselves imprinted and monoallelically expressed regardless of the parental expression of the imprinted gene they regulate. Most of these antisense transcripts are noncoding and thought to have some regulatory function (Reik and Walter 2001). Two of the best studied examples of reciprocally-imprinted antisense transcripts are *Air* and *Kcnq1ot1*, which overlap with the maternally-expressed *Igf2r* and *Kcnq1* genes, respectively (Lee, DeBaun et al. 1999; Smilinich, Day et al. 1999; Lyle, Watanabe et al. 2000). Both of these antisense RNAs originate in introns of the respective sense strand genes. The *Air* antisense transcript includes some of the *Igf2r* promoter (Lyle, Watanabe et al. 2000), but this is not the case for *Kcnq1ot1* (Engemann, Strodicke et al. 2000). The promoter regions of the antisense transcripts are CpG-rich and are differentially methylated on the inactive maternal allele. Deletions of both of these DMRs leads to a loss of expression of the antisense transcript and a loss of imprinting of *Igf2r* and *Kcnq1* (Wutz, Smrzka et al. 1997) (Horike, Mitsuya et al. 2000). The exact mechanism of how the antisense transcript interferes with the expression of the sense gene is not known, but various scenarios could be envisioned including the antisense transcript causing alterations to the chromatin structure, DNA methylation, promoter exclusion or even RNA interference.

Figure 1.6 Reading mechanisms in imprinted genes

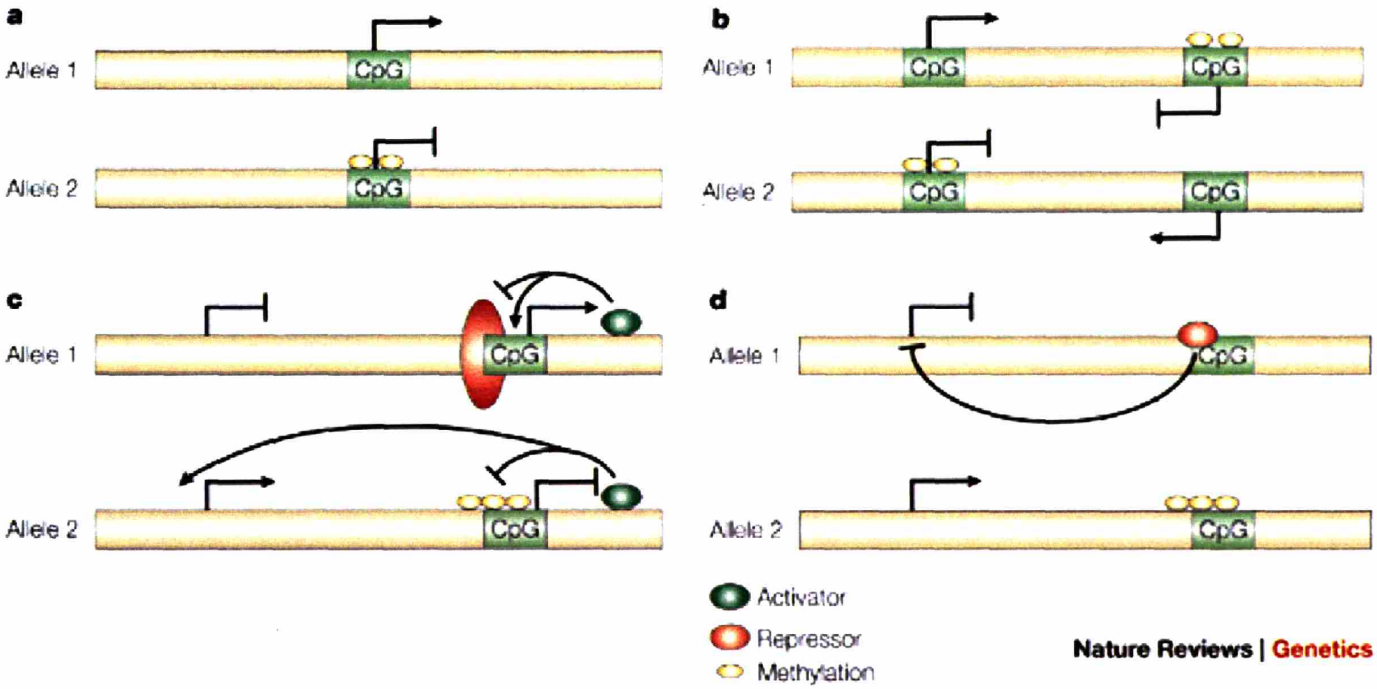


Figure 6 I Reading mechanisms in imprinted genes.
a I Differential silencing by CpG island or promoter methylation. b I Regulation by anti-sense transcripts in conjunction with CpG island or promoter methylation; c I Allele-specific regulation of neighbouring genes by dif-ferential methylation of boundary elements within a CpG island. Factors such as CCCTC-binding factor (CTCF) (red disc) bind to the unmethylated allele and block the access of upstream promoters to down-stream enhancers (green), leading to transcriptional repression of the upstream gene. d I Differential methylation results in differential binding of silencing factors (red, in this case methylation-sensitive), which repress the promoter in cis. Adapted from Reik and Walter 2001.

It is also possible that the antisense transcripts have no role by themselves but instead reflect the activity of other repressor sequences, such as silencers and boundary elements (Reik and Walter 2001). This might explain how the deletion of the *Kcnq1ot1* DMR leads not only to the loss of repression of *Kcnq1,* but also *Cdkn1c*, a neighboring non-overlapping imprinted gene (Horike, Mitsuya et al. 2000).

Boundaries

A recent observation that endoderm specific enhancers can be shared between the physically linked genes *Igf2* (paternally expressed) and *H19* (a maternally expressed non-coding gene), suggests that chromatin boundaries might be involved in imprinted gene regulation. The region upstream of *H19* is paternally methylated and this is associated with expression of the neighbouring *Igf2* gene. When this region in deleted from the maternal allele, the normally silent *Igf2* gene is expressed (Thorvaldsen, Duran et al. 1998). This led to the model that the DMR associated with *H19* is a chromatin boundary element that is in a 'closed' state when unmethylated but is in an 'open' state when methylated. Several DNaseI hypersensitive sites have been found on the unmethylated maternal *H19* DMR region and these are not present on the methylated paternal allele (Hark and Tilghman 1998; Khosla, Aitchison et al. 1999). In addition, the previously characterized chromatin boundary element CTCF, which has been shown to be important for the function of the chick *globin* gene boundary element, binds to the maternal but not to the paternal *H19* DMR (Bell and Felsenfeld 2000) (Hark, Schoenherr et al. 2000; Kanduri, Pant et al. 2000; Szabo, Tang et al. 2000). Furthermore, deletion studies have shown that loss of the CTCF-binding motifs in the *H19* DMR abolishes the observed

boundary effect (Bell and Felsenfeld 2000) (Hark, Schoenherr et al. 2000; Kanduri, Pant et al. 2000; Szabo, Tang et al. 2000).

Silencers or enhancer competition.

A number of imprinted genes have DMRs associated with the expressed allele and this has led to the suggestion that these regions contain silencers (ie elements bound by repressors) that are inactivated by methylation (Sasaki, Jones et al. 1992; Brandeis, Kafri et al. 1993; Stoger, Kubicka et al. 1993; Feil, Walter et al. 1994). This model has proved correct for the DMR1 of the *Igf2* gene, which is paternally expressed and methylated in a number of fetal tissues such as the heart, kidney and lung. The *Igf2* DMR1 functions as a maternal silencer in a subset of these tissues, as its deletion in mice results in repression of expression (Constancia, Dean et al. 2000).

1.1.2.4.2 Long range or imprint control regions.

The existence of imprint control regions (ICRs) was first proposed following molecular and genetic studies of imprinting disorders and targeted mutagenesis analyses in mice (Buiting, Saitoh et al. 1995; Leighton, Ingram et al. 1995; Yang, Adamson et al. 1998). It was observed that the expression of certain imprinted genes is dependent upon a signal that acts over large distances. Patients with the imprinting disorder PWS have small microdeletions of the promoter and the first exon of the *SNRPN* gene, which results in the loss of expression of not only *SNRPN,* but at least three other paternally-expressed genes that lie on the same chromosome. Similarly, AS patients were found to have deletions a few kilobases upstream of where the PWS microdeletions are found. For

presentation of the disease phenotype, paternal transmission of the deletion is required for PWS, whereas AS requires maternal transmission. The ability of these deletions to alter the expression and methylation patterns of multiple imprinted genes that lie as far away as 1 megabase, is referred to as 'epigenotype spreading' (Buiting, Saitoh et al. 1995). In the PWS patients, these genes (which are normally paternally-expressed) are found silenced and methylated. Correspondingly, in AS patients, maternal transmission of the deletion results in the expression and demethylation of genes that are otherwise repressed. These results have been recapitulated in the mouse model of the PWS deletion (Yang, Adamson et al. 1998). Based on these observations it has been proposed that the ICR region functions to switch the regional epigenotype in the germline (i.e. in PGCs during embryogenesis) or alternatively, it plays a role in the maintenance of the epigenotype (Reik and Walter 2001). However, at this point it is still unknown whether epigenotype spreading occurs in the germline or postzygotically. In addition, little is known about the factors that mediate this phenomenon.

1.2 Cancer biology

1.2.1 DNA methylation and cancer

Over the last five years it has become clear that the traditional model of cancer initiation and progression, involving multiple changes in chromosomal instability, activation of oncogenes, silencing of tumor suppressors and the inactivation of DNA repair machinery, is not only caused by genetic mutations but also by epigenetic abnormalities (Feinberg and Tycko 2004). During cancer progression, it has been noted that the genome undergoes both global hypomethylation and regional hypermethylation

at CpG islands (Feinberg and Tycko 2004). Alteration to the methylation state of the genome can eventually have both genetic and epigenetic consequences for the cell and carcinogenesis.

1.2.1.1 Hypomethylation

A large body of evidence now exists to show that hypomethylation of DNA leads to the activation of otherwise silent genes (Feinberg and Vogelstein 1983). CpG islands that are known to be methylated in somatic tissues have recently been shown to be hypomethylated in cancer, causing the activation of nearby genes (Strichman-Almashanu, Lee et al. 2002). Examples of genes that are affected by promoter CpG demethylation include the oncogene *H-Ras* (Feinberg and Vogelstein 1983), *Cyclin D2* (Oshimo, Nakayama et al. 2003), *maspin* in gastric carcinoma (Akiyama, Maesawa et al. 2003), *CA9* in human renal-cell carcinoma and human papillomavirus 16 (HPV16) in cervical cancer (Badal, Chuang et al. 2003).

Recently, DNA hypomethylation has been shown to cause genomic instability. This link was made when Wilm's tumors, ovarian and breast carcinomas were found to have significant demethylation within the pericentric region of satellite repeat sequences (Qu, Grundy et al. 1999). This demethylation is frequently associated with unbalanced chromosomal translocations with the breakpoint occurring in the pericentric regions of chromosome 1 and 16 (Qu, Grundy et al. 1999). These unbalanced translocations are often the only detectable abnormality found and result in loss-of-heterzygosity (LOH) for chromosome 16 and a characteristic anaplasia (Yeh, Wei et al. 2002).

The most direct evidence for genomic instability due to hypomethylation has come from the study of cells that express very low levels of *Dnmt1*. DNA hypomethylation caused by an insufficiency of Dnmt1 leads to increased mitotic recombination and LOH due to chromosomal translocations (Eden, Gaudet et al. 2003; Gaudet, Hodgson et al. 2003). It should be noted however, that a transgene in hypomethylated ES cells showed no evidence of LOH. This discrepancy could be due to the chromosomal location of the transgene and/or the differentiation state of the cell (Chan, van Amerongen et al. 2001).

1.2.1.2 Hypermethylation

As discussed earlier, DNA methylation at CpG islands is believed to be the major mechanism for maintaining the silent epigenetic state. In support of this, demethylating drugs result in the reactivation of silenced genes. The role of hypermethylation in cancer progression was pioneered with studies relating to the classic tumor-suppressor retinoblastoma (*RB*) gene. The Horsthemke study in the 1989 showed that the *RB* promoter is methylated in a significant number of retinoblastomas (Greger, Passarge et al. 1989). The magnitude of this epigenetic silencing was appreciated when it was demonstrated that there was a 92% reduction of *RB* expression in tumors with promoter hypermethylation (Ohtani-Fujita, Fujita et al. 1993; Greger, Debus et al. 1994). More recent studies have confirmed promoter hypermethylation at numerous other loci in cancer cells supporting the notion that epigenetic gene inactivation contributes to cancer progression. Key tumor suppressor proteins such as $p16^{Ink4a}$, MLH1, VHL and E-cadherin were all shown to be eliminated in both cell lines and in primary cancers by an

epigenetic pathway that correlates with dense CpG methylation of their respective promoters (Herman, Latif et al. 1994; Gonzalez-Zulueta, Bender et al. 1995; Graff, Herman et al. 1995; Cunningham, Christensen et al. 1998).

1.2.1.3 Loss of imprinting

Evidence for a role of human imprinted genes in cancer formation came in the 1980s when several independent studies reported a strong parent-of-origin bias in LOH for chromosome 11p15 alleles in Wilm's tumor and rhabdomyosarcoma. The finding that there was consistently a loss of maternal alleles and a duplication of paternal alleles, led to the suggestion that loss of imprinting (LOI) was the likely cause (Schroeder, Chao et al. 1987; Pal, Wadey et al. 1990) (Scrable, Cavenee et al. 1989; Williams, Brown et al. 1989). In addition, studies of familial Beckwith-Widemann Syndrome (BWS), which causes prenatal overgrowth and leads to a predisposition of various embryonal tumors including Wilm's tumor (Brown, Williams et al. 1990), also indicated a parent-of-origin effect, as the overgrowth phenotype was only seen after maternal transfer (Brown, Williams et al. 1990). More direct evidence came from mapping BWS to 11p15 and the demonstration that chromosomal rearrangements in this region in patients with BWS were all of maternal origin (Mannens, Hoovers et al. 1994). More recent cancer studies have focused on LOI at single loci, including *Igf2*, *Igf2r* and *Peg3*.

1.2.1.3.1 Insulin-like growth factor-2

LOI leading to biallelic expression of *IGF2* in Wilm's tumors was discovered in the early 1990s (Ogawa, Eccles et al. 1993; Rainier, Johnson et al. 1993). Additional

studies demonstrated that this abnormality was always linked to a gain of DNA methylation in the closely linked *H19* gene (Moulton, Crenshaw et al. 1994; Steenman, Rainier et al. 1994). Overexpression of *Igf2* is known to induce growth, however recent work has suggested that the mechanism of tumor progression may actually be due to an inhibition of apoptosis. Hanahan and colleagues found that knocking out one allele of *Igf2*, which prevents biallelic expression, arrested cancer progression in a B-lymphocyte tumor model by increasing apoptosis (Christofori, Naik et al. 1994; Christofori, Naik et al. 1995). In more recent mouse studies, it was shown that the number of intestinal tumors that form in $Apc^{+/min}$ mutant mice was increased when *Igf2* was biallelically expressed (Sakatani, Kaneda et al. 2005). Taken together, these studies suggest that a loss of normal imprinting at the *Igf2/H19* locus contributes to cancer progression.

1.2.1.3.2 Insulin-like growth factor-2 receptor and peg3

The mannose 6 phosphate/insulin-like growth receptor (Igf2r) is an intriguing protein with multiple ligands and multiple functions. Approximately 90-95% of the receptor is located intracellularly, with 5-10% being on the cell surface. It is known to be essential for the transport of newly-synthsized lysosomal enzymes from the trans-golgi network to the lysosomes. More recently however, the loss of this receptor has been described in some tumor types. Jirtle and colleagues have clearly demonstrated LOI and LOH at the *Igf2r* locus in breast, lung and liver tumors (De Souza, Hankins et al. 1995; De Souza, Hankins et al. 1995; Hankins, De Souza et al. 1996; Yamada, De Souza et al. 1997; Kong, Anscher et al. 2000). However, not all tissues display a loss of *Igf2r*

following cancer transformation, raising the possibility that in these tumors, *Igf2r* expression confers a selective advantage.

Peg3 is a paternally imprinted gene that encodes a protein with 12 C_2H_2 Kruppel-like zinc fingers and two proline-rich repeat domains (Kuroiwa, Kaneko-Ishino et al. 1996; Relaix, Weng et al. 1996). Peg3 physically interacts with tumor necrosis factor (TNF)-receptor-associated factor 2 (TRAF2), which is involved in the TNF-nuclear factor pathway (Relaix, Wei et al. 1998) and mediates apoptosis via p53 (Relaix, Wei et al. 2000). *Peg3*-deficient mice are viable, albeit smaller than their wild-type siblings, and adult female mutants display impaired maternal behaviors such as nest building, gathering pups and keeping them warm (Li, Keverne et al. 1999). Interestingly, *PEG3* maps to 19q13.4, a region that contains a putative tumor suppressor of oligodendrogliomas (Maegawa, Yoshioka et al. 2001; Sanson, Leuraud et al. 2002). Consistent with this, *PEG3* has tumor suppressor activity in transfected cells and is epigenetically silenced in gliomas (Kohda, Asai et al. 2001) (Maegawa, Yoshioka et al. 2001).

1.2.2 Genetic mechanisms of cancer.

The cancer phenotype is characterized by a number of features. Compared with many normal tissues, cancer cells are highly sensitized to apoptotic signals and survive only because they have acquired lesions that prevent or impede cell death. Cancers are also able to invade and colonize the surrounding tissues. By comparing cells that are derived from cancer patients with those from normal individuals and by utilizing mouse

models of key cancer regulators, it has been possible to gain significant insights into the molecular and cellular nature of the cancer phenotype.

1.2.2.1 DNA repair, cell-cycle checkpoints and cancer.

DNA-repair and cell-cycle checkpoint pathways allow cells to deal with both endogenous and exogenous sources of DNA damage. In addition to directly repairing DNA breaks or adducts, cells respond to DNA damage by halting cell-cycle progression or by undergoing programmed cell death. How much an individual is exposed to these agents and how their cells respond to DNA damage are critical determinants of whether that individual will go on to develop cancer.

1.2.2.1.1 DNA Repair

The DNA contained in every mammalian cell is under constant attack by agents that can either directly damage one of its three billion bases or break the phosphodiester backbone on which the base resides. Because there are various types of DNA lesions that can occur, a variety of different repair mechanisms exist to combat the damage.

DNA can be damaged in at least three ways. Firstly, energy by free oxygen radicals can break the phosphodiester bonds in the backbone of the DNA helix. When two of these are close to each other but on the opposite strand, a double-strand break (DSB) is present in the DNA and the cell faces a challenging repair (Doll and Peto 1981). Secondly, alkylating chemical moieties can modify purine bases and thirdly, inhibitors of DNA topoisomerases can lead to enhanced single or DSBs depending on which topoisomerase is inhibited and on the phase of the cell cycle (Froelich-Ammon and

Osheroff 1995). Different mechanisms are required to repair the damage to the DNA backbone or to the DNA bases, and the challenges of repairing the DNA can vary in the different phases of the cell cycle. To optimally repair DNA damage, the cell must also control other cellular processes before or during the repair, such as DNA replication or mitosis. Cells that are damaged when they are in the middle of the process of DNA replication face this challenge by halting or slowing replication until the damage has been repaired (Kastan and Bartek 2004).

The first steps leading to an inhibition of the cell-cycle following DNA damage include activation of the phosphatidyl-inositol-3-OH kinase-like kinases (PIKKs), ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3 related). The ATM kinase is primarily activated following DNA damage, whereas the ATR kinase seems to be critical for mediating the cellular response to an arrest in DNA replication-fork progression (caused by DNA damage or other stresses). Due to the many types of lesions that can result from direct DNA damage and from an arrest of DNA replication forks, ATM and ATR seem to participate together in many cellular-stress responses (Abraham 2001; Shiloh and Kastan 2001).

To efficiently transduce the alert signal and initiate a global cellular response to DNA damage, proximal checkpoint kinases ATM and ATR cooperate closely with two other classes of proteins, the checkpoint mediator (or adaptor) proteins and the transducer kinases CHK1 and CHK2 (Bartek and Lukas 2003; Shiloh 2003).

The study of checkpoint mediators is an emerging field, with the precise mechanism of action largely unknown. Thus far, there are three mediator checkpoint factors (MDC1, 53BP1 and BRCA1) involved in facilitating the signaling of ATM and

one protein, claspin known to modulate ATR (DiTullio, Mochan et al. 2002; Wang, Matsuoka et al. 2002; Goldberg, Stucki et al. 2003; Lou, Minter-Dykhouse et al. 2003; Manke, Lowery et al. 2003; Shang, Bodero et al. 2003; Yu, Chini et al. 2003; Chini and Chen 2004). As most of the mediators are initially recruited to the site of DNA damage independently of ATM and ATR, they might be involved in identifying these lesions or recruited through their interaction with candidate DNA-damage sensors (Goldberg, Stucki et al. 2003; Kastan and Bartek 2004).

The transducing kinases CHK1 and CHK2 are activated by ATM and ATR phosphorylation and in turn regulate downstream checkpoint targets, affecting the machinery involved in DNA repair, apoptosis and the cell cycle (Bartek and Lukas 2003). Most prominent among their targets, tanscription factor p53 and the family of the cyclin/CDK activator proteins CDC25 (Bartek and Lukas 2003).

1.2.2.1.2 Cell cycle checkpoints

There are four successive phases of a typical mammalian cell cycle: M phase consists of nuclear division followed by cytoplasmic division, after which the daughter cells begin interphase of a new cell cycle. Interphase starts with the G1 phase in which biosynthetic activities resume after cellular division. The S phase begins when DNA synthesis starts and ends when the DNA content of the nucleus has doubled, resulting in chromosome replication. The cell then enters G2 phase with continued biosynthetic activities until mitosis starts initiating M phase.

During unperturbed proliferation, a mammalian cell can only withdraw from the cell-cycle in the early- to mid-G1 phase in response to growth-factor deprivation or

growth inhibitory signals. This must occur before the cell passes through a restriction point controlled by the transcription factors retinoblastoma (RB) and E2F. After this point, the cell is committed to a round of DNA replication and cell division. Given the critical significance of error-free DNA replication and chromosome segregation for the maintenance of genomic integrity and the prevention of cancer, it is not surprising that these stages of the cell cycle are protected by a number of check-point effector mechanisms (Bartek, Bartkova et al. 1997; Sherr and McCormick 2002).

The dominant checkpoint response to DNA damage in the G1 phase of the cell cycle is the ATM/CHK2-p53/$p21^{Cip1}$ pathway, which is capable of inducing sustained and sometimes permanent G1 arrest (Kastan, Lim et al. 2000; Bartek and Lukas 2003). The intra-S-phase checkpoint network, which is activated by genotoxic insults, causes largely transient, reversible inhibition of the DNA origins-of-replication that have not yet been initiated. There are two parallel branches of this checkpoint that slow down the ongoing DNA synthesis, both of which are controlled by the ATM/ATR signaling machinery (Bartek and Lukas 2003; Bartek, Lukas et al. 2004). Finally, the G2 checkpoint prevents cells from initiating mitosis when they experience DNA damage during G2, or when they progress into G2 with some unrepaired damage inflicted during the previous S or G1 phases (Nyberg, Michelson et al. 2002; Xu, Kim et al. 2002). The critical target of the G2 checkpoint is the mitosis promoting activity of the cyclin B/CDK1 kinase. CyclinB/CDK1 activation is inhibited after various stresses by ATM/ATR, CHK1/CHK2 and the inactivation of the CDC25 family of phosphatases, responsible for activating CDK1 (Smits and Medema 2001).

1.2.2.2 Key tumor suppressors and their role in the cell cycle.

The proteins p53, Rb, $p16^{Ink4a}$, $p19^{Arf}$ and $p21^{Cip1}$ reside at the core of a signaling network that principally governs cell-cycle entry and exit. Loss-of-function of *$p16^{Ink4a}$*, *$p19^{Arf}$*, *Rb* and *p53* occurs frequently in cancers, prompting speculation that biochemical pathways regulated by these proteins must be disabled in order for normal cells to be transformed into tumor cells **(Fig 1.7)**.

1.2.2.2.1 Tumor suppressor p53.

The p53 tumor suppressor belongs to a small family of structurally and functionally related proteins that includes two other members p63 and p73 (Melino, De Laurenzi et al. 2002). Whereas p63 and p73 play important roles in embryonic development (Irwin and Kaelin 2001), p53 has evolved in higher organisms to prevent tumor development. Activation of *p53* occurs in response to several malignancy-associated stress signals, resulting in the inhibition of cell growth (Balint and Vousden 2001). Several cellular responses can be invoked by p53, including cell-cycle arrest, senescence, differentiation and apoptosis, with the option chosen dependent on many factors that are both intrinsic and extrinsic to the cell (Vousden and Lu 2002). Under some circumstances, p53 also contributes to the repair of genotoxic damage, potentially allowing for the release of the rehabilitated cell back into the proliferating pool. However, in most cases induction of p53 leads to an irreversible inhibition of cell growth, most decisively by activating apoptosis.

Figure 1.7 Cell cycle circulatory

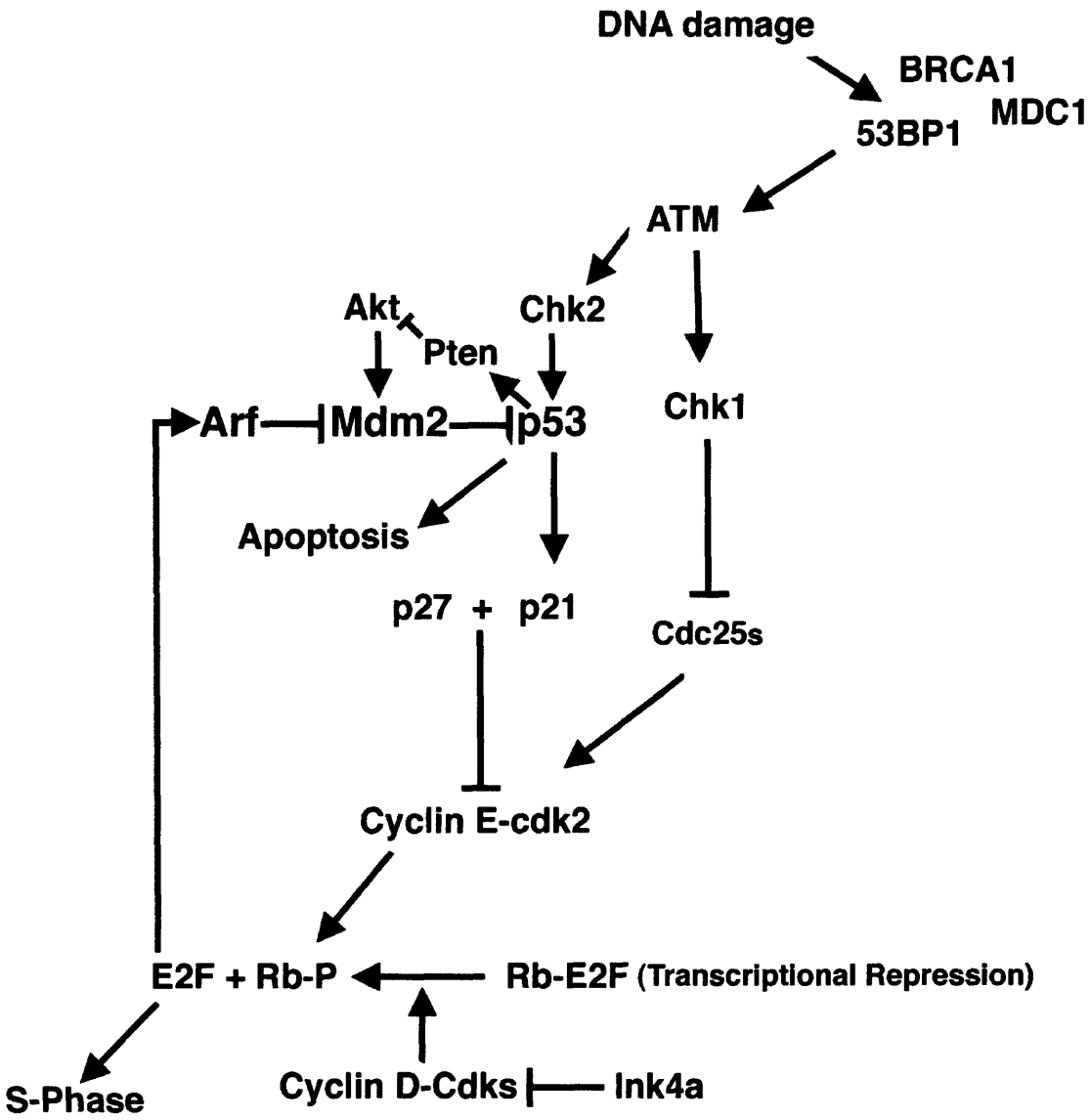


Figure 1.7 Cell Cycle Circulatory
Many of the key regualtors associated with cell cycle arrest at the G1-S phase transition.

Mutations of the p53 gene

Loss of p53 function in cancer can occur by a number of mechanisms, including lesions that prevent induction of p53, mutations within the gene itself or mutations in the downstream targets of p53 (Harris and Levine 2005). Analyses of many tumors have shown that *p53* is mutated in about half of all cancers, resulting in a loss of apoptotic function. Tumor-associated mutations in *p53* are predominantly point mutations that result in single amino-acid substitutions. The mutational spectrum seen in *p53* is very different from other tumor suppressor genes in which large deletions or frame-shift mutations tend to result in the complete loss of protein expression (Vousden and Lu 2002). The result of *p53* mutation by single amino acid substitutions is that many tumor cells retain the ability to express the mutant p53 protein. These point mutant proteins are often more stable than wild-type p53 and levels accumulate at very high levels in the tumor cell. One explanation for the selection of such mutations is that the mutant p53 proteins can act as dominant-negative inhibitors of wild-type p53, which functions as a tetramer (Ko and Prives 1996; de Vries, Flores et al. 2002; Olive, Tuveson et al. 2004). In addition to the dominant-negative inactivation of wild-type p53, there is evidence that some of the tumor associated mutant p53 proteins acquire new transforming functions that contribute to tumor development (Sigal and Rotter 2000).

Mutations in regulators of p53 and downstream targets

Human tumors that do not display mutations in *p53* frequently harbor defects in either the pathways that allow for the stabilization of p53 in response to stress, or in the

effectors of the apoptotic activity of p53 (Woods and Vousden 2001). Two related proteins, MDM2 and MDM4, have critical roles in regulating p53 activity (Jones, Roe et al. 1995; Montes de Oca Luna, Wagner et al. 1995; Parant, Chavez-Reyes et al. 2001). MDM2 participates in an autoregulatory loop with p53 and functions as an ubiquitin ligase that targets p53 for degradation. Defects in pathways that inhibit MDM2 activity are common in tumors that do not possess mutations in the *p53* gene (Vousden 2002).

Apoptotic activities of p53

p53 is a transcription factor that activates the expression of genes that contain p53-binding sites within their regulatory regions and a number of target genes have been identified (Vousden and Lu 2002). Many of these genes can be divided into classes that might mediate a specific p53 function, one of the largest of these groups are those genes involved in the activation of apoptosis. p53 can induce the expression of numerous apoptotic genes that can contribute to the activation of both death-receptor and mitochondrial apoptotic pathways. In addition, p53 can also affect the efficiency of survival signaling (Vousden and Lu 2002).

Identification of pro-apoptotic transcriptional p53 targets revealed several members of the Bcl-2 family (Bax, Puma, Noxa and Bid) as the best gene targets for triggering the mitochondrial pro-apoptotic pathway inducing the release of Cytochrome-c and the eventual activation of Caspase-3 (reviewed in Schuler and Green 2005). In addition, members of the apoptotic death receptor pathway, Fas, Pidd and Killer/Dr5 were also up regulated in response to p53, ultimately activating the same effector Caspase-3 resulting in cellular apoptosis (reviewed in Ozoren and El-Deiry 2003).

In addition to the mitochondrial and death receptor apoptotic pathways the choice between cell death and survival is strongly dependent on the activity of 'survival signals' such as soluble ligand binding or direct interaction with neighboring cells or the extracellular matrix(Vousden and Lu 2002). p53-induced apoptosis can be rescued by the activation of Akt kinase which is able to interact with and stabilize Mdm2, enhancing the degradation of p53(Sabbatini and McCormick 1999). Furthermore, through indirect evidence it is thought that Akt may down regulate the expression of *Puma,* which as discussed earlier is a mediator of the apoptotic response through the mitochondrial pathway (Han, Flemington et al. 2001). Interestingly, the inhibition of p53 by Akt is counteracted by the ability of p53 to induce expression of Pten, a phosphatase that can inhibit the activation of Akt (Stambolic, MacPherson et al. 2001). The induction of Pten has been shown to be essential for p53 mediated apoptosis in mouse cells, underscoring the importance of survival signaling in determining the final outcome of the p53 response.

The ability to engage various apoptotic pathways via several routes is likely to be particularly important for the tumor-suppressor activity of p53, as the selective pressure to lose pro-apoptotic gene function is extremely high during tumor development.

1.2.2.2.2 The retinoblastoma protein

Retinoblastoma is a rare childhood cancer of the retina that initiates during development as a result of RB inactivation in the developing retina. RB encodes a 110-kDa nuclear phosphoprotein that binds and inhibits members of the E2F transcription factor family. E2F transcription factors regulate other genes required for S-phase and cell

cycle progression (Classon and Harlow 2002). The binding of RB to E2F is regulated by the phosphorylation of cyclin/cyclin-dependent kinases. RB acts as a tumor suppressor, in part, by inhibiting cell-cycle progression past the G1/S restriction point. Inactivation of RB removes the constraint on cell-cycle control, which results in deregulated cell proliferation. Two other RB family members have been identified, p107 and p130 (also known as retinoblastoma-like 1 and retinoblastoma-like 2, respectively). These proteins have functions in common with RB, but also possess unique functions (Classon and Dyson 2001).

1.2.2.2.3 $p19^{Arf}$ and $p16^{Ink4a}$

The *Ink4a-Arf* locus encodes two tumor suppressors, Ink4a ($p16^{Ink4a}$) and Arf ($p19^{Arf}$ in mice and $p14^{Arf}$ in humans) whose expression enhances the growth-suppressive functions of Rb and p53, respectively. The genetic organization of the *Ink4a-Arf* locus is unusual because the two protein products are encoded in part by common nucleotide sequences that are read in alternative reading frames (Sherr 2001). Activation of $p16^{Ink4a}$ and $p19^{Arf}$ is commonly associated with cellular senescence-- a form of cell-cycle arrest that is thought to be irreversible and was initially linked to the replicative exhaustion of human fibroblasts in culture. Although replicative senescence is triggered by telomere malfunction, a phenotypically similar endpoint can be produced much more rapidly in response to activated oncogenes, DNA damage, oxidative stress and inappropriate cell-culture conditions (Shay and Wright 2001).

The $p19^{Arf}$ protein binds directly to, and inactivates, Mdm2, and this is turn triggers a p53-dependent transcriptional program that leads either to G1-phase arrest or to

apoptosis. The $p19^{Arf}$ protein accumulates in the nucleolus of cells and can mobilize Mdm2 to this compartment, segregating Mdm2 from p53. $p19^{Arf}$ binding to Mdm2 also antagonizes its E3 ubiquitin ligase activity for p53, thereby preventing p53 turnover (Sherr 2001).

E2F transcription factors regulate genes that promote entry into the S-phase of the cell-cycle. By binding to E2Fs on chromatin, Rb-family members (Rb, p107 and p130) recruit histone deacetylases and chromatin remodeling factors to E2F-responsive promoters to repress gene expression. Phosphorylation of Rb by cyclin D- and cyclin E-dependent kinases (Cdks), releases E2Fs from the negative constraints of Rb, enabling execution of the E2F transcriptional program. In turn, $p16^{Ink4a}$ can inhibit cyclin D-dependent kinases, thereby maintaining Rb-E2F repression and restricting cell proliferation (Sherr 2001)

1.2.2.2.4 Cdk interacting protein p21 ($p21^{Cip1}$)

$p21^{Cip1}$ is a well-characterized cyclin-dependent kinase (cdk) inhibitor that belongs to the Cip/Kip family of cdk inhibitors (Gartel, Serfas et al. 1996). It mainly inhibits the activity of cyclin/cdk2 complexes and negatively modulates cell cycle progression (Brugarolas, Moberg et al. 1999). In addition, $p21^{Cip1}$ can bind to proliferating cell nuclear antigen thereby blocking DNA synthesis (Waga, Hannon et al. 1994).

$p21^{Cip1}$ is a transcriptional target of p53 and plays a crucial role in mediating growth arrest when cells are exposed to DNA damaging agents such as doxorubicin and (Brugarolas, Moberg et al.) gamma irradiation (el-Deiry, Harper et al. 1994; el-Deiry,

Tokino et al. 1995). It has been shown that overexpression of $p21^{Cip1}$ results in G1-, G2-, or S-phase arrest (Ogryzko, Wong et al. 1997; Radhakrishnan, Feliciano et al. 2004). Conversely, $p21^{Cip1}$-deficient cells fail to undergo cell cycle arrest in response to p53 activation after DNA damage (Waldman, Kinzler et al. 1995). Furthermore, $p21^{Cip1}$ and p53 are essential to sustain the G2 checkpoint after DNA damage in human cells (Bunz, Dutriaux et al. 1998).

Apart from p53, a variety of other factors including Sp1/Sp3, Smads, Ap2, signal transducers and activators of transcription (STAT), BRCA1, E2F-1/E2F-3, and CAAT/enhancer binding protein (Cooper, Woolley et al.) are known to activate $p21^{Cip1}$ transcription (reviewed in Gartel and Tyner 1999). In addition to its role in DNA damage response, $p21^{Cip1}$ is also implicated in terminal differentiation, replicative senescence, and protection from p53-dependent and -independent apoptosis (reviewed in Gartel and Tyner 2002).

1.2.2.3 Tumorigenesis

The process of malignant transformation occurs in discrete histopathological steps, many of which correlate with specific genetic alterations. After the experiments of the 1980s, it became clear that a single mutation was rarely, if ever, sufficient to accomplish the entire process of transformation. Subsequent studies using transgenic mice showed that pairs of oncogenes, that were capable of collaborating to immortalize and then transform cells *in vitro,* could drive tumor formation with far more rapid kinetics than single oncogenes (Land, Parada et al. 1983; Ruley 1983; Sinn, Muller et al. 1987; Thompson, Southgate et al. 1989). It is now clear that there are a limited number

of molecular pathways that when disrupted, contribute to most, if not all cancers. In humans, colon cancer studies suggest that 4-6 mutations are required to reach this state, but fewer seem to be required in mice (Hahn and Weinberg 2002).

1.2.2.3.1 Immortalization of human and mouse cells

Most normal human cells are mortal, lack telomerase activity and show telomere shortening with passage in culture. By contrast, most cells that are derived from human cancer patients are immortal, express telomerase and have stable telomere lengths. *In vitro* experiments with human cells demonstrate that they have limited replication lifespan and enter irreversible growth arrest after extended passage, termed replicative senescence (Hayflick and Moorhead 1961). Introduction of viral oncogenes, such as SV40 Large T antigen (LgT antigen), bypasses senescence but the cells remain mortal (Shay, Pereira-Smith et al. 1991; Bond, Haughton et al. 1999). LgT antigen acts by binding to, and inactivating, Rb and p53 (Rundell and Parakati 2001). The continued passage of LgT-expressing post-senescent cells results in a second barrier being reached, termed crisis, which is characterized by widespread apoptosis and genome-wide instability caused by shortened telomeres (Counter, Avilion et al. 1992; Sedivy 1998). However, activation of telomerase or its catalytic domain hTERT will immortalize post-senescent human cells (Bodnar, Ouellette et al. 1998; Vaziri and Benchimol 1998; Yang, Chang et al. 1999). An accumulation of work now exists to indicate that three pathways involving: telomere shortening, p53 and Rb control replicative senescence and immortality in humans, and play a central role in almost all cancers (Hahn and Weinberg 2002).

In contrast to human cells, studies with mouse embryo fibroblasts (MEFs) have shown that genetic inactivation of either *p19^{Arf}* or *p53* alone is sufficient for cellular immortalization (Zindy, Eischen et al. 1998) (Harvey and Levine 1991; Kamijo, Zindy et al. 1997). As primary MEFs are grown in culture they eventually undergo growth arrest, in which levels of p19^{Arf} (and therefore p53) are elevated (Zindy, Eischen et al. 1998). The spontaneous inactivation of either of these genes allows the outgrowth of clones of immortalized MEFs (Harvey and Levine 1991; Kamijo, Zindy et al. 1997). Current research does not indicate that telomere shortening plays a role in limiting the cellular lifespan of cells derived from inbred mice. Despite this difference with human cells, mouse models have provided essential insights into the biology of human cancer and continue to be an invaluable tool to understand the mechanisms of tumorigenesis.

1.2.3 Perspectives on cancer biology

Our understanding of the molecular basis for cancer formation has rapidly advanced in the last 20 years. Emerging from a seemingly impenetrable thicket of genetic complexity has come the realization that, for most part, all cancers are controlled by a common set of mechanisms. However, cancer biologists are facing a new era in which they must unite with the field of epigenetics, a discipline that has long been in the shadows of human cancer genetics. It is now clear that the epigenetic mechanisms that control hypomethylation, hypermethylation, and LOI play a significant role in tumor progression (Feinberg and Tycko 2004). Thus, integrating the epigenetics of tumorigenesis into the mainstream of cancer biology will be one of next challenges for the field.

1.3 Acknowledgments

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Chapter 2:

Global Loss of Imprinting Leads to Widespread Tumorigenesis in Adult Mice.

T.M Holm[1,2], L. Jackson-Grusby[1,3], T. Brambrink[1], Y. Yamada[1,3], W. M. Rideout 3rd[1,3], R. Jaenisch[1,2†]

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1. Whitehead Institute for Biomedical Research, Cambridge, MA 02142, USA
2. Massachusetts Institute of Technology, Cambridge, MA 02142, USA
3. Present addresses: Children's Hospital Boston and Harvard Medical School Pathology Departments, Boston, USA (LJG), Department of Tumor Pathology, Gifu University, Japan (YY), AVEO Pharmaceutical Inc, Cambridge, USA (WMR)

† To whom correspondence should be addressed. E-mail: jaenisch@wi.mit.edu

2.1 Abstract

Loss of imprinting (LOI), commonly observed in human tumors, refers to loss of monoallelic gene regulation normally conferred by parent-of-origin specific DNA methylation. To test the function of LOI in tumorigenesis, we developed a model using transient demethylation to generate imprint-free mouse embryonic stem cells (IF-ES cells). Embryonic fibroblasts derived from IF-ES cells (IF-MEFs) display TGFβ resistance, reduced $p19^{Arf}$ and p53 expression, and form tumors in SCID mice. IF-MEFs exhibit spontaneous immortalization, and cooperate with H-ras in cellular transformation. Chimeric animals derived from IF-ES cells develop multiple tumors arising from the injected IF-ES cells within 12 months. These data demonstrate LOI alone can predispose cells to tumorigenesis, and identify a pathway through which immortality conferred by LOI lowers the threshold for transformation.

2.2 Notes

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2.3 Introduction.

Cancer has traditionally been thought to be caused by a series of genetic mutations in cancer-susceptibility genes, which include oncogenes, tumor-suppressor genes and genes causing genetic instability. Until recently, this rationale has remained relatively unchallenged. However, it is now clear that epigenetic changes (changes to the DNA maintained by cell division other than alterations to the nucleotide sequence) play a critical role during tumorigenesis (Feinberg and Tycko 2004). The genome of cancer cells is characterized by localized hypermethylation in CpG islands, resulting in the silencing of tumor suppressor gene expression (Baylin and Bestor 2002). In addition, global genomic and CpG island hypomethylation are associated with both benign and malignant tumors (Feinberg and Vogelstein 1983; Gama-Sosa, Slagel et al. 1983)). Mechanistically these epigenetic changes are known to cause both oncogene activation and chromosomal instability resulting in loss of heterozygosity (Feinberg and Vogelstein 1983) (Chen, Pettersson et al. 1998) (Gaudet, Hodgson et al. 2003).

Imprinted genes display a characteristic parent-of-origin specific DNA methylation pattern that results in only a single allele being expressed (either the paternal or maternal allele). DNA methylation that maintains the monoallelic expression of imprinted genes is established during gametogenesis and is important for fetal growth regulation and perinatal development (Bartolomei 2003) (Reik and Walter 2001). Although imprinting persists in the adult, the requirements for proper imprinting in the context of normal tissue homeostasis is not well understood.

Loss of imprinting (LOI), either biallelic expression or complete silencing of imprinted genes, has been implicated in the progression of several tumors (Ogawa, Eccles et al. 1993; Rainier, Johnson et al. 1993). For instance, aberrant biallelic expression of the *Insulin-like growth factor-2* (*IGF2*) gene, a significant risk factor for human colorectal carcinogenesis, is thought to promote tumorigenesis by inhibiting apoptosis (Cui, Cruz-Correa et al. 2003). In addition *PEG3*, $p57^{kip2}$, and *IGF2R* all display LOI that leads to their silencing in oligodendrogliomas, breast cancer, and hepatocellular carcinomas, respectively (De Souza, Yamada et al. 1997; Kobatake, Yano et al. 2004; Trouillard, Aguirre-Cruz et al. 2004). Although there is evidence that LOI at the *IGF2* locus promotes tumorigenesis in Beckwith-Wiedemann syndrome (DeBaun, Niemitz et al. 2002), for the majority of human tumors is has not been clear if LOI plays a causal role in cancer or is merely a consequence of altered epigenetic regulation in already transformed cells. Furthermore, in all studies to date, the effects of LOI on cancer progression have been restricted to either single loci or have examined the consequences of a global imbalance of imprinting in parthenogenetic or androgenetic cells (Hernandez, Kozlov et al. 2003). Thus, the effect of genome-wide LOI on tumor formation has not been addressed.

Here, we use conditional alleles of *Dnmt1*, encoding a DNA methyl transferase, to transiently remove all DNA methylation marks from the genome of embryonic stem (ES) cells. Reactivation of *Dnmt1* expression resulted in a restoration of global DNA methylation but failed to re-methylate imprinted genes. From these imprint-free (IF) ES cells we derived murine embryonic fibroblasts (MEFs) and examined their growth characteristics. The IF-MEFs displayed a number of characteristics of transformed cells including increased growth rate, immortality, and resistance to growth inhibition by TGFβ.

When injected into SCID mice, the IF-MEFs formed tumors with long latency. Overexpression of oncogenic V12 H-Ras in the IF-MEFs significantly shortened tumor latency. Chimeric animals that were generated with the IF-ES cells developed widespread tumors by 12 months of age, with the cancers being derived from the IF-ES cells. Our findings suggest a causal link between LOI and cancer and demonstrate that imprinting plays a critical tumor suppressor role in the adult.

2.4 Results.

2.4.1 Generation of IF and CTL-ES cells.

To investigate the role of LOI in the progression of cancer we took advantage of previous observations in which mouse embryonic stem (ES) cells deficient in *Dnmt1* (encoding an enzyme that maintains global DNA methylation) were found to lack global DNA methylation, including the methylation associated with imprinting (Li, Beard et al. 1993). Although loss of *Dnmt1* through a conditional knockout allele has no effect on ES cell viability, it is essential in differentiated cells (Jackson-Grusby, Beard et al. 2001). Lethality and global DNA methylation have been rescued following re-expression of a *Dnmt1* cDNA in the *Dnmt1* mutant ES cells(Tucker, Beard et al. 1996). However because of the low *Dnmt1* level in the "rescued" cells, the genome was incompletely re-methylated resulting in abnormalities such as genomic instability (Chen, Pettersson et al. 1998; Gaudet, Hodgson et al. 2003). To circumvent these shortcomings, and to generate ES cells expressing normal and properly regulated levels of *Dnmt1*, we devised an alternative strategy in which *Dnmt1* was first conditionally inactivated and then reactivated by sequential exposure of the cells to the cre and flp recombinases.

Figure 2.1 Conditional alleles of *Dnmt1*

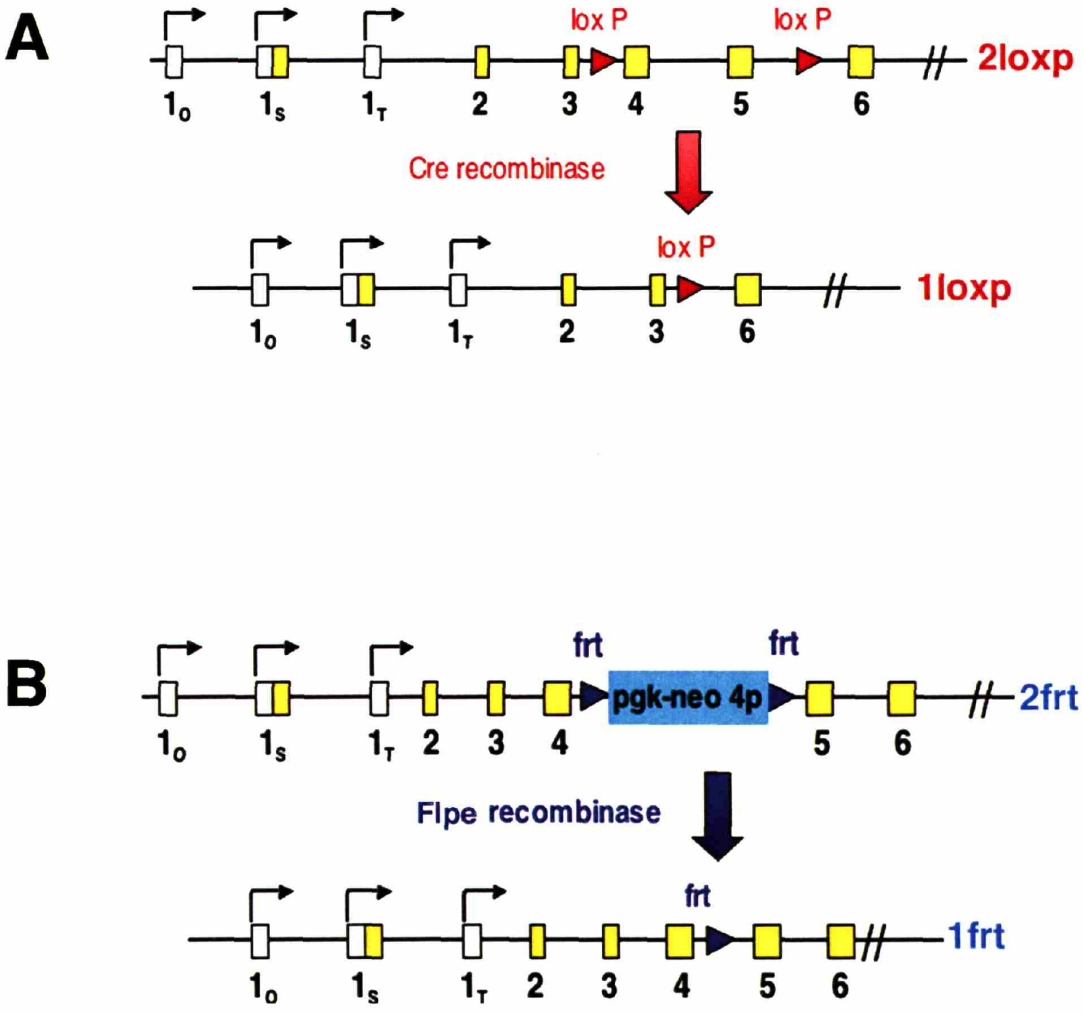


Figure 2.1 Conditional alleles of ***Dnmt1***
(A) Conditional inactivation of *Dnmt1* 2LoxP allele. Shows the recombination of *Dnmt1* to 1LoxP when exposed to Cre-recombinase. (B) Conditional activation of *Dnmt1* 2Frt allele. Shows the recombination of *Dnmt1* allele to 1Frt and the removal of the stop cassette when exposed to Flipe-recombinase.

To do this we constructed an inactive *Dnmt1* allele (2-Frt) by inserting a STOP cassette flanked by two Frt sites between the fourth and fifth exons of the *Dnmt1* gene. This mutant allele is revertible and becomes re-activated following Flpe-mediated recombination (**Fig 2.1B**). Mice carrying the 2Frt allele were generated by injection of targeted ES cells into mouse blastocysts, and the subsequent animals were bred to the *Dnmt1* 2lox(Jackson-Grusby, Beard et al. 2001) and a *ROSA26* Flp reporter allele (Possemato, Eggan et al. 2002) (**Fig 2.1A**). ES cell lines containing the *Dnmt1* conditional 2-Lox and *Dnmt1* 2-Frt alleles, and the Flp beta-geo reporter allele were derived from these animals (**Fig 2.2A**). When the 2-Lox/2-Frt ES cells were transfected with a Cre plasmid, deletion of exons 4 and 5 create the null *Dnmt1* 1-Lox allele resulting in *Dnmt1* deficiency (**Fig 2.2B**). Consistent with this, the 1-Lox/2-Frt cells displayed global genome demethylation as demonstrated by Southern blot analysis of centromeric regions, LINE, and IAP elements and (**Fig 2.3A-C**). The demethylated cells were then transfected with a Flpe plasmid, causing the STOP cassette to be excised (2-Frt -> 1-Frt), the *Dnmt1* allele to be reactivated, and genome methylation to be restored (**Fig 2.2A-B**; these cells are herein referred to as 'IF-ES cells')(Buchholz, Angrand et al. 1998). To serve as a control, we transfected 2-Lox/2-Frt ES cells with flpe first and Cre second, a manipulation that maintains *Dnmt1* activity at each step and has no effect on genomic methylation level and imprinting (referred to as 'CTL-ES cells)(**Fig 2.2A**).

Figure 2.2 Generation of IF and CTL-ES cells

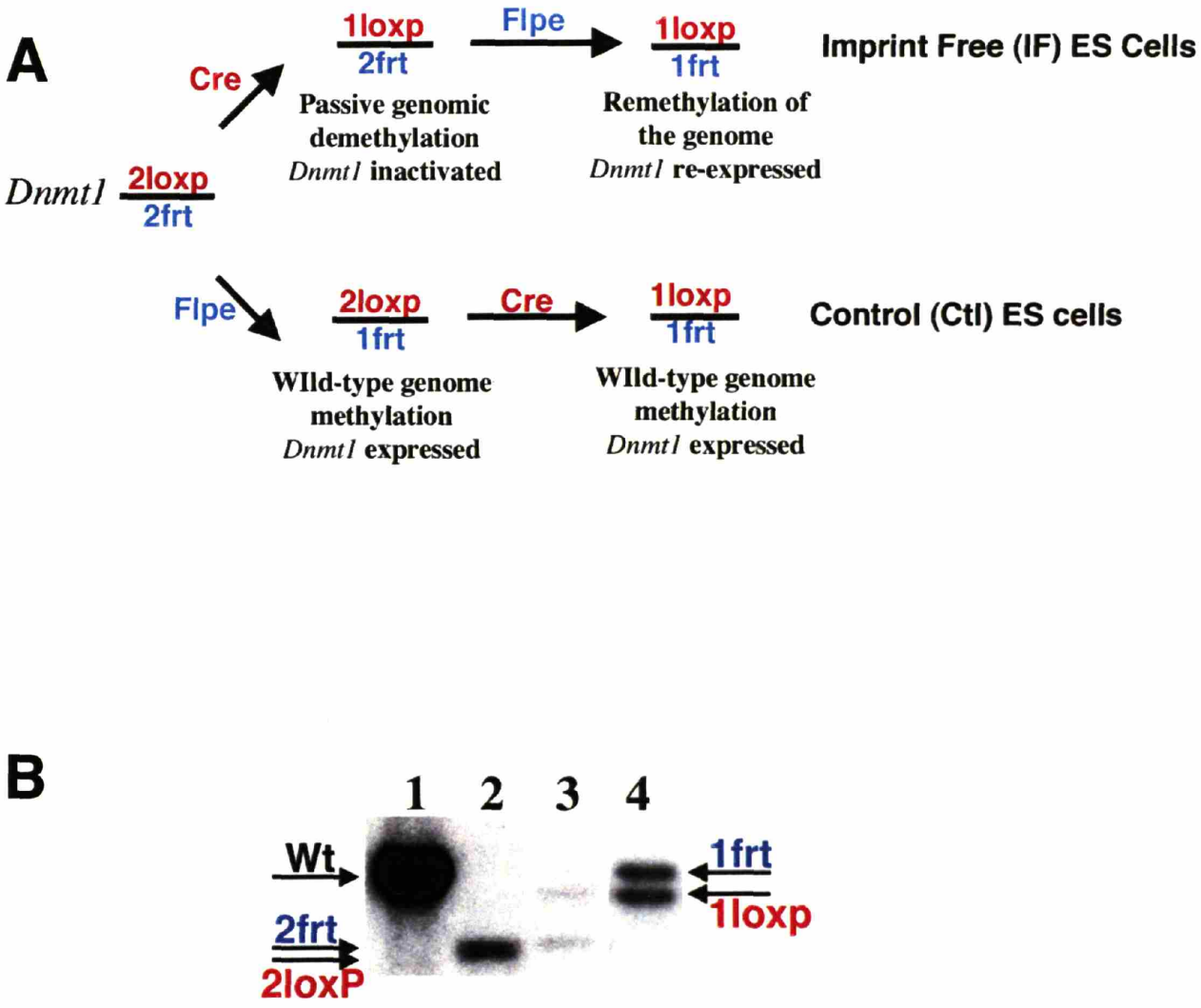


Figure 2.2 Generation of IF and CTL-ES cells.
(A) Schematic representation of the steps used to create the IF-ES cells. Red and blue indicate the *Dnmt1* genotype. CTL-ES cells were generated by reverse exposure to the recombinases. (B) Genomic southern blot analysis confirming the recombined *Dnmt1* alleles. Lane 1, Wild-type *Dnmt1*; Lane 2, 2LoxP/2Frt *Dnmt1*; Lane3, 1LoxP/2Frt *Dnmt1*; Lane 4, 1LoxP/1Frt *Dnmt1* .

Figure 2.3 Genomic methylation state of IF and CTL cells

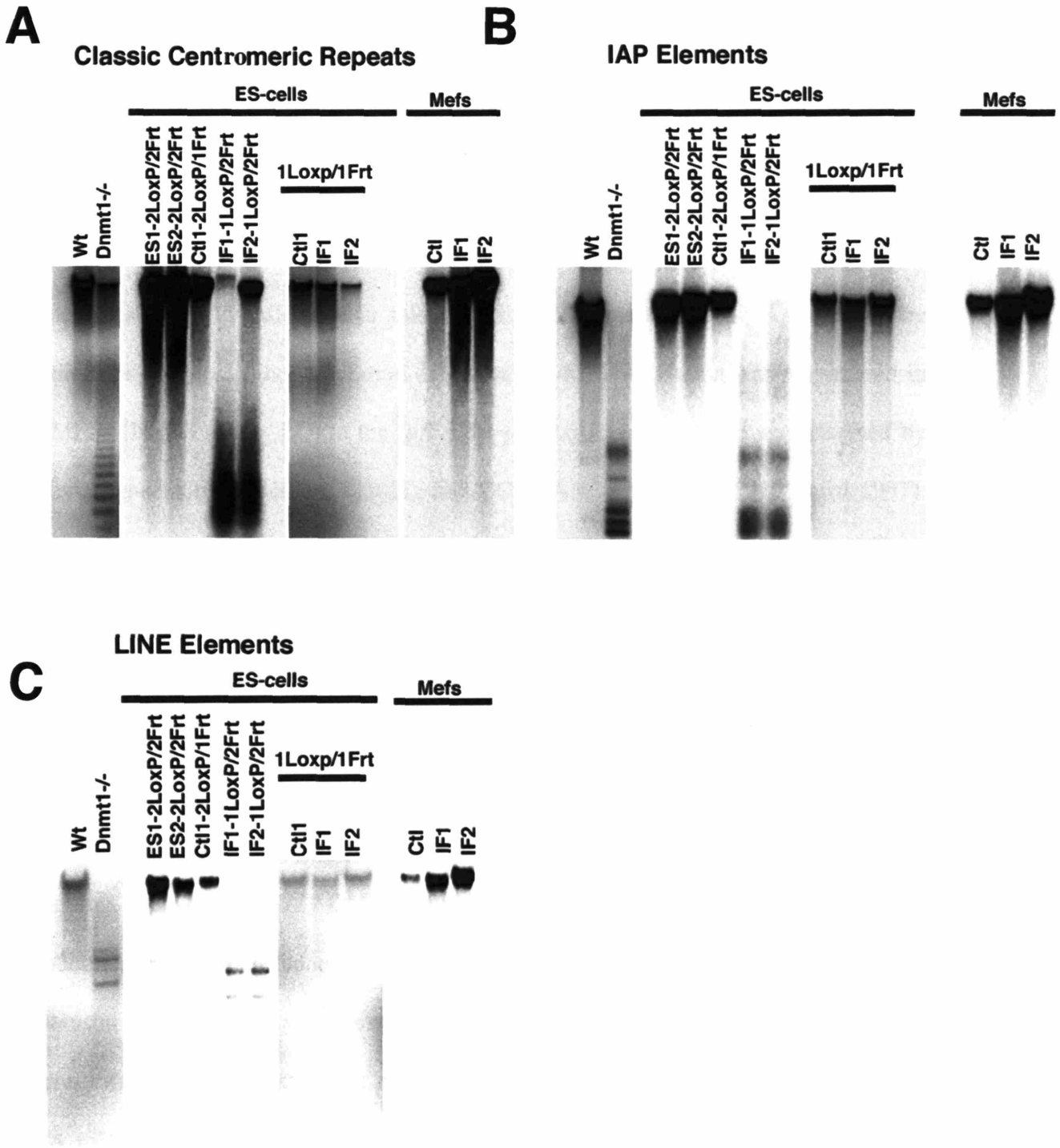


Figure 2.3 Genomic methylation.
(A) The methylation status of the classic centromeric repeats from genomic DNA of control, ES and MEF cells. (B) The methylation status of IAP elements from genomic DNA of control, ES and MEF cells. (C) The methylation status of LINE elements from genomic DNA of control, ES and MEF cells.

2.4.2 Expression and methylation of IF and CTL-MEFs.

To confirm that our strategy had successfully erased imprinting, we examined the methylation status of selected imprinted genes in imprint-free ES cells and murine embryonic fibroblasts (MEFs). To generate the MEFs, wild-type blastocysts were injected with either IF- or CTL-ES cells and fibroblasts were isolated from E13.5 chimeras using G418-resistance to select for ES cell derived MEFs. As assessed by Southern blot analysis, the restoration of normal global methylation was maintained in the MEFs (**Fig2.3 A-C**). LOI at the *Igf2r*, *Snrpn*, *Peg3,* and *Igf2* loci was assessed by methylation-sensitive Southern blotting and COBRA analysis(Xiong and Laird 1997). While normal imprinted methylation of *Igf2r*, *Snrpn*, and *Peg3* was found in the CTL-ES cells and CTL-MEFs, these genes lacked methylation in IF-ES cells and IF-MEFs, consistent with LOI. In contrast, COBRA analysis revealed that the Differentially Methylated Region (DMR) of the *H19-Igf2* locus, a regulatory element that controls expression of *H19* and *Igf2*, was variably methylated in IF-ES cells and IF-MEFs (**Fig2.4**). Although this result was contrary to expectation in our model, it was not surprising, since the *Igf2-H19* DMR is highly susceptible to *de novo* methylation when the methylation status of the genome has been altered (Tucker, Beard et al. 1996; Chen, Pettersson et al. 1998; Biniszkiewicz, Gribnau et al. 2002; Baqir and Smith 2003) whereas the methylation status of other imprinted regions is more stable. Loss of the DNA methylation associated with imprinting is expected to either erase or result in biallelic expression of imprinted genes depending on whether the methylation of the

Figure 2.4 Characterization of methylation status of imprinted genes

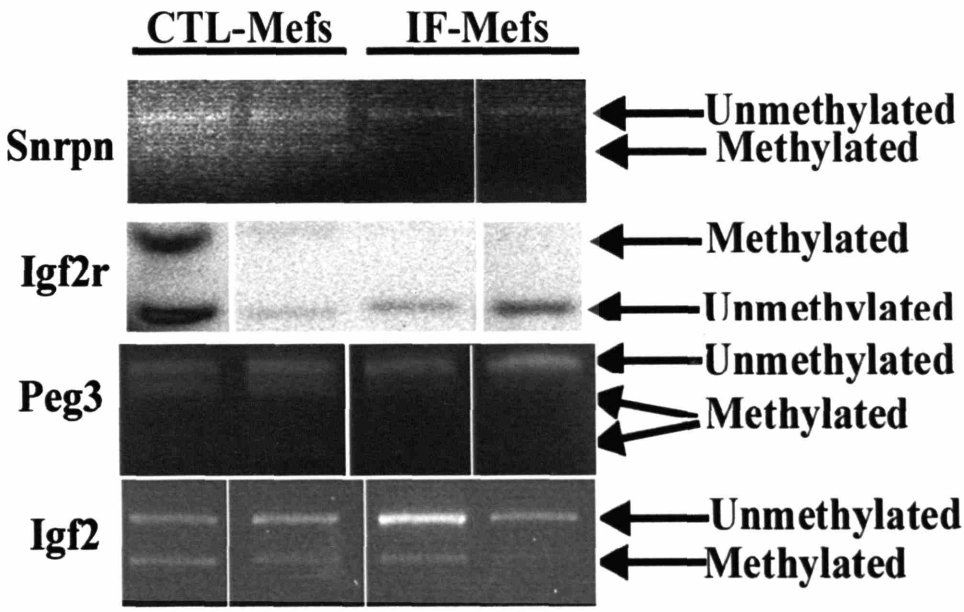


Figure 2.4. Characterization of Methylation status of IF- and CTL-MEFs.
Methylation status of imprinted genes in CTL and IF-MEFs was carried out by methylation sensitive Southern blot analysis for *Igf2r* and COBRA anaylsis for *Igf2, Snrpn* and *Peg3*.

Figure 2.5 Characterization of expression status of IF- and CTL-MEFs

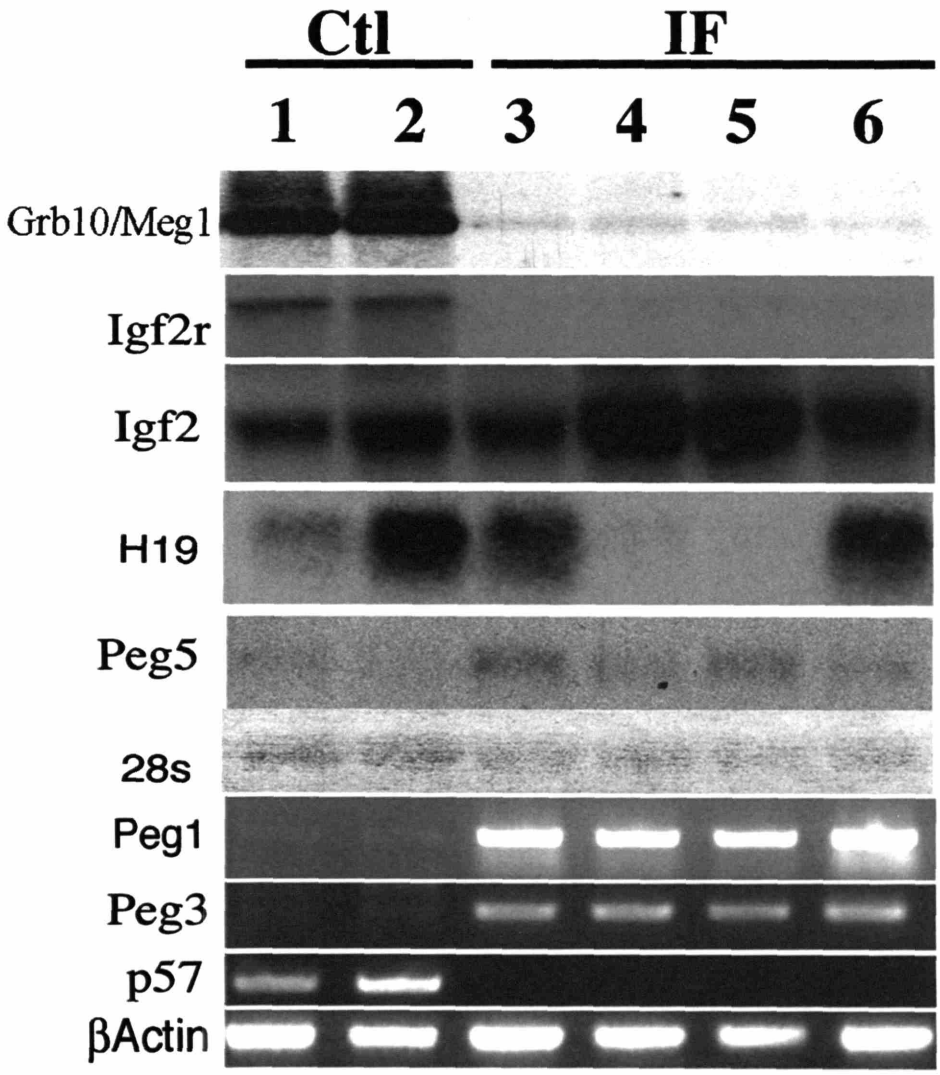


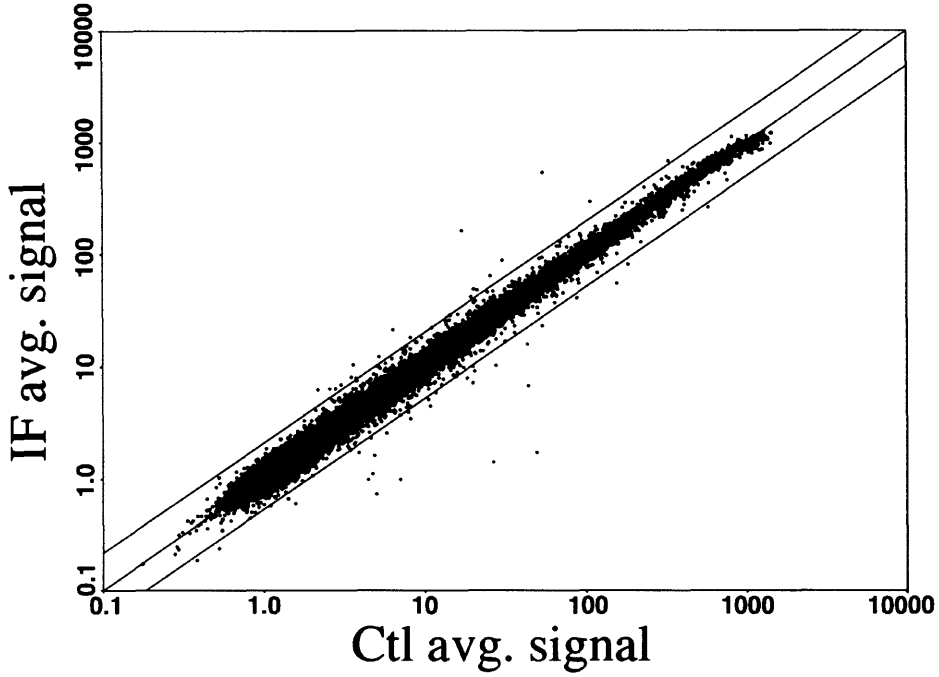
Figure 2.5. Characterization of Expression status of IF- and CTL-MEFs.
Confirmation of the expression of selected imprinted genes by northern blot and RT-PCR analysis. Lanes 1 and 2 CTL-MEFs; Lanes 3, 4, 5 and 6 IF-MEFs.

respective imprinting box or differentially methylated region (DMR) has a positive or negative effect on transcription (Reik and Walter 2001).

To assess the effect of LOI on gene expression, we examined the gene expression profiles of IF-MEFs and CTL-MEFs using microarrays. This analysis revealed a tight clustering for most genes, indicating that there was very little difference in expression patterns between the two populations (Pearson coefficient-r = 0.9919) (**Fig 2.6A**). However, the expression of a small number of genes, mostly corresponding to known imprinted genes, was altered (**Fig 2.6B**). The remaining genes were known or likely downstream targets of imprinted genes (data not shown), although we cannot rule-out the possibility that some represent non-imprint-related genes independently affected by our demethylation/remethylation strategy. In agreement with our finding that the *Igf2-H19* DMR was variably methylated, we found ~2-fold higher level of *Igf2* and no *H19* expression in 2 of 4 IF-MEFs samples as compared to CTL-MEFs, and the other two IF-MEFs were unchanged relative to the CTL-MEFs. To validate the microarray data, expression of *Meg1/Grb10*, *Igf2r*, *Igf2*, *H19*, *Peg5*, *Peg1*, *Peg3* and $p57^{kip2}$ was examined in the imprint-free and control MEFs by Northern blot analysis and RT-PCR, as described in Experimental Procedures (**Fig 2.5**). This analysis confirmed that the IF-MEFs were negative for *Igf2r*, *Meg1/Grb10*, $p57^{kip2}$ and variable for *H19*, but displayed increased levels of *Peg3*, *Peg5* and variable *Igf2* compared to CTL-MEFs. Our findings suggest that the strategy of conditionally inactivating and then reactivating *Dnmt1* successfully erased the methylation associated with imprinting, but not global genome methylation, resulting in widespread alterations in the expression of imprinted genes.

Figure 2.6 Micro Array Analysis of IF- and CTL-MEFs

A



B

Fold Regulation-UP		Fold Regulation-Down	
Impact	↑1.7	Igf2r	↓2.5
Plagl1	↑2.2	Grb10/Meg1	↓10
Sgce	↑1.6	Ctgf	↓2.7
Peg12/Frat	↑1.4	p57kip2/cdkn1c	↓3.5
Ndn	↑1.6	Zim1	↓not expressed
Peg3	↑1.8	Thrombospondin	↓2.1
Igf2	var	H19	var

Figure 2.6 Micro array analysis of IF- and CTL-MEFs.
(A) Characterization of IF- and CTL-MEFs by microarray analysis. Full documentation can be found at (http://www.ebi.ac.uk/arrayexpress; accession number E-MEXP-450). (B) Selected imprinted genes that are up- or down-regulated in IF-MEFs by microarray analysis.

2.4.3 Altered growth properties of IF-MEFs.

Many imprinted genes such as *Igf2* and *Grb10/Meg1* have been implicated in embryonic growth control (Reik and Walter 2001). We therefore examined whether IF-MEFs displayed altered growth properties compared to CTL-MEFs. To analyze the cell cycle in the MEFs, DNA content was measured by flow cytometry. The majority (~70%) of the CTL-MEFs were in the G1 phase of the cell cycle with approximately 13% and 17% in the S- and G2/M-phases, respectively. In contrast, under the same culture conditions, the IF-MEFs displayed a higher mitotic index with a lower proportion of cells in the G1- (46%) and S-phases (7%) and a pronounced increase in the proportion of cells in G2/M (47%) (**Fig 2.7**). To determine if this altered cell cycle profile was indicative of altered growth, we counted the total number of IF- and CTL-MEFs during an 8-day interval and calculated the growth rate during the exponential phase of proliferation (2-4 days after plating, **Fig 2.8A**). The viability of the exponentially growing cells was approximately 95% as assessed by Trypan blue dye exclusion. This analysis showed that the IF-MEFs grew significantly faster than the control fibroblasts (**Fig 2.8A**), consistent with a shortened cell cycle.

Microarray analysis revealed the down regulation in IF-MEFs of *Igf2r*, *Tsp1* and $p57^{kip2}$ (**Fig 2.6B**), three genes known to positively regulate transforming growth factor-β (TGFβ) activation and signaling. These gene expression changes coupled with the increased growth rates in IF-MEFs suggested a potential inability of IF-MEFs to respond to TGFβ, a pleiotropic cytokine that inhibits the growth of a diverse range of cell types (Roberts and Wakefield 2003).

Figure 2.7 Cell cycle characteristics of IF-MEFs

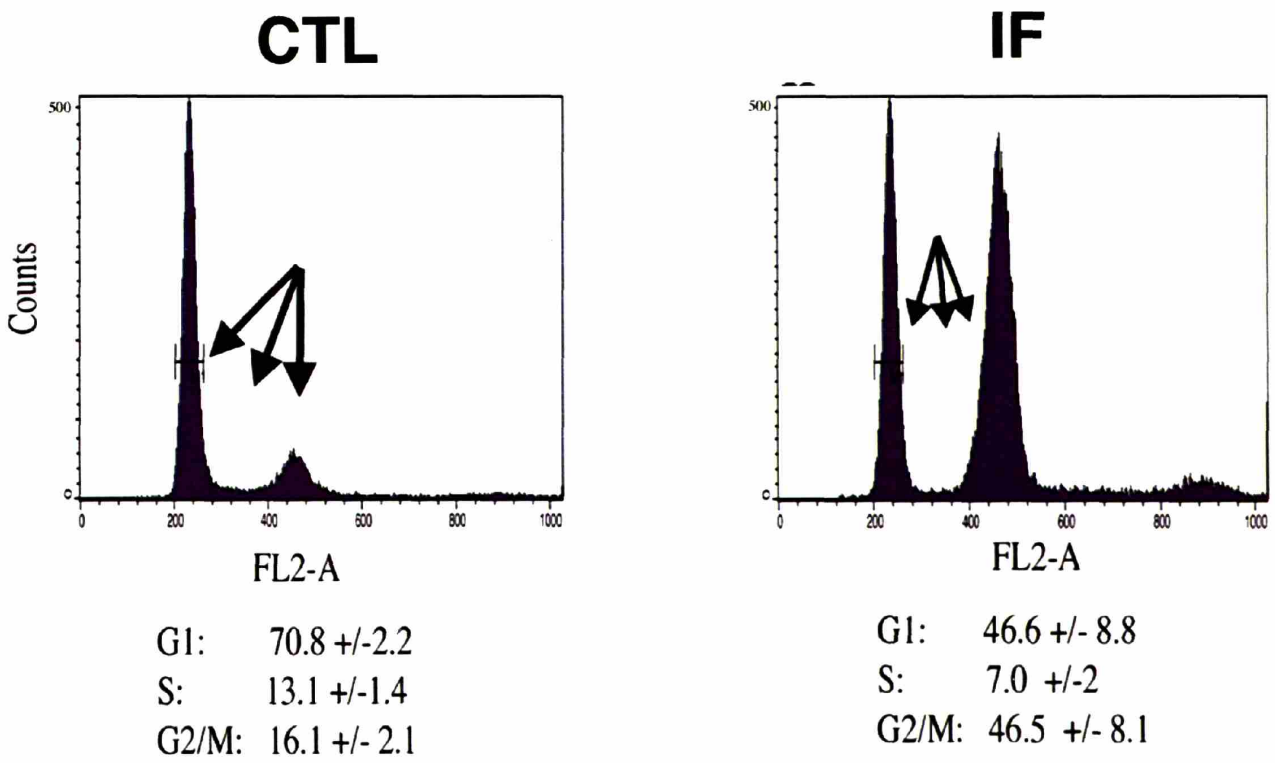


Figure 2.7 Cell cycle characteristics of IF-MEFs.
Cell cycle analysis of IF and Ctl-MEFs by flow cytometry. A representative example of propidium iodide stained MEFs is shown for both IF and Ctl. The three arrows indicate the peaks that represent cells in G1, S and G2/M phase of the cell cycle respectively. Below each graph is the quantification of cells in each stage of the cell.

Figure 2.8 Growth characteristics of IF-MEFs

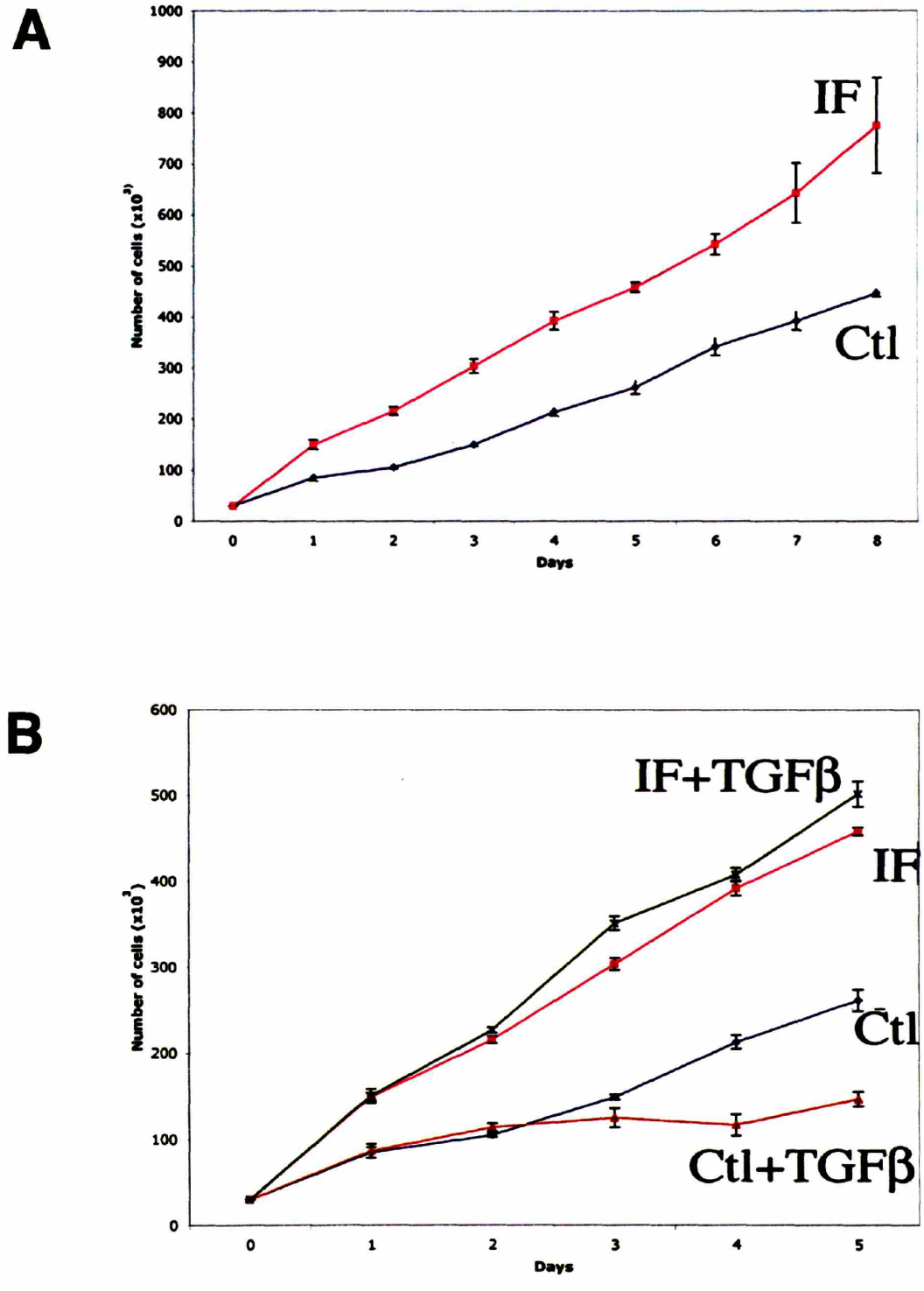


Figure 2.8 Growth Characteristics of IF-MEFs.
(A) Growth Rate of IF and CTL-MEFs. The graph shows the faster growth rate of IF-MEFs compared to CTL-MEFs over a period of 8 days. (B) Lack of response of IF-MEFs to the growth inhibitory cytokine TGFβ.

To investigate whether LOI altered the ability of MEFs to respond to TGFβ growth-inhibition, we exposed IF- and CTL-MEFs to TGFβ (0.1 ng/ml) 24 hours after plating. Within 48 hours, the CTL-MEFs had undergone growth arrest (**Fig 2.8B**), whereas the IF-MEFs continued to proliferate normally (**Fig 2.8B**). These results suggest that IF-MEFs are no longer responsive to the growth-inhibitory effects of TGFβ, a property shared by malignant cells.

2.4.4 Immortalization and transformation of IF-MEFs.

Wild-type MEFs have a limited life-span in cell culture and eventually undergo senescence. To determine the life-span of the IF-MEFs, we used the 3T3 protocol (Todaro and Green 1963), which involves continuous passaging of the cells. By passage 8, CTL-MEFs had undergone senescence and appeared as large, flat non-dividing cells. In contrast, IF-MEFs appeared to be spontaneously immortalized as they maintained a constant proliferation rate and grew for at least 20 passages without showing any indication of undergoing senescence (**Fig 2.9**). Among many of the changes associated with cellular senescence in culture is the increased expression of cell cycle regulators such as $p16^{Ink4a}$ and $p19^{Arf}$ that are thought to be critical in inducing permanent G_0/G_1 arrest (Kamijo, Zindy et al. 1997; Stein and Dulic 1998). Figure 2.10 shows that the level of $p16^{Ink4a}$ increased in later passages of IF-MEFs, comparable to that in CTL-MEFs. The $p21^{Cip1}$ cell cycle inhibitor has been shown to have a similar function as $p16^{Ink4a}$ in senescing human cells, although its role in mouse cells is less clear (Brown, Wei et al. 1997; Pantoja and Serrano 1999).

Figure 2.9 IF-MEFs are immortal

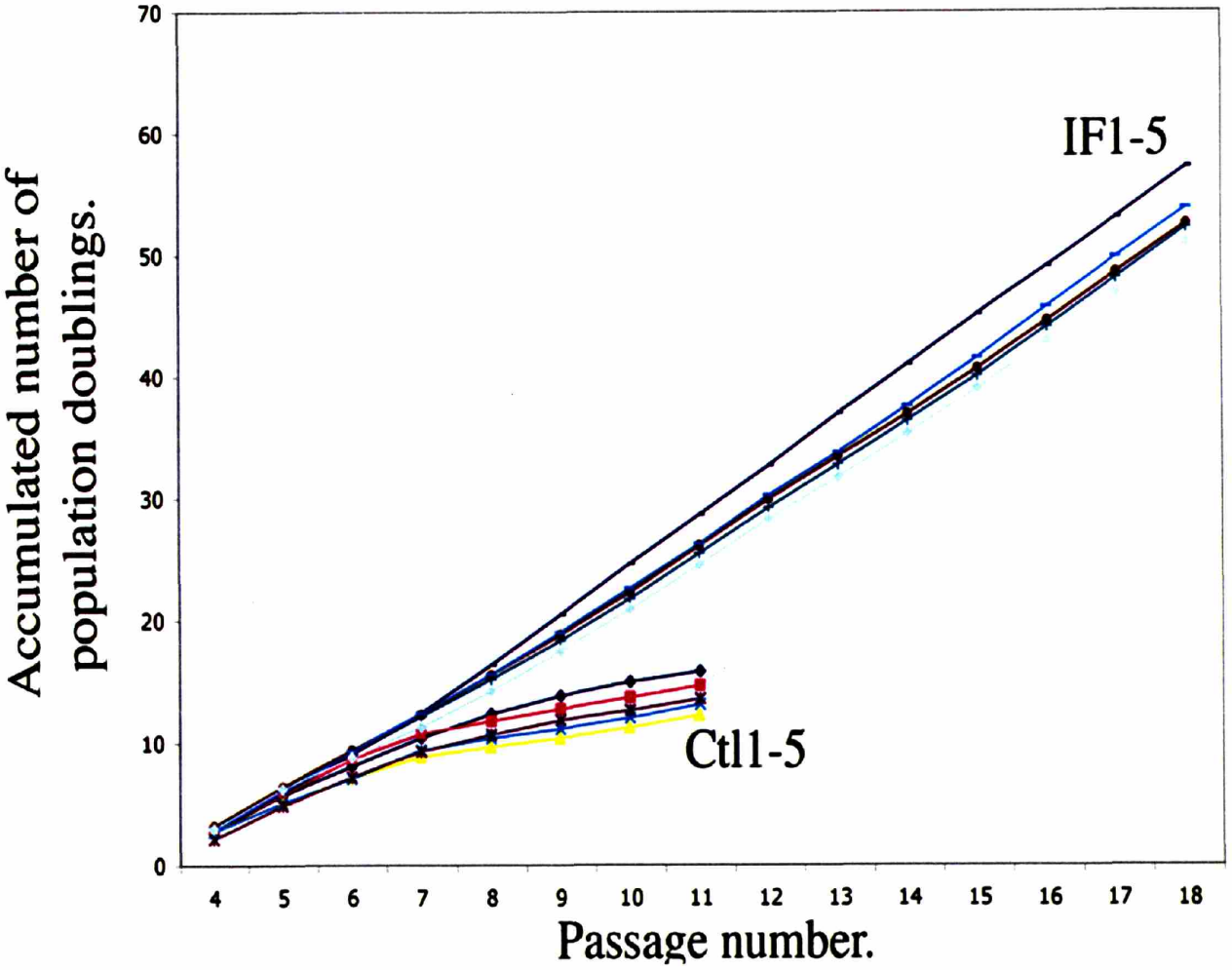


Figure 2.9 IF-MEFs are immortal. Immortalization was determined by accumulated number of population doublings over continued passages.

Figure 2.10 Analysis of Cell Cycle regulators

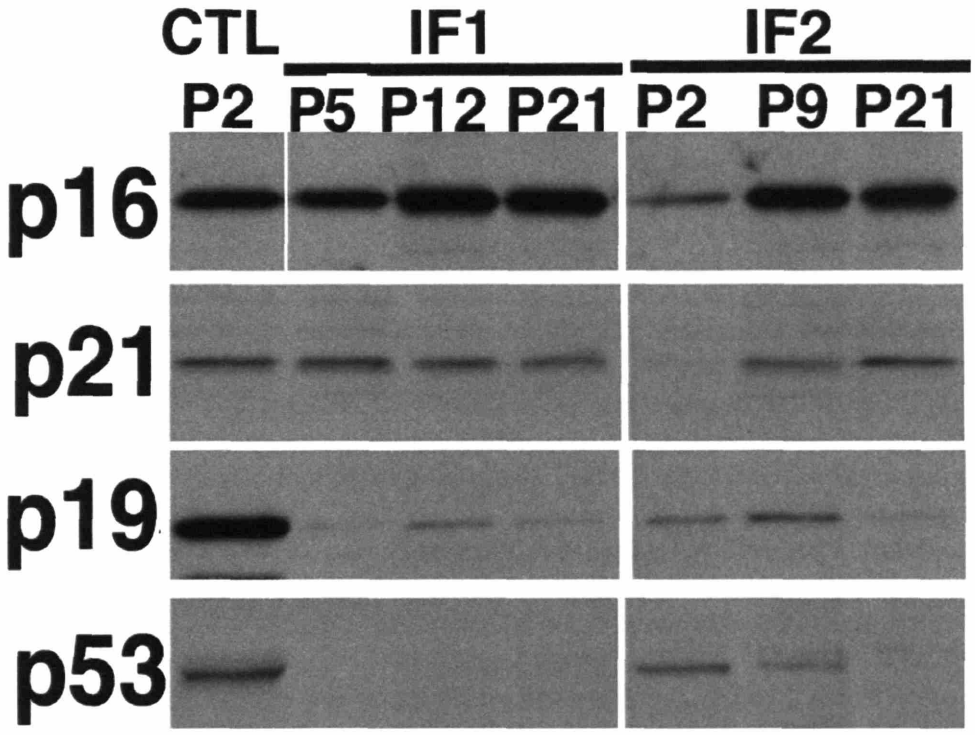


Figure 2.10 Western blotting analysis of cell cycle regulators in CTL-MEFS and IF-MEFS at increasing passages.

Like $p16^{Ink4a}$, the levels of $p21^{Cip1}$ remained constant or increased in the late passages of IF-MEFs (**Fig 2.10**). In contrast, the level of $p19^{Arf}$ did not increase in later passages of IF MEFs and were significantly less than in CTL-MEFs (**Fig 2.10**). Because $p19^{Arf}$ is known to bind to and sequester MDM2 and to inhibit the MDM2-dependent degradation of p53 (Sherr and Weber 2000), we examined the level of p53 in early- and late- passages IF-MEFs. In early passages, p53 was present but, as expected from the decreased level of $p19^{Arf}$, p53 expression decreased significantly at later passages (**Fig 2.10**).

Immortalization is an essential prerequisite for malignant transformation in mammals (Hahn and Weinberg 2002). Therefore we tested the transformation potential of IF-MEFs using the classic foci formation assay. This assay involves the maintenance of confluent cells over a 3-week period without passaging. In the rare incidence that a cell becomes transformed it escapes quiescence and forms a dense outgrowth known as a focus. When CTL-MEFs were grown under these conditions, 1.3 ± 0.6 foci were found per plate (**Fig 2.11 A-Ctl & B-Ctl**). In contrast, IF-MEFs formed approximately 35 ± 2.4 foci (**Fig 2.11 A-IF & B-IF**), consistent with LOI predisposing the IF-MEFs to spontaneous transformation.

Igf2 is an imprinted gene that often exhibits LOI and aberrant biallelic expression in human tumors (Cui, Cruz-Correa et al. 2003). To determine if biallelic expression of *Igf2* was solely responsible for the immortality and the increased rate of spontaneous transformation of the IF-MEFs, we overexpressed *Igf2* in CTL-MEFs following retrovirus mediated transduction.

Figure 2.11 Transformation of IF-MEFs

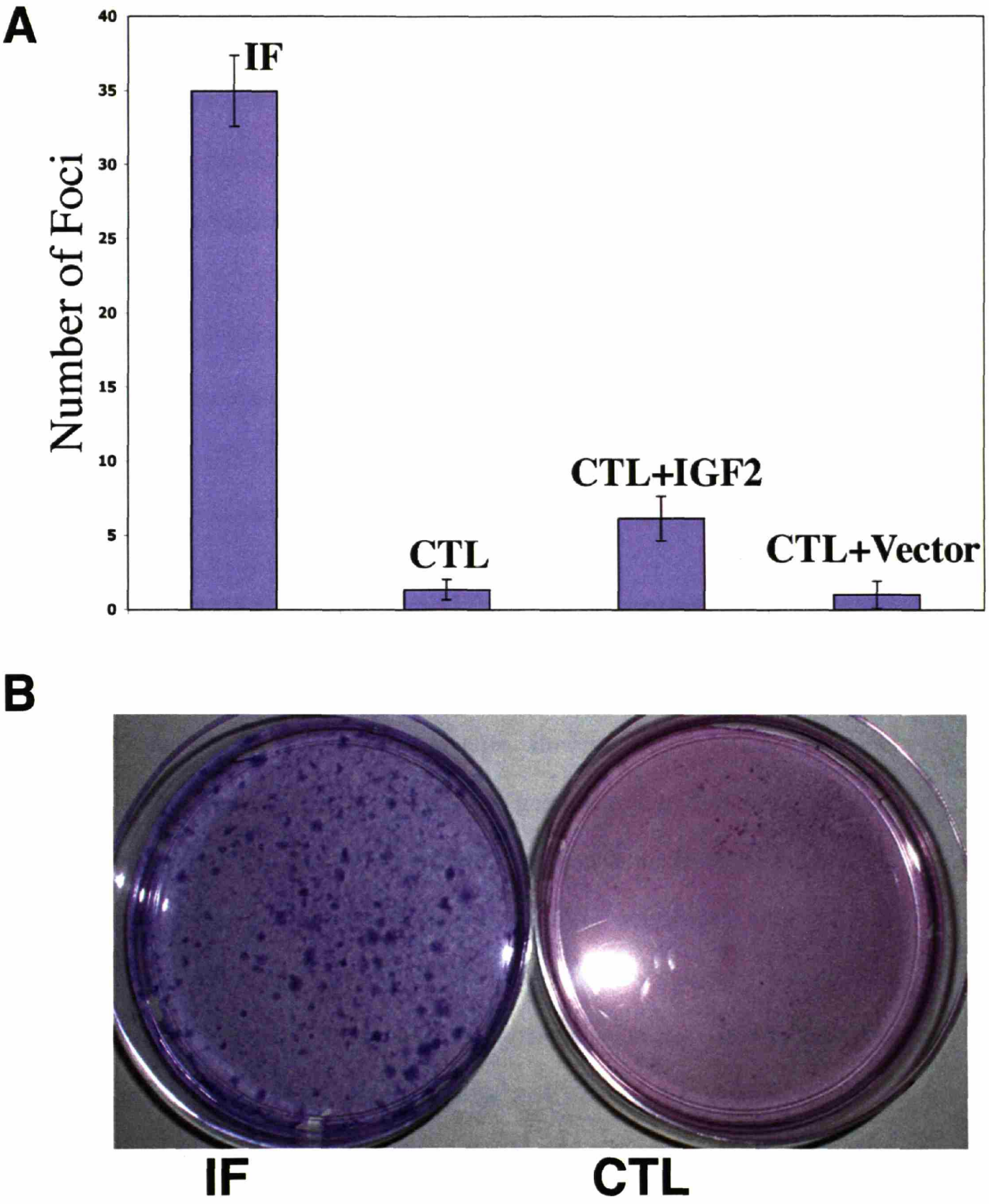


Figure 2.11 Transformation of IF-MEFs.
(A) Spontaneous transformation of IF-MEFs measured by the foci assay. IF-MEFs 34.9 ± 2.4; CTL-MEFs 1.3 ± 0.6; CTL-Igf2 6.1 ± 1.5; CTL-Vector 1.0 ± 0.93. (B) Representative plate demonstrating the spontaneous transformation present in the IF-MEF foci assay.

While *Igf2* overexpression in CTL-MEFs led to an increased growth rate, it only led to immortalization after extensive passaging as previously reported (data not shown and (Hernandez, Kozlov et al. 2003). In the foci formation assay, *Igf2*-infected CTL-MEFs (at low passage number) formed approximately six fold more foci than vector-only infected CTL-MEFs (6.1 ± 1.5 and 1 ± 0.9 foci, respectively) but failed to induce the level of spontaneous transformation observed with IF-MEFs. These data combined with the northern and micro-array analysis suggest that the variable expression levels of *Igf2* contribute to the ability of the cells to spontaneously transform. However, LOI of additional genes is needed to achieve the full extent of immortalization and transformation seen in IF-MEFs.

Forced overexpression of the oncogenes SV40 Large T antigen (LgT) and a constitutively active growth signal, such as that provided by V12 H-Ras, in wild-type MEFs is sufficient to cause cellular transformation. However, MEFs V12 H-Ras alone will not become transformed, but instead are either immortalized by LgT or are induced to undergo senescence (Hahn and Weinberg 2002). Given that the IF-MEFs displayed a greater potential for spontaneous transformation, we asked whether the effect of LOI could replace the function of either LgT or active H-Ras in this process. IF- and CTL-MEFs were infected with a LgT retroviral vector or an empty vector and drug selection was used to select for transduced cells. The cells were plated at low density and focus formation was assessed after a 14-day culture period. As expected, LgT-infected CTL-MEFs formed a greater number of foci as compared to the vector-only CTL-MEFs, similar to previous reports.

Figure 2.12 Morphology of IFHRas-MEFs

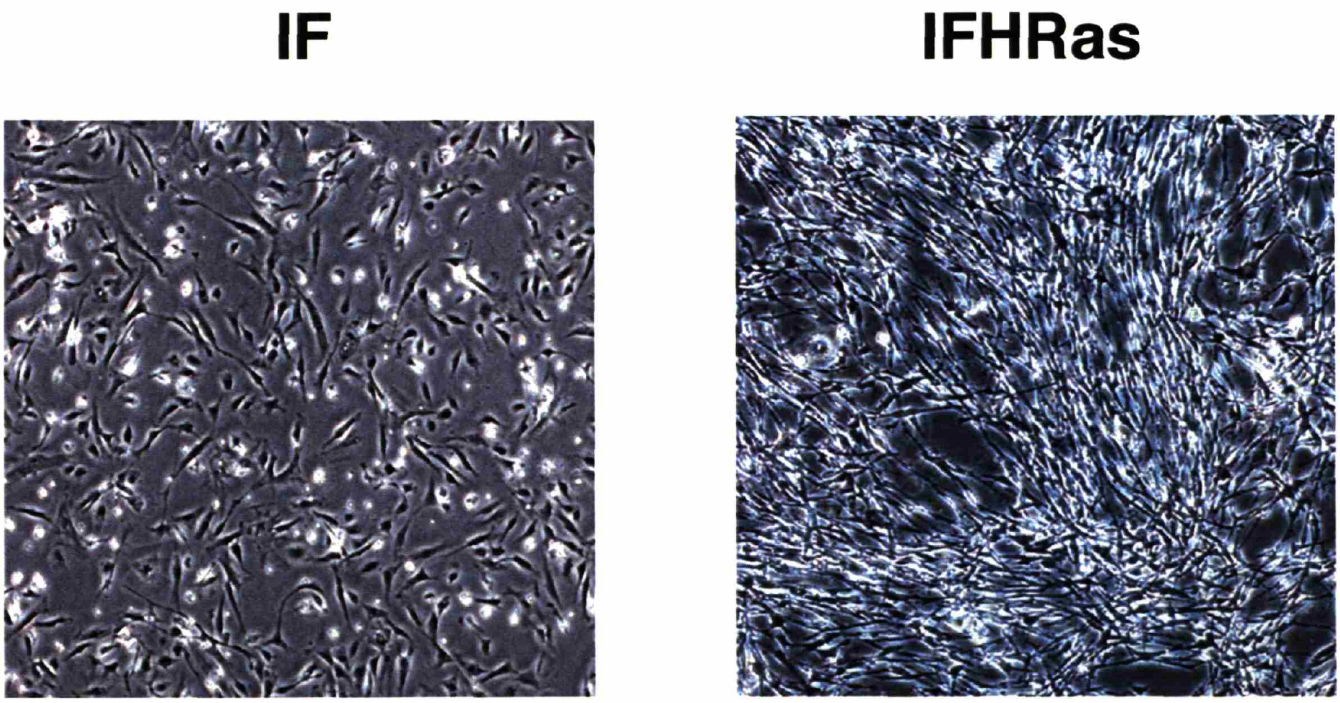
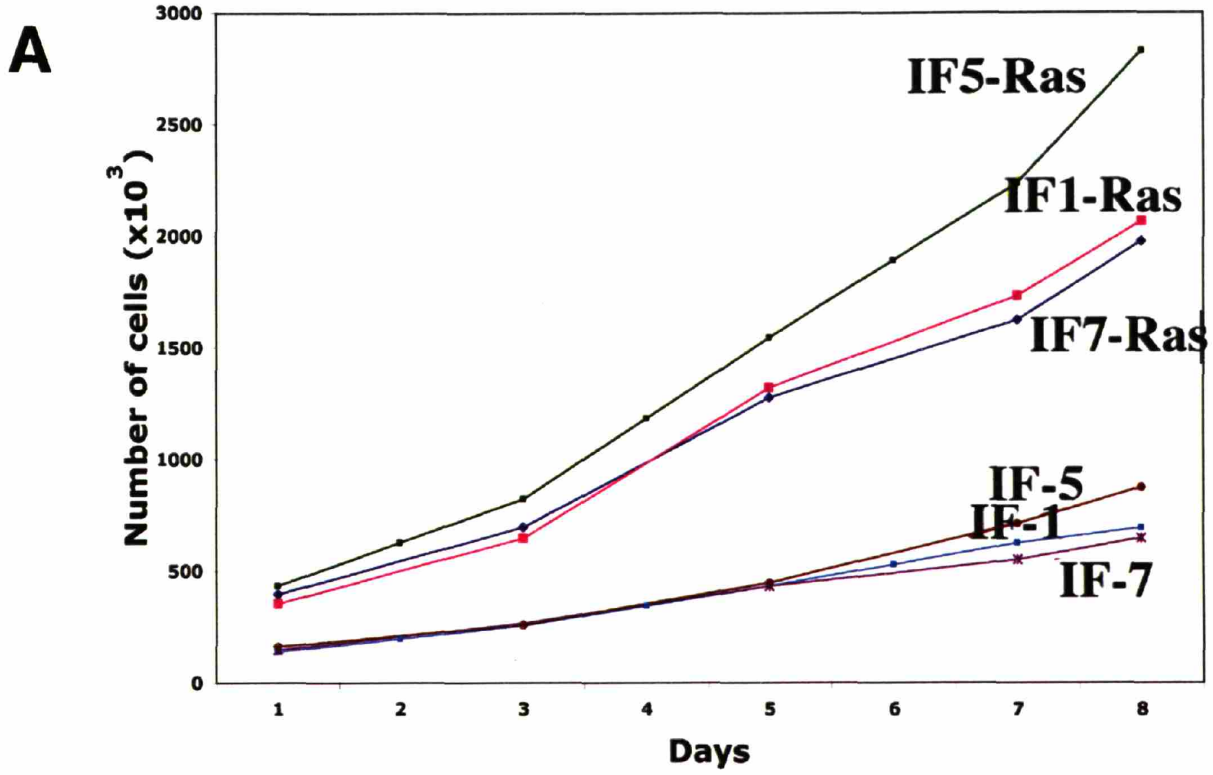


Figure 2.12 IF-MEFs infected with V12-HRas (IF-Ras) show altered morphology when compared to IF-MEFs (IF) alone.

Figure 2.13 Growth rate and Foci formation of IFRas-MEFs



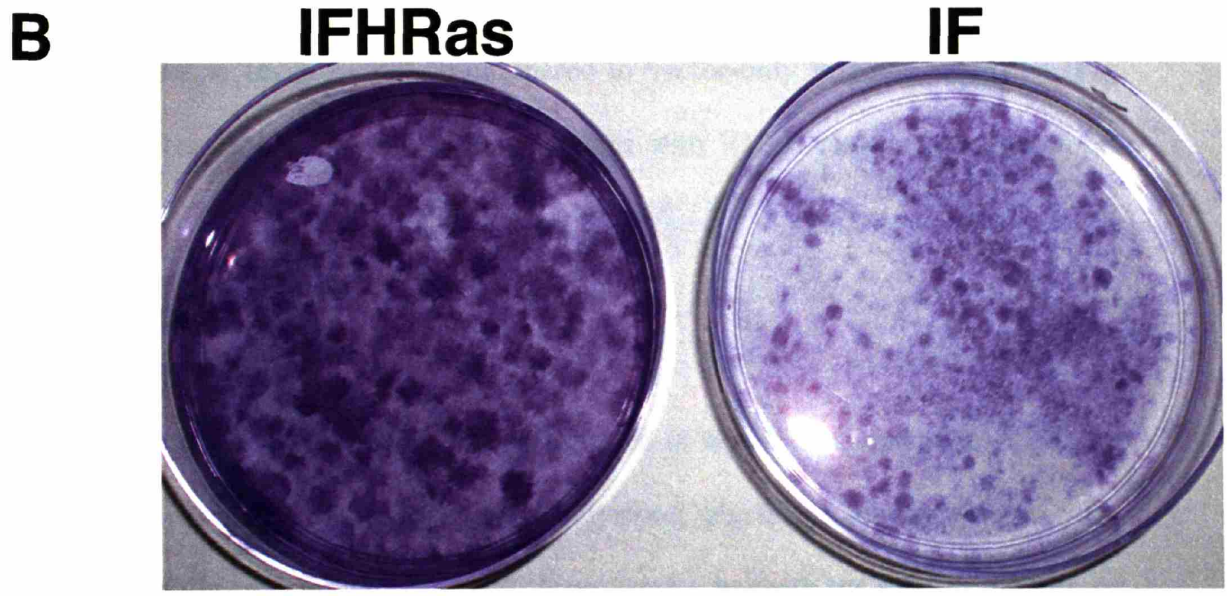


Figure 2.13
(A). IF-HRas-MEFs grow at a significantly faster rate than IF-MEFs. (B) Representative plate demonstrating spontaneous transformation in the low density foci assay comparing IF and IF-HRas.

Although the vector-only infected IF-MEFs formed more foci than the respective CTL-MEFs, no further increase in foci numbers were observed in the LgT-infected IF-MEFs (data not shown). Thus, LOI and LgT expression do not cooperate in cellular transformation.

To ask whether LOI can replace the function of LgT and cooperate with activated H-ras, we examined the effect of V12 H-Ras expression on IF-MEFs transformation frequency. CTL- and IF-MEFs were infected with either a V12 H-Ras or an empty retrovirus vector. Expression of V12 H-Ras in CTL-MEFs induced an immediate inhibition of cell growth and quiescence after passaging (data not shown), consistent with previous studies (Serrano, Lin et al. 1997). In contrast, IF-MEFs infected with V12 H-Ras lost contact inhibition, exhibited a significant change in morphology and continued to proliferate at a significantly higher rate than the vector-only control IF-MEFs (**Fig 2.12; 2.13**). In the focus formation assay, the V12 H-Ras-infected IF-MEFs displayed a massive increase in focus formation compared to vector-only infected IF-MEFs (**Fig 2.13**). These results indicate that LOI cooperates with V12 H-Ras to fully transform MEFs.

2.4.5 Tumor formation

The tumorigenic potential of cells was tested *in vivo* by sub-cutaneous injection into Severe Combined Immuno-deficient (SCID) mice, which lack mature lymphocytes. In this assay, fully transformed cells will form tumors beneath the skin at the site of injection.

Table 2.1 Incidence of MEF derived fibrosarcomas in SCID mice.

MEFs	SCID Mouse Fibrosarcomas	
	8-12 Days	**4-5 Months**
CTL	**0/6**	**0/6**
CTL+LgT	**0/6**	**0/6**
IF	**0/6**	**2/6**
IF+LgT	**0/6**	**2/6**
IF+Ras	**7/7**	**N/A**

Table 2.1 Incidence of MEF-derived fibrosarcomas in SCID mice.

2 x 10^6 MEFs were injected into three independent sites and growth of tumors was assessed after the defined time point.

Using this assay, we compared the tumorigenic potential of CTL- and IF-MEFs infected with either LgT or empty vector and IF-MEFs infected with V12 H-Ras. No tumors were found in SCID mice after 4-5 months following injection with CTL-MEFs (0/7) or LgT-infected CTL-MEFs (0/7). Tumors were observed in SCID mice injected with IF-MEFs (2/6), or LgT-infected IF-MEFs (2/6) after 5 months. In contrast, all SCID mice injected with V12 H-Ras-infected IF-MEFs (7/7) formed large fibrosarcomas within 14 days post-injection (**Table 2.1**). These findings are consistent with cooperation between LOI and oncogenic Ras to induce a fully transformed phenotype in MEFs. It should be noted that, due to the senescence that occurred in V12 H-Ras infected CTL-MEFs there were insufficient cells to perform the tumor formation assay in SCID mice.

To further examine the role LOI plays in oncogenesis *in vivo*, we created chimeric mice using IF- and CTL-ES cell lines (using two independently-derived ES cell lines for each condition). The level of chimerism contributed by the IF-ES cells ranged from 5-20% in the adults, whereas CTL-ES cells contributed up to 100%, as judged by coat color. Chimeras were sacrificed at 9, 12 and 18 months of age and their tissues were examined histologically for abnormalities. Strikingly, about half of the IF-chimeras at 9 months of age (n= 3/5) and all of the chimeras at 12 and 18 months of age showed the presence of hepatocellular tumors and intestinal adenomas (**Table 2.2; Fig 2.14 A-E**). The hepatocellular carcinomas, and in some cases adenomas, averaged approximately 5 x 6 mm in size (n=6; data not shown) by 18 months of age.

Table 2.2 The incidence of tumors in IF-chimeras.

IF Tumor Incidence	9 Months	12 Months	18 Months
Hepatocellular Carcinomas	**4/5**	**7/7**	**4/4**
Intestinal Adenomas/Carcinomas	**3/5**	**3/3**	**4/4**
Testicular Seminoma	**0/5**	**0/7**	**1/4**
Lymphoma	**0/5**	**1/7**	**0/4**
Leukemia	**0/5**	**1/7**	**0/4**

Other cancers were also observed, for instance in one IF-chimera the normal architecture of the testis was disrupted by a proliferation of homogenous cells consistent with testicular seminoma (**Fig 2.14E**). Another IF-chimera displayed splenomegaly and histological analysis of the bone marrow revealed a phenotype consistent with a leukemia (**Fig 2.14D**). In a 12-month old IF-chimera, a large tumor with histology consistent with lymphoma was isolated (**Fig 2.14C**). In contrast to these results, control chimeras appeared healthy at all time points, and when sacrificed at 24 months of age displayed no tumors (n=6/6; up to 100% chimerism assessed by coat color contribution).

To confirm that the cancers in the IF-chimeras were derived from the IF-ES cells, genomic DNA from tumors was analyzed by PCR for the presence of the *neo* fragment of the beta-geo gene contained in the IF-ES cells but not in the BDF1 host tissue. The *neo* gene was amplified from all of the tumors examined, consistent with an IF-ES cell origin for these neoplasms (**Fig 2.14F**). Taken together, tumorigenic potential of both IF-ES chimeras and IF-MEFs provides strong evidence that LOI promotes transformation both *in vitro* and *in vivo*.

In humans, the imprinted genes $p57^{kip2}$, *Igf2r*, and *Igf2* have been implicated in the tumorigenesis of multiple cancers (Mills, Falls et al. 1998; Feinberg and Tycko 2004). We therefore examined the expression of these genes in tumors from the IF-chimeras. Similar to our results in IF-ES cells and IF-MEFs, transcripts for $p57^{kip2}$ and *Igf2r* were absent in the tumors (**Fig 2.15A** *Igf2r, $p57^{kip2}$* Lanes1-4), whereas variable levels of *Igf2* expression was found (**Fig 2.15A** *Igf2* Lanes1-4).

Figure 2.14 Histological analysis of IF tumors

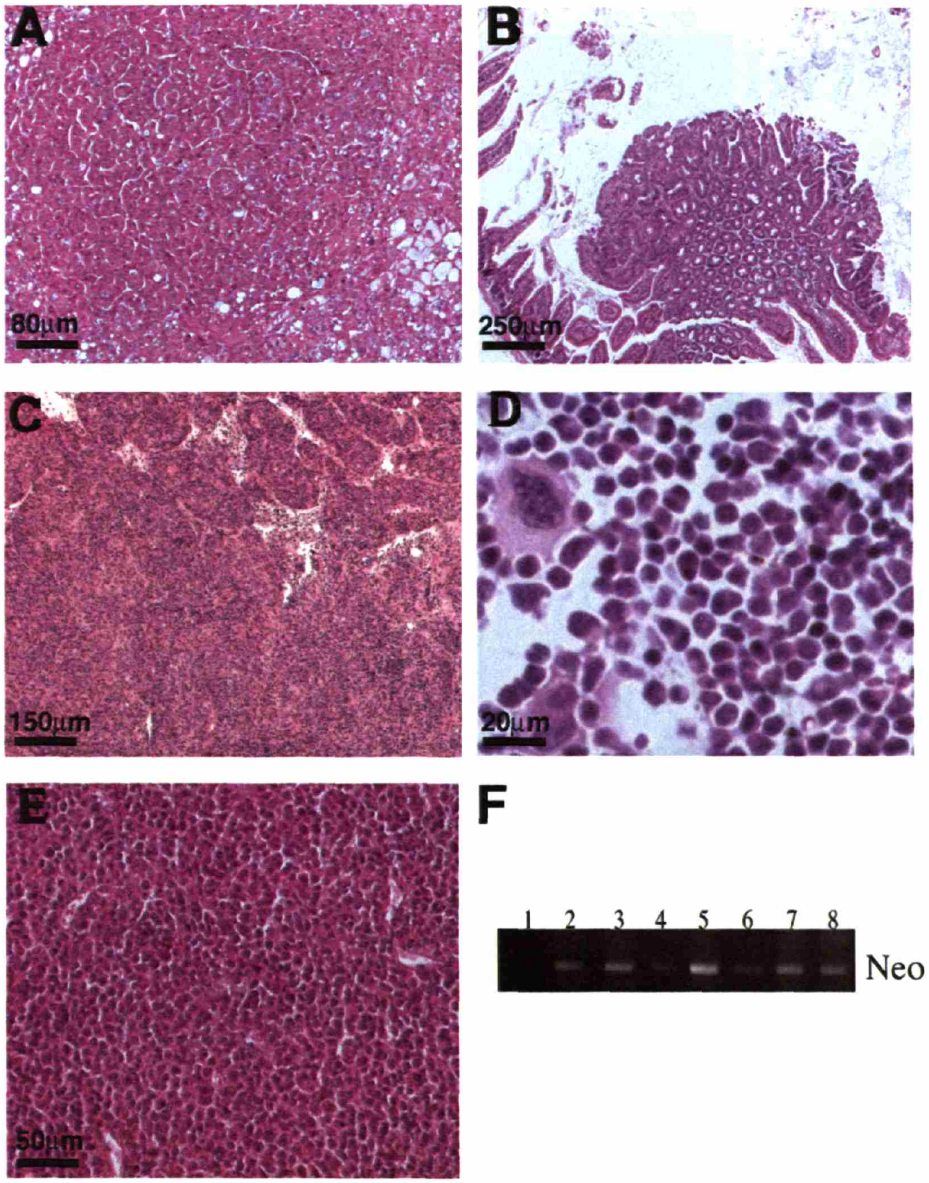


Figure 2.14 Histological analysis of IF tumors.
Histological analysis of tumor formation in IF chimeras stained with H&E. (A) Sections of an IF hepatecellular carcinoma/adenoma. (B) Intestinal adenoma/carcinoma, (C) Lymphoma, (D) Chronic myeloid-like leukemia, (E) Testicular seminoma. (F) Genomic PCR to detect the presence of the IF neomycin marker gene. Lane 1, BDFI wildtype host blastocyst; Lane 2, hepatocellular tumor 1; Lane 3, hepatocellular tumor 2; Lane 4, hepatocellular tumor 3; Lane 5, testicular seminoma; Lane 6, intestinal adenoma; Lane 7, Chronic myeloid-like leukemia; Lane 8, Lymphoma.

Figure 2.15 Expression analysis of IF tumors

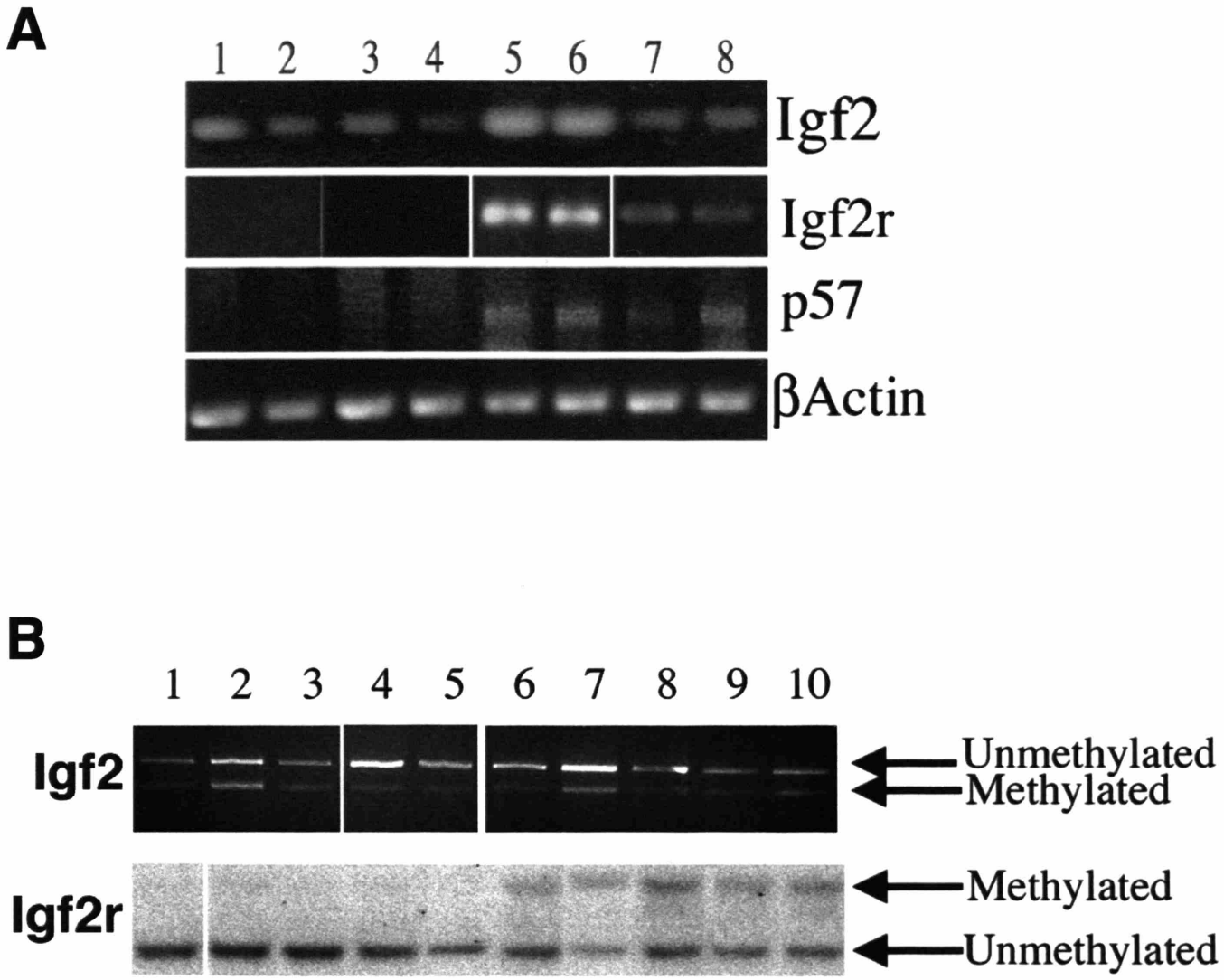


Figure 2.15 Expression analysis of IF tumors.
(A) RT-PCR expression analysis of selected imprinted genes in IF tumors. Lane 1, hepatocellular carcinoma; Lane 2, testicular seminoma; Lane 3, chronic meyloid-like leukemia; Lane 4, lymphoma; Lane 5, wild-type liver; Lane 6, wild-type testis; Lane 7, wild-type bone marrow; Lane 8, wild-type spleen. (B) Methylation status of selected imprinted genes in IF tumors by COBRA (*Igf2*) and methylation sensitive southern blotting (*Igf2r*) analysis. Lane 1, hepatocellular carcinoma; Lane 2, intestinal adenoma; Lane 3, testicular seminoma; Lane 4, chronic myeloid-like leukemia; Lane 5, lymphoma; Lane 6, wild-type Liver; Lane 7, wild-type intestine; Lane 8, wild-type testes; Lane 9, wild-type spleen; Lane 10, wild-type bone marrow.

In agreement with our findings in IF-MEFs (**Fig 2.4**), the tumors displayed methylation of the *H19-Igf2* locus (**Fig 2.15 B** *Igf2* Lanes 1-5). These results indicate that the tumors observed in the chimeras display a loss of transcripts for the putative tumor suppressors $p57^{kip2}$ and *Igf2r* and variable expression levels of the *Igf2* oncogene, similar to that observed in the parental IF-ES cells and IF-MEFs.

2.4.6 No tumors after germline transmission.

Imprinted genomic methylation is established during gametogenesis (Tucker, Beard et al. 1996), predicting that the breeding of adult IF-chimeras should "re-set" the methylation state and result in progeny with normal imprinting. To test whether restoration of normal imprinting would revert the tumorigenic phenotype, consistent with an epigenetic basis for these cancers, IF-chimeric mice from the two independently derived IF-ES and CTL-ES cells were crossed to C57B6 mice. Progeny inheriting the ES cell derived genome, as determined by coat color, were aged and examined for tumors at autopsy. Careful histological analyses failed to detect tumors at 18 or 24 months of age (n=0/8) with the exception of a single lung adenoma detected at 24 months of age (n=1/5) in a CTL mouse (**Table 2.3**). Due to the age of this animal this is most likely a spontaneous tumor and not caused by genetic manipulation of the parental ES cell line. The reversibility of the tumorigenic potential of IF-cells following germline passage supports the epigenetic basis of these cancers, and argues against the tumor formation in the IF chimeras was due to an oncogenic mutation incurred during the culturing of the ES cells.

Table 2.3 Tumor incidence after germline transmission.

Tumor Incidence after Germline Transmission	18 Months	24 Months
Control	0/3	1/2
IF	0/4	0/4

2.5 Discussion.

It has been known for some time that altered DNA methylation is associated with both benign and malignant tumor states. Considerable attention has focused on the effect of global hypo- or hypermethylation of DNA in tumors, whereas considerably less is known about the importance of imprinting during cancer progression. The goals of this study were to engineer mice with global LOI and then examine the involvement of imprinting in tumorigenesis. By sequentially eliminating and then reestablishing *Dnmt1* expression in ES cells we generated cells which possessed normal global DNA methylation but lacked the methylation associated with imprinting (with the exception of the *Igf2/H19* locus). Gene expression analyses confirmed the success of our approach, with imprinted genes being either silenced or upregulated ~2-fold in IF-MEFs compared with control cells. An analysis of the growth and cell cycle characteristics of IF-MEFs revealed that LOI conferred a number of characteristics possessed by transformed cells, including a higher growth rate, a shortened cell cycle time, cellular immortality, resistance to TGFβ, and foci formation on a confluent monolayer. Consistent with this, IF-MEFs formed tumors in SCID mice and more significantly, adult chimeric mice derived from IF-ES cells developed tumors in multiple tissues. These data demonstrate that in addition to regulating normal embryonic growth, imprinting plays a much wider role in tissue homeostasis by providing an essential tumor suppressor function in the adult.

Previous studies have implicated altered imprinting at the *Igf2/H19* locus with cancer formation. In the case of Wilms tumors in the rare Beckwith-Wiedmann syndrome (BWS), specific loss of normal imprinting on the maternal *H19* allele leads to biallelic expression of *IGF2* expression and is thought increase the number of pre-malignant nephrogenic precursors (Okamoto, Morison et al. 1997; Ravenel, Broman et al. 2001). In recent mouse studies, it was shown that the number of intestinal tumors that form in $Apc^{+/min}$ mutant mice was increased when *Igf2* was biallelically expressed (Sakatani, Kaneda et al. 2005). These studies suggested that a loss of normal imprinting at the *Igf2/H19* locus contributes to Wilms tumors and APC-induced intestinal tumors. However, it is unlikely that deregulated *Igf2* expression was solely responsible for the transformed phenotype we observed caused by global LOI. In our study, the *Igf2/H19* DMR was variably *de novo* methylated following re-expression of *Dnmt1*. This was an expected finding because *Igf2/H19* DMR, unlike other imprinted loci, has been shown to be highly susceptible to *de novo* methylation during cancer progression (Feinberg and Tycko 2004), following reactivation of high levels of *Dnmt1* in $Dnmt1^{-/-}$ mutant cells (Biniszkiewicz, Gribnau et al. 2002), and during *in vitro* cultivation of preimplantation embryos (Latham, Doherty et al. 1994; Mann, Lee et al. 2004). As a result of stochastic methylation at the *Igf2/H19* DMR in both the IF-MEFs and tumors, the expression of *Igf2* (and *H19*) was variable (ranging from normal to approximately biallelic levels in the IF-MEFs). Therefore it is unlikely that the enhanced tumorigenic potential of cells with LOI can be solely attributed to altered expression of *Igf2*. Consistent with this, re-activating monoallelic expression of *Igf2r,* which functions in the inactivation of Igf2, only slightly reduces the level of spontaneous transformation of IF-MEFs (T.M.H and R.J,

unpublished results). Similarly, overexpression of *Igf2* in CTL-MEFs was not sufficient to induce the high rate of spontaneous transformation that was observed with IF-MEFs at low passage number (4-6), although it led to an increased growth rate. We note that at higher passage numbers (>15), MEFs forced to overexpress *Igf2* undergo spontaneous transformation, which is likely the result of secondary mutations that accrue during extensive passaging (Hernandez, Kozlov et al. 2003). Based on these observations, we conclude that imprinted loci other than *Igf2/H19* and *Igf2r* are primarily responsible for the altered growth characteristics and transformed phenotype of cells with LOI.

Immortalization is an essential prerequisite for the formation of a tumor cell, as the introduction of an oncogene into a mammalian cell that is not immortalized will induce senescence or apoptosis due to the activation of anti-neoplastic defense mechanisms (Hahn and Weinberg 2002). It is for this reason that pairs of cooperating oncogenic mutations are needed to transform mouse cells, as the second oncogene is usually required to neutralize the protective pathways triggered by the first oncogene. For instance, overexpression of oncogenic V12 H-Ras alone in MEFs leads to an upregulation of $p19^{Arf}$ and premature growth arrest. Senescence can be avoided in these cells by inhibiting the $p19^{Arf}$-p53 pathway (Serrano, Lin et al. 1997). Similarly, our data suggest that LOI confers immortality to MEFs by inactivating the $p19^{Arf}$-p53 pathway. Western blot analysis revealed a significant decrease in the levels of $p19^{Arf}$ and p53 in IF-MEFs. Consistent with this, overexpression of LgT in IF-MEFs, which binds to and inactivates p53, did not enhance tumor formation in the SCID assay. In contrast, overexpression of V12 H-Ras in IF-MEFs failed to induce growth arrest and instead cooperated with LOI to induce tumors with short latency in SCID mice. Given the role

of $p19^{Arf}$ in protecting p53 from MDM2-dependent degradation, it is possible that the reduction in p53 in IF-MEFs results indirectly from a loss of $p19^{Arf}$. At present, the imprinted gene/s responsible for the reduction in $p19^{Arf}$ and p53 protein levels are unknown and further study is required for their identification.

Deregulated TGFβ signaling is a common feature of malignant cells. In the early stages of tumorigenesis, TGFβ inhibits cell growth by inducing apoptosis and arresting the cell cycle. As cells progress toward a fully malignant tumor phenotype they become resistant to the growth inhibiting effects of TGFβ, while other TGFβ responses remain fully operative (Roberts and Wakefield 2003). At present, the molecular mechanisms by which cancer cells acquire this selective resistance are unclear. Our results indicate that LOI provides one mechanism by which cells can become resistant to the growth inhibitory effects of TGFβ. At least three imprinted genes have been implicated in TGFβ signaling (Tsibris, Segars et al. 2002). TSP-1 and IGF2R act as non-signaling receptors for TGFβ and facilitate the cleavage and activation of the latent (preproprotein) ligand (Godar, Horejsi et al. 1999; Murphy-Ullrich and Poczatek 2000). LOI-induced loss of *Tsp-1* and *Igf2r* expression, resulting in a failure to process latent TGFβ, is unlikely to be responsible for the lack of TGFβ responsiveness as IF-MEFs also failed to respond to active, cleaved ligand. $p57^{kip2}$ is a more likely candidate, as this gene encodes a putative tumor suppressor that belongs to the Cip/Kip family of cyclin-dependent kinase (CDK) inhibitors and acts as a negative regulator of the cell cycle(Deshpande, Sicinski et al. 2005). In human hematopoietic cells, $p57^{kip2}$ is upregulated by TGFβ and is essential for mediating its cytostatic effects on blood cells (Scandura, Boccuni et al. 2004). Whether $p57^{kip2}$ plays a similar role in MEFs has yet to be determined. A third possibility comes

from the recent discovery that p53 plays a key role in TGFβ-induced growth arrest (Cordenonsi, Dupont et al. 2003; Takebayashi-Suzuki, Funami et al. 2003). *p53*$^{-/-}$ MEFs show a defective cytostatic response to TGFβ and an inability to upregulate the CDK inhibitor p21^{Cip1} (Cordenonsi, Dupont et al. 2003). Furthermore, p53 interacts with Smad2 and Smad3, downstream mediators of TGFβ signaling, where it appears to function as part of a transcriptional complex (Liberati, Datto et al. 1999; Labbe, Letamendia et al. 2000; Cordenonsi, Dupont et al. 2003; Seoane, Le et al. 2004). It is tempting to speculate that the mechanism by which LOI inhibits the p19^{Arf}-p53 pathway to immortalize MEFs is related to its ability to confer resistance to TGFβ-induced growth arrest. Further study is required to unravel the importance of p53 in LOI-induced tumorigenesis.

Consistent with LOI rendering MEFs highly susceptible to transformation, all chimeric mice derived from IF-ES cells developed tumors in multiple tissues. This suggests that global LOI has a causal role in promoting cancer. The most common cancers were hepatocellularcarcinomas and intestinal adenomas. It is unclear why this particular spectrum of cancer was observed in the IF-chimera mice. It is possible, however, that tumorigenesis may be due to the dysregulation of imprinted tumor suppressor genes and oncogenes specific to each organ. The long latency of tumor development indicates that LOI sensitizes cells to cancer and that additional somatic events are required for tumor development. Offspring derived from chimeric mice that had inherited the ES cell derived IF genome were cancer free. This indicates that the aberrant state of genomic imprinting in the IF-ES cells is fully reversible after passage through the germ line, consistent with the fact that the methylation associated with

imprinting can only be established in germ cells. If the tumorigenic phenotype in the LOI model was caused by a random mutational event during ES culture, then tumors would be expected to also arise in the F1 progeny. Although it is possible that an oncogene or tumor suppressor gene may have been improperly methylated (and thereby became abnormally expressed) during the generation of the IF-ES cells, we would have expected to detect such an abnormality from the microarray analysis. Furthermore, the likelihood of such an event occurring in two independently derived IF-ES cell lines but not the CTL-ES lines is small.

In summary, the ability of a cell to become immortal is a prerequisite for transformation and ultimately tumor formation. By manipulating the maintenance methylase *Dnmt1* we were able to generate ES cells that lack the methylation marks required for maintaining imprinting, leading to global LOI. Our study provides strong evidence that LOI predisposes cells to cancer. The data support a model in which LOI at key loci encoding tumor suppressors and oncogenes provides the first step towards tumor formation by conferring cellular immortality. Furthermore, our results are consistent with subsequent genetic alterations, such as constitutive activation of a mitogenic signal, providing the next step necessary for a fully transformed phenotype both *in vitro* and *in vivo*.

2.6 Experimental Procedures

2.6.1 2-Frt flanked *Dnmt1* inactive allele.

We constructed an inactive *Dnmt1* allele (2-Frt) by inserting a STOP cassette flanked by two Frt sites between the fourth and fifth coding exons in the *Dnmt1* gene. This allele becomes re-activated following Flp-mediated recombination (Fig 1).

2.6.2 Generation of imprint free and control ES cells.

All experiments on live vertebrates were preformed in accordance with relevant institutional and national guidelines and regulations. Massachusetts Institute of Technology's committee on animal care has approved the experiments and has confirmed that all experiments conform to the relevant regulatory standards. The most recent review and approval received on November 4th 2004.

Mice heterozygous for the inactive 2Frt *Dnmt1* allele were crossed to mice homozygous for the conditional 2LoxP *Dnmt1* allele(Jackson-Grusby, Beard et al. 2001) and the *ROSA26*-β-geo Flp reporter allele (Possemato, Eggan et al. 2002). Fertilized embryos were dissected at E0.5, cultured until the blastocyst stage, and explanted for ES cell line derivation as described (Hochedlinger and Jaenisch 2002). ES cells were cultured as previously described (Li, Bestor et al. 1992). Two independently derived ES cell lines tested positive for both the 2Frt and the 2LoxP *Dnmt1* alleles. These cells were exposed to Cre-recombinase through lipofectamine mediated, transient transfection. After short-term puromycin selection positive, clones were picked, expanded and their genomic DNA extracted. Clones were analyzed for Cre mediated recombination of the *Dnmt1*

(2LoxP to 1LoxP) allele by southern blotting. Positive clones were expanded and the process repeated with exposure to Flpe-recombinase to excise the stop cassette and reactivate *Dnmt1* expression. The resultant doubly recombined *Dnmt1* ES cells were termed IF (Fig1). Control ES cells (CTL) were generated by exposure to the same recombinases but in the reverse order (Fig 1). Genomic DNA was extracted from ES cells at each stage of recombination for each *Dnmt1* genotype 2Loxp/2Frt, 1LoxP/2Frt, 2LoxP/1Frt and 1LoxP/1Frt. Southern blotting analysis was carried out after methylation sensitive digestion with HpaII. Repetitive regions and elements known to be methylated were probed, these included classic centromic satellite repeats, IAP and LINE element (Biniszkiewicz, Gribnau et al. 2002)(Fig 3).

2.6.3 Derivation of primary embryonic fibroblast cell lines from IF- and CTL-ES cells.

MEFS were derived from day 13.5 embryos obtained by injecting IF and CTL-ES cells into wild-type BDF1 blastocysts and implanted into foster mothers. IF and CTL MEFs derived from chimeric embryos were selected for two passages with the appropriate antibiotics. MEFs were frozen at passage 2 or 3 and with the exception of long-term culture were used for all experiments before passage 6. Only pure populations were used as assayed by genomic southern or PCR. MEFs and 293T ecotrophic packaging cell line were grown in DMEM supplemented with 10% FBC, 5mM Glutamine and penicillin/streptomycin. All the cells used tested negative for mycoplasma.

2.6.4 Characterization of the methylation and expression status of IF- and CTL-MEFs.

Genomic DNA was extracted from MEFs isolated from different chimeric fetuses and methylation analysis of selected imprinted genes was preformed by two different methods. Methylation sensitive enzymatic digestion followed by southern blotting was used to probe for *Igf2r* methylation (Biniszkiewicz, Gribnau et al. 2002). COBRA analysis was used to analyze the methylation status of *Igf2 Snrpn* and *Peg3* (Lucifero, Mertineit et al. 2002). Expression analysis of selected imprinted genes was carried out by two different methods using total RNA extracted from different clones of CTL- and IF-MEFs. *Igf2r, H19, Igf2, Peg5* and *Grb10* expression was analyzed by Northern blotting using cDNA probes to the open reading frame of these genes. $p57^{kip2}$, *Peg1*, and *Peg3* expression was analyzed by RT-PCR using the following primers. $p57^{kip2}$ forward ctgacctcagacccaattcc and reverse gttctcctgcgcagttctct. *Peg1* forward gctggggaagtagctcagt and reverse tttcttcttagcaagggcca. *Peg3* reverse cttctggaagccgacattatc and forward cctgatcaatgggttccttg. $\beta-Actin$ forward ggtcagaaggactcctatgtgg and reverse tccctctcagctgtggtggt

2.6.5 Microarray analysis of IF- and CTL-MEFs.

Arrays were manufactured from the Mouse Genome Oligo Set V3 (Operon), containing 31,769 oligo probes. Oligos were printed onto CodeLink Activated Slides (Amersham) using an Omnigrid 100 printer (Genemachines). Printing and post processing of arrays was carried out according to the manufacturers instructions, using a

modified print buffer containing 250mM phosphate buffer (pH 8.5), with 0.00025% N-Lauroylsarcosine.

For expression profiling, 5μg of total RNA from independent IF-MEF (n=6) and CTL-MEF (n=2) populations were reverse transcribed using a T7-oligo-d(T)-primer (TCTAGTCGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGT$_{21}$N). Second strand cDNA was synthesized as previously described (Gubler and Hoffman 1983). Double stranded cDNAs were purified and in vitro transcribed with the Microarray RNA Target Synthesis Kit T7 (Roche). Unlabelled cRNAs were purified and 1μg cRNA per channel was labelled with 1μl Cy5 or Cy3 using the Micromax ASAP RNA labelling kit (Perkin Elmer). Arrays were hybridized, washed and dried according to the Agilent 60-mer oligo microarray processing protocol.

Arrays were scanned with a GenePix400B Scanner (Axon Instruments) and expression data was extracted by Genepix Pro 6 Software (Axon). Data normalization within and between arrays was performed with the Limma software package of the Bioconductor project (Smyth 1983).

2.6.6 Retroviral infection assay

MEFs were infected with a high titer of retrovirus stocks produced by transient transfection of 293T cells with an ecotrophic packaging virus. pBabe-blast or pWZL-blast cells containing the retroviral infection were selected out with Blactocydin. The pBabe-blast and pWZL-blast vectors containing constitutively active V12H-Ras or Large T antigen cDNA, respectively were both gifts of R. Weinberg (The Whitehead Institute).

2.6.7 Immunoblotting

Whole-cell extracts of exponentially growing cells were prepared in lysis buffer (65 mM Tris pH7, 1% NP40, 2 mM EDTA, 100 mM NaCl) containing the Complete cocktail of proteases inhibitors (Roche), and protein concentrations were determined with the BioRad *D/C* protein assay reagent. Immunoblot analysis of p53 (Oncogene, AB-3, 1 : 500), $p16^{Ink4a}$ (Santa Cruz, M-156, 1 : 1000), $p21^{Cip1}$ (Santa Cruz, F-5, 1 : 1000) $p19^{Arf}$ (Novus Biological, AB80-100, 1 : 500) was performed with 60 µg of proteins run on 12.5% acrylamide gels transferred to Immobilon-P (Millipore). Secondary antibodies coupled to HRP were purchased from Jackson ImmunoResearch Laboratories and used at 1 : 10000. Detection was performed by chemiluminescence.

2.6.8 Growth Curves, immortalization and transformation assays

All experiments were repeated at least 3 times using cells from a minimum of 5 different fetuses for each genotype. For growth curves 3 x 10^4 cells were plated into 12 well dishes and fed every 2^{nd}day. Serial 3T3 cultivation was conducted as described. To test the ability of the cells to form multiple layers (foci formation assay), 1x10^5 cells were plated in triplicate for each of the different genotypes and grown for 28 days the medium was changed every second day. Plates were then fixed in ice-cold methanol and stained with crestal violet. Low density foci assays were conducted by plating 1.3 x 10^3 MEFs as described (Sage, Mulligan et al. 2000). To test tumorigenicity, exponentially growing cells were resuspended in PBS and 1 x 10^6 cells were injected subcutaneously into SCID mice. Tumor development was monitored for up to 6 months. IF and CTL-MEFs infected with pBabe V12HRas-blastoiciden, pBabe-blastociden, pWZL Large T antigen-

blastociden and pWZLGFP-blastociden were selected for three days in 2ug/ml blasticiden and then replated to be expanded for foci formation assays and injection into SCID mice.

2.6.9 TGFβ and the cell cycle.

Cells were plated at 3 x 10^4 in 12 well plates from 6 different embryos for each genotype in triplicate and 24 hours later exposed to TGFβ at a concentration of 0.1nM. Cells were then counted each day for 5 days. Cells were prepared for cell cycle analysis as described (Brugarolas, Bronson et al. 1998). Samples were processed using FACScan apparatus (Becton Dickinson) and the data were analyzed using ModFit LT software (Becton Dickinson).

2.6.10 Generation of imprint free chimeric mice and histological analysis

IF- and CTL-ES cells were injected into wild-type BDF1 blastocysts. Chimeric animals were born and allowed to age for time points, 9, 12, 18 months. Tumors were removed at necropsy from chimera animals and fixed in formalin for sectioning and later stained with H & E

2.6.11 PCR, RT-PCR and methylation tumor analysis

Genomic DNA from all tumors was extracted and tested for the presence of the neomycin gene using the following primers, forward AAG CCG GTC TTG TCG ATC AG and reverse GAT ATT CGG CAA GCA GGC AT. The methylation status of genomic DNA associated with *Igf2* and *Igf2r* was assessed by COBRA and methylation sensitive digestion and southern blotting respectively (Biniszkiewicz, Gribnau et al. 2002;

Lucifero, Mertineit et al. 2002). RNA was extracted from tumors using the Qiagen RNasy Kit and reverse transcriptase PCR was preformed using the following primer pairs for these genes. *Igf2* forward tgcttctcatctctttggcc and reverse ggcacagtatgtctccagga. *Igf2r* forward gtgtggtatttttatgtatagttagg and reverse aaatatcctaaaaatacaaactacac. $p57^{kip2}$ forward ctgacctcagacccaattcc and reverse gttctcctgcgcagttctct. β-Actin forward ggtcagaaggactcctatgtgg and reverse tccctctcagctgtggtggt.

Chapter 3:

Global loss-of-imprinting leads to a failure in embryonic development at mid-gestation

L. Jackson-Grusby*[1,3], T.M Holm*[1,2], W. M. Rideout 3[rd1,3], Y. Yamada[1,3]

J. Dausman[1] and R. Jaenisch[1,2†]

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* These authors contributed equally to this work

3. Whitehead Institute for Biomedical Research, Cambridge, MA 02142, USA

4. Massachusetts Institute of Technology, Cambridge, MA 02142, USA

3. Present addresses: Children's Hospital Boston and Harvard Medical School Pathology Departments, Boston, USA (LJG), Department of Tumor Pathology, Gifu University, Japan (YY), AVEO Pharmaceutical Inc, Cambridge, USA (WMR)

† To whom correspondence should be addressed. E-mail: jaenisch@wi.mit.edu

3.1 Abstract

Imprinting, a mammalian adaptation whereby subsets of genes are differentially expressed depending on their parental origin, is thought to be maintained by DNA methylation. The 'parental conflict' hypothesis has been proposed to explain imprinting and is based on a conflict between the mother and father over the allocation of maternal resources to the embryonic offspring. To examine the effect of global loss-of-imprinting (LOI) on development, we generated imprint-free embryonic stem (IF-ES) cells by conditionally inactivating and then reactivating the DNA methyltransferase, *Dnmt1*. In addition, we erased the imprint methylation marks in androgenetic ES cells (IFAT-ES) and examined the developmental potential of IF-ES and IFAT-ES cells by tetraploid complementation, relative to control ES cells. IF-ES and IFAT-ES cells were unable to support development to full-term gestation and the resulting embryos ceased developing at E11.5 and E9.5-10.5, respectively. Interestingly, IFAT-embryos developed further than control AT-embryos, which failed to develop beyond at E8.5. Using chimera studies, we showed that both IF- and IFAT-ES cells retained pluripotency in contrast to AT-ES cells. The chimera studies also demonstrated that LOI induces an overgrowth phenotype in IF-embryos, which likely contributes to their lethality. Restoration of normal monoallelic expression of the imprinted gene *Igf2r*, a negative regulator of embryonic growth, extended development of IF-embryos to day E12.5, but was unable to rescue the overgrowth phenotype or lethality. Furthermore, our results indicate that androgenetic ES cells that are devoid of imprint methyl marks are not equivalent to IF-ES, suggesting that mechanisms other than DNA methylation may maintain imprinting.

3.2 Notes

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3.3 Introduction

Nuclear transfer experiments have demonstrated that the maternal and paternal genomes are nonequivalent, and both are required for normal development of the mouse embryo (Barton, Surani et al. 1984; McGrath and Solter 1984; Surani, Barton et al. 1984). The differences between the paternal and maternal genome are inherited from the gametes in which parental imprinting of the genome is established. Genomic imprinting is classically defined as the differential expression of the paternal or maternal allele of a small set of genes. Among the 40 or so known imprinted genes, some such as *H19*, *Igf2r* and $p57^{kip2}$, are expressed when inherited from the mother while others, such as *Snrpn*, *Peg1* and *Peg3*, are expressed when inherited from the father.

DNA methylation patterns are established and maintained during development by three distinctive DNA cytosine methyltransferases (Dnmt1, Dnmt3a, and Dnmt3b) (Bird and Wolffe 1999). Recently, a third member of the Dnmt3 family, the DNA methyltransferase-like protein Dnmt3L, was shown to be required for the establishment of maternal methylation imprints in mouse (Bourc'his, Xu et al. 2001; Hata, Okano et al. 2002). Although Dnmt3L lacks the conserved residues known to be involved in DNA

methyltransferase activity and is inactive on its own, it has been shown that Dnmt3L physically interacts with Dnmt3a and Dnmt3b and is necessary for maternal and paternal *de novo* methylation in gametogenesis (Bourc'his, Xu et al. 2001; Hata, Okano et al. 2002; Bourc'his and Bestor 2004; Kaneda, Okano et al. 2004; Suetake, Shinozaki et al. 2004; Hata, Kusumi et al. 2005).

Dnmt1 is believed to function primarily as a maintenance DNA methyltransferase that is required for stable inheritance of tissue-specific methylation patterns. Inactivation of *Dnmt1* in mice leads to global loss-of-methylation and bi-allelic expression or silencing of imprinted genes (Li, Bestor et al. 1992; Li, Beard et al. 1993; Caspary, Cleary et al. 1998). Thus, DNA methylation is believed to be the epigenetic mechanism that controls imprinting in mammals. The presence of differentially methylated regions (DMRs) between paternal and maternal alleles in almost all imprinted genes provides a molecular basis for the regulation of allele-specific expression (Neumann and Barlow 1996; Ferguson-Smith and Surani 2001). DNA methylation is a reversible epigenetic process that can be reprogrammed during embryogenesis and gametogenesis (Reik, Dean et al. 2001). Parental methylation imprints are erased in the primordial germ cells during embryogenesis and are re-established during gametogenesis in male and female germ cells independently (Reik and Walter 2001). In addition, the DMRs of imprinted genes must resist the genome-wide demethylation that takes place soon after fertilization and the wave of *de novo* methylation that occurs after implantation (Monk, Boubelik et al. 1987).

Imprinted genes appear to function in a wide range of developmental processes such as the regulation of embryonic growth, placenta function, and maternal behavior.

While various theories have been proposed to explain the evolution and function of imprinting (Moore and Haig 1991) (Barlow 1995) (Jaenisch 1997) (Tilghman 1999), the 'parental conflict' hypothesis by Moore and Haig is the most widely accepted. The 'parental conflict' proposes that imprinting arose in polyandrous (multiple fathers per litter) mammals as a result of a conflict between the father and the mother over the allocation of maternal resources to the offspring. It is envisioned that the father's objective is to achieve maximal flow of nutrients to its offspring to effect maximal growth. Thus, many of the paternally-expressed imprinted genes are involved in placental growth or function. Conversely, the mother's objective is to spread resources evenly to her offspring. Ultimately, this tug of war results in a growth-rate that is a compromise between the two parental objectives. The 'parental conflict' hypothesis makes a specific prediction that if imprinting were eliminated from both the paternal and the maternal genomes, there would be no ill effects and the 'imprint free' genome would support normal embryonic development (Moore and Haig 1991; Jaenisch 1997).

In this study, we have examined the effect of loss-of-imprinting (LOI) on embryonic development by using conditional alleles of *Dnmt1* to remove the imprinted methyl marks from the genome of both biparental (wild-type) and androgenetic (AT) embryonic stem (ES) cells. The developmental potential of these imprint-free (IF) cells were then examined by tetraploid complementation and chimera studies. Our analysis showed that LOI was embryonic lethal in a biparental background and caused an overgrowth phenotype that could not be rescued by reactivating expression of the negative growth regulator, *Igf2r*. In an AT background, LOI extended development compared with unmanipulated AT-embryos and restored pluripotency to IFAT-ES cells.

However, IFAT-embryos died at an earlier stage than biparental IF-embryos, suggesting that other mechanisms may exist to maintain non-equivalency between the paternal and maternal genomes.

3.4 Results

3.4.1 Imprint free and Control ES cells.

We have previously reported a technique to generate IF-ES cells by sequentially inactivating and then restoring the expression of conditional alleles of *Dnmt1* using cre and flpe recombinases (Holm et al., 2005). This strategy results in global LOI while still preserving the genome-wide methylation of repetitive DNA elements. As a control, the ES cells were exposed to flpe then cre (in the reverse order) resulting in the preservation of Dnmt1 activity and normal methylation patterns **(Fig 3.1)**.

3.4.2 Effect of LOI on embryogenesis

In our earlier study, the effect of LOI on embryogenesis was not examined. To investigate this, embryos derived from the IF-ES cells were generated by tetraploid complementation. No full-term pups were ever recovered from these experiments, suggesting that LOI resulted in embryonic lethality. Timed dissections were then performed to determine the developmental stage the IF-embryos were achieving. IF-embryos with a normal gross morphology were found from E8.5-10.5. At E11.5, IF-embryos displayed mixed characteristics of both E10.5 and E11.5 wild-type embryos **(Fig 3.2; Table 3.1)**. For example, IF-embryos at this stage displayed retina pigmentation and limb bud development that was characteristic of the E11.5 stage.

Figure 3.1 Generation of IF-ES cells

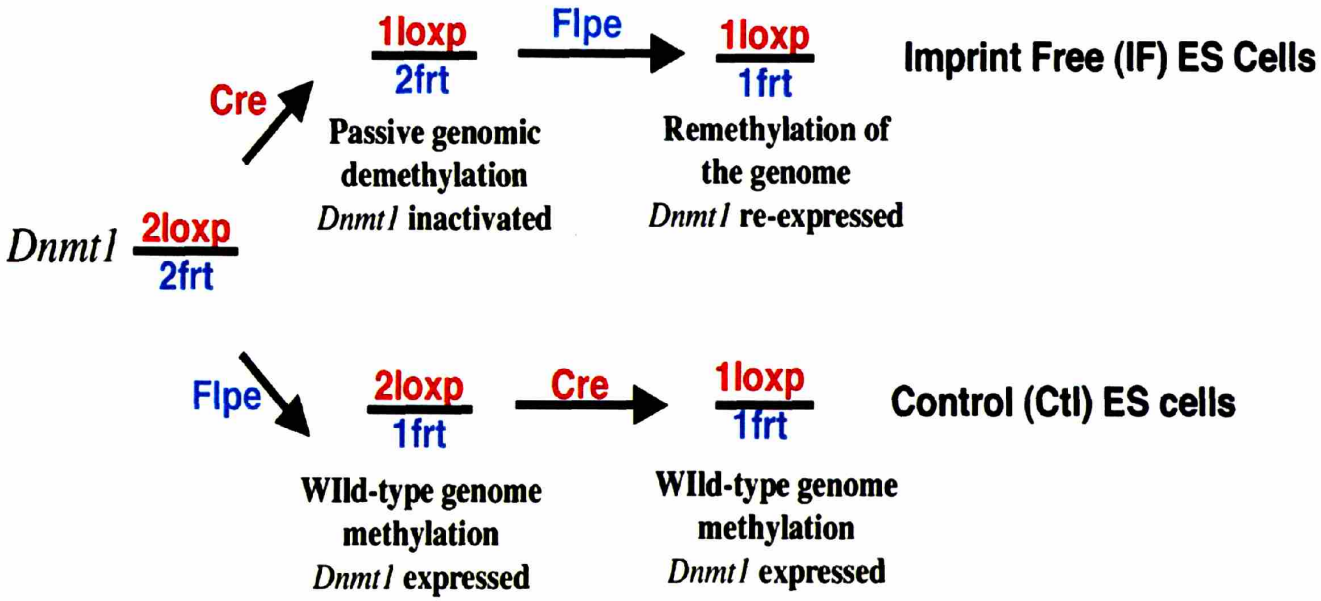

Figure 3.1
Schematic representation of the steps used to create the IF-ES cells. Red and blue indicate the *Dnmt1* genotype. CTL-ES cells were generated by reverse exposure to the recombinases.

Figure 3.2 Development of embryos with different epigenetic status.

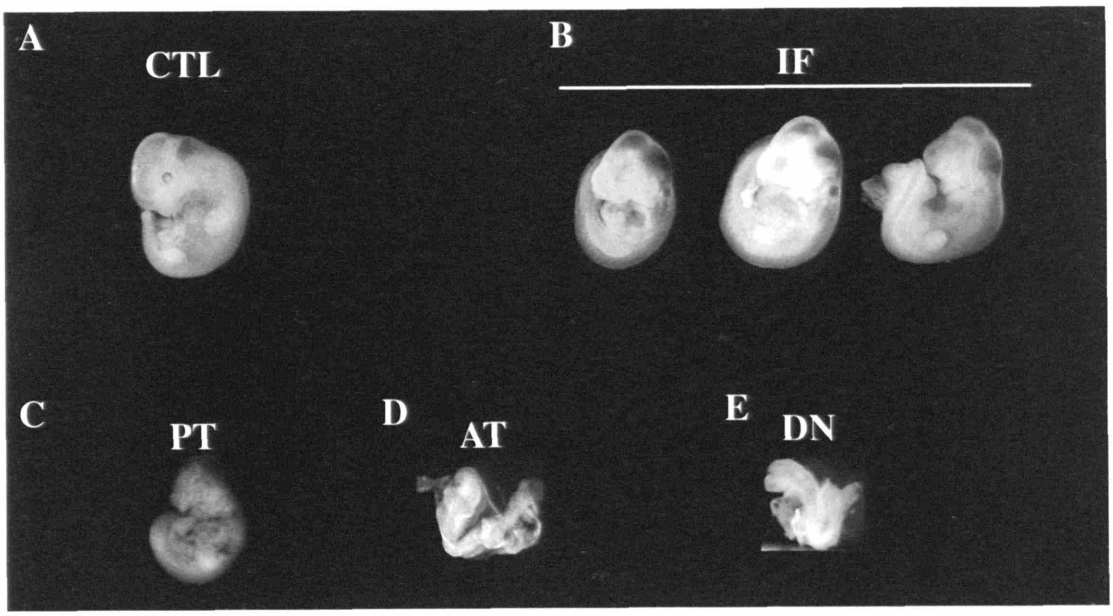

Figure 3.2 Development of embryos derived from ES cells with different epigenetic status using tetraploid complementation.

(A) Embryo derived from CTL-ES cells at day E11.5. (B) Embryos derived from IF-ES cells at day E10.5-11.5. (C) Embryo derived from PT-ES cells at day E9.5-10.5. (D) Embryo derived from AT-ES cells at day E8.5. (E) Embryo derived from Dnmt1 null (DN) 2Frt/1LoxP ES cells at day E8.5.

However, the overall morphology of IF-embryos at E11.5 more closely resembled that of an E10.5 biparental wild-type embryo, although IF-embryos were qualitatively larger than expected for the E10.5 stage. The larger size of IF-embryos may be indicative of an overgrowth phenotype or alternatively the IF-embryos may have stalled their development between E10.5 and E11.5 but continued to increase in size. Dissections of IF-embryos after the E11.5 time point revealed no further advancement in embryonic development and necrosis became evident at E14.5. We conclude from these data that embryos lacking imprinting fail to develop beyond the E10.5-11.5 stage, thus indicating that imprinting is essential for embryogenesis.

IF-embryos represent a state in which the methyl marks of imprinting are removed. We next wanted to compare the embryonic lethal phenotype of IF-embryos to those of embryos with different states of genome methylation. To do this, we generated ES cells with uniparental patterns of imprinting (parthenogenetic and androgenetic), as well as ES cells that were completely devoid of imprinting and repetitive sequence methylation (*Dnmt1* null embryos). To create the parthenogenic (PT) ES cells, we first generated PT blastocyts by activating oocytes with strontium and cytochalasin B, and then used these to derive an ES cell line with maternal-only imprinting. Tetraploid complementation was then used to make PT-embryos from these parthenogenetic ES cells. Timed dissections revealed that PT-embryos survived to E9.5-E10.5, as previously reported (McGrath and Solter 1984; Surani, Barton et al. 1984; Spindle, Sturm et al. 1996), and appeared grossly normal (**Fig 3.2; Table 3.1**).

Table 3.1

Embryos generated from different epigenetic states

Status of embryonic imprinting.	Number of embryos examined.
Generated by tetraploid complentation	
Biparental-IF (IF)	19
Androgenetic (AT)	8
Androgenetic-IF (IFAT)	4
Parthenogenetic (PT)	7
Dnmt1 null 2F/1L (DN)	3
Day E13.5 chimeras	
Biparental-IF (IF)	29
Biparental-CTL (CTL)	28
Androgenetic (AT)	9
Androgenetic-IF (IFAT)	15

To generate androgenetic (AT) ES cells, we removed the maternal pronucleus from a fertilized egg and then fused the zygote to a second male pronucleus that was transplanted just under the *zona pellucida*. AT-embryos generated by tetraploid complementation were found to develop up to the E8.5 stage, but not beyond this time point (**Fig 3.2; Table 3.1**).

To create *Dnmt1*-null embryos (DN), the ES cell line carrying the cre- and flpe-conditional alleles of *Dnmt1* was exposed to cre alone (resulting in inactivation of Dnmt1 activity), and then used as a source of cells for tetraploid complementation. Similar to the androgenetic embryos, DN-embryos developed up to E8.5 but not beyond (**Fig 3.2; Table 3.1**), suggesting that the loss of genome-wide DNA methylation mediated by Dnmt1 perturbs embryogenesis to a similar extent as having a paternal-only pattern of imprinting. Taken together, these data highlight the importance of correct DNA methylation patterns for embryonic development and indicate that the severity of developmental defects caused by altered DNA methylation patterns follows the phenotypic order of DN=AT>PT>IF.

3.4.3 LOI and chimera studies

Chimera studies have shown that cells with uniparental patterns of imprinting contribute unequally to tissues in the embryo, suggesting that the imprint status of the ES cell affects its pluripotency (Thomson and Solter 1988; Allen, Logan et al. 1995). To determine whether LOI affects ES cell pluripotency, IF-ES cells (carrying a LacZ reporter) were injected into biparental wild-type blastocysts and chimeric embryos at E13.5 were analyzed. Staining for LacZ activity revealed that IF-ES cells contributed to

Figure 3.3 IF-Chimera studies

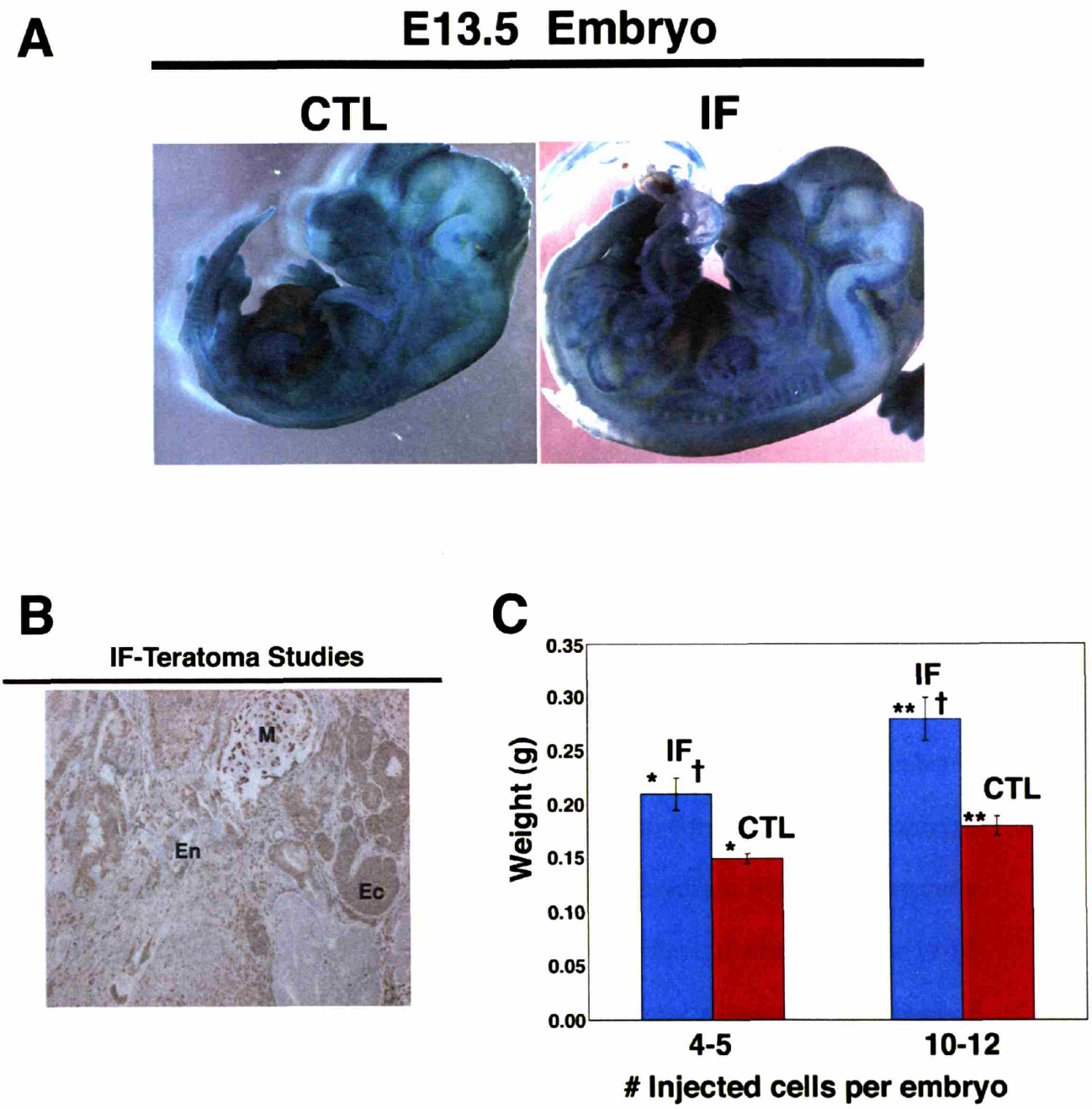

Figure 3.3 IF-Chimera studies.
(A) Day E13.5 chimeric embryos cut in sagittal section and stained for the presence of the IF and CTL- ES cell marker βgalactosidase. (B) Teratoma tumor formation from WT/IF ES cells immunohistochemically stained for GFP. M= Mesodermal (Cartilage); En= Endodermal (Glandular epithelium/GI); Ec= Ectodermal (Neural) (C) IF-Embryos are a larger size. Weight distribution of IF and CTL chimeric animals . P values: * = 2.1 x 10-5, ** = 6.8 x 10-5, † = 5.6 x 10-3.

all tissues of the chimera with the same level of efficiency as control ES cells (**Fig 3.3A; Table 3.1**). To explore this more closely, we injected wild-type and IF-ES cells (carrying the GFP reporter) under the skin of severe combined immunodeficient (SCID) mice and assayed teratoma formation. As an internal control, to ensure that the teratomas were capable of generating tissue derivatives from all three germ layers, a 1:1 mixture of IF-ES cells and wild-type ES cells were injected into the SCID mice. After 14 days, the teratomas were dissected, sectioned, and immunohistochemically stained for GFP. The IF-ES cells, like the co-injected wild-type ES cells, were found to give rise to tissue derivatives from all three germ layers, including glandular epithelial cells (endoderm), neuronal cells (ectoderm) and cartilage (mesoderm; En, Ec and M in **Fig 3.3B**). Taken together, these results indicate that LOI does not result in a loss of ES cell pluripotency or biased contribution.

In the course of performing the IF chimera experiments, it was noticed that the greater the number of IF-ES cells that contributed to the chimera, the larger the embryos appeared (**Fig 3.3A**). To more accurately quantitative this, either a small (4-5) or large (10-12) number of IF-ES cells or control ES cells were injected into wild-type blastocysts and the resulting embryos were weighed at E13.5. Regardless of whether 4-5 or 10-12 IF-ES cells were injected, there was a statistically significant increase in the weight of chimeric IF-embryos compared with chimeras derived from control ES cells (**Fig 3.3C**).

3.4.4 Reactivation of the *Igf2r* allele in IF-embryos

We and others have previously found that despite a loss of the methyl marks that maintain imprinting, the *Igf2* locus becomes variably re-methylated and therefore re-

Figure 3.4 Reactivation of *Igf2r* expression

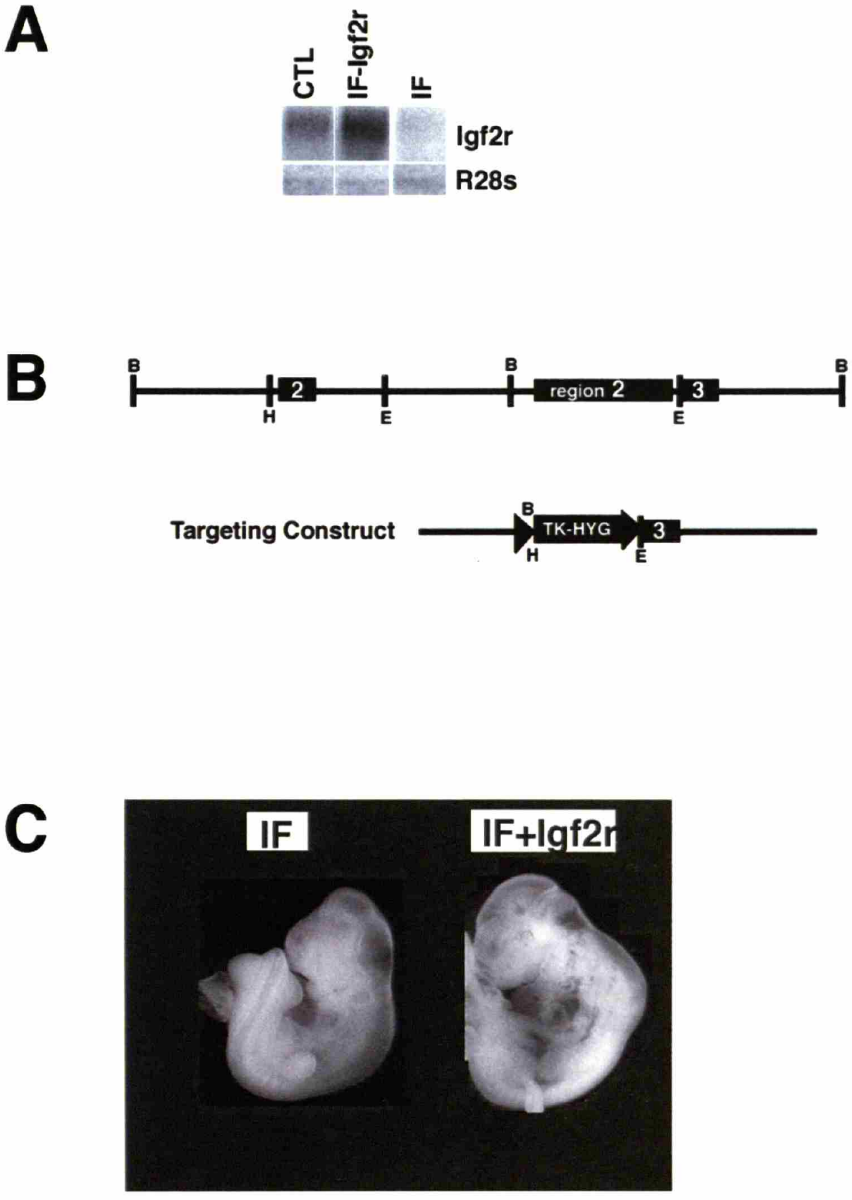

Figure 3.4 Reactivation of *Igf2r* expression
(A) Confirmation of *Igf2r* expression by northern blot analysis of RNA extracted from Day 7 retinoic acid differentiated CTL, IF+Igf2r and IF-ES cells. (B) Targeting construct for reactivation of *Igf2r* allele (Wutz et al 1997). B=BamH1, H=HinDIII, E=EcoRI. (C) Development of embryos derived from both IF and IF+Igf2r ES cells using tetraploid complementation. IF embryonic development to day E10-11.5. IF+Igf2r embryonic development to day E12.5.

expressed in IF cells (Tucker, Beard et al. 1996) (Biniszkiewicz, Gribnau et al. 2002) (Holm et al., 2005). Consistent with this, we found expression of *Igf2* in IF-embryos, contrary to what one would expect in a LOI state (data not shown). In contrast, the *Igf2r* locus, which encodes a negative regulator of Igf2 that acts by sequestering excess ligand, was not expressed in IF-embryos, as expected (**Fig 3.4A**). Based on these observations, we hypothesized that the overgrowth phenotype associated with LOI may be related to an imbalance in the expression of *Igf2* and *Igf2r*. To test this, we removed the differentially methylated region (DMR) in intron two of the *Igf2r* locus by gene targeting in IF-ES cells (**Fig 3.4B**). Deletion of this DMR is known to result in re-activation of *Igf2r* expression (Wutz, Smrzka et al. 1997). Successful re-activation of monoallelic *Igf2r* expression in the targeted IF-ES cells (referred to herein as IF+*Igfr2*) was confirmed by Northern blot analysis following retinoic acid-induced differentiation (**Fig 3.4A**). The IF+*Igfr2* ES cells were then used to generate embryos by tetraploid complementation. No full-term pups were recovered from these experiments and timed dissections revealed that the IF+*Igfr2* embryos developed until E12.5, before becoming necrotic and partially reabsorbed (**Fig 3.4C**). Grossly, the IF+*Igfr2* embryos displayed an overgrowth phenotype similar to IF-embryos (data not shown). To more accurately quantitate this, we made IF+*Igfr2* chimeric embryos and compared their weights with IF-ES and control ES chimeras. This analysis revealed no significant statistical difference between the weights of IF+*Igfr2* and IF chimeras (**Fig 3.5A**). In order to obtain further evidence that the growth of IF cells was unaffected by re-activating *Igf2r* expression, we derived murine embryonic fibroblasts (MEFs) from IF and IF+*Igfr2* embryos and examined their growth rates *in vitro*. IF and IF+*Igfr2* MEFs were found to have similar growth rates to

Figure 3.5 Growth characteristics of IF+Igf2r chimeric embryos and MEFs

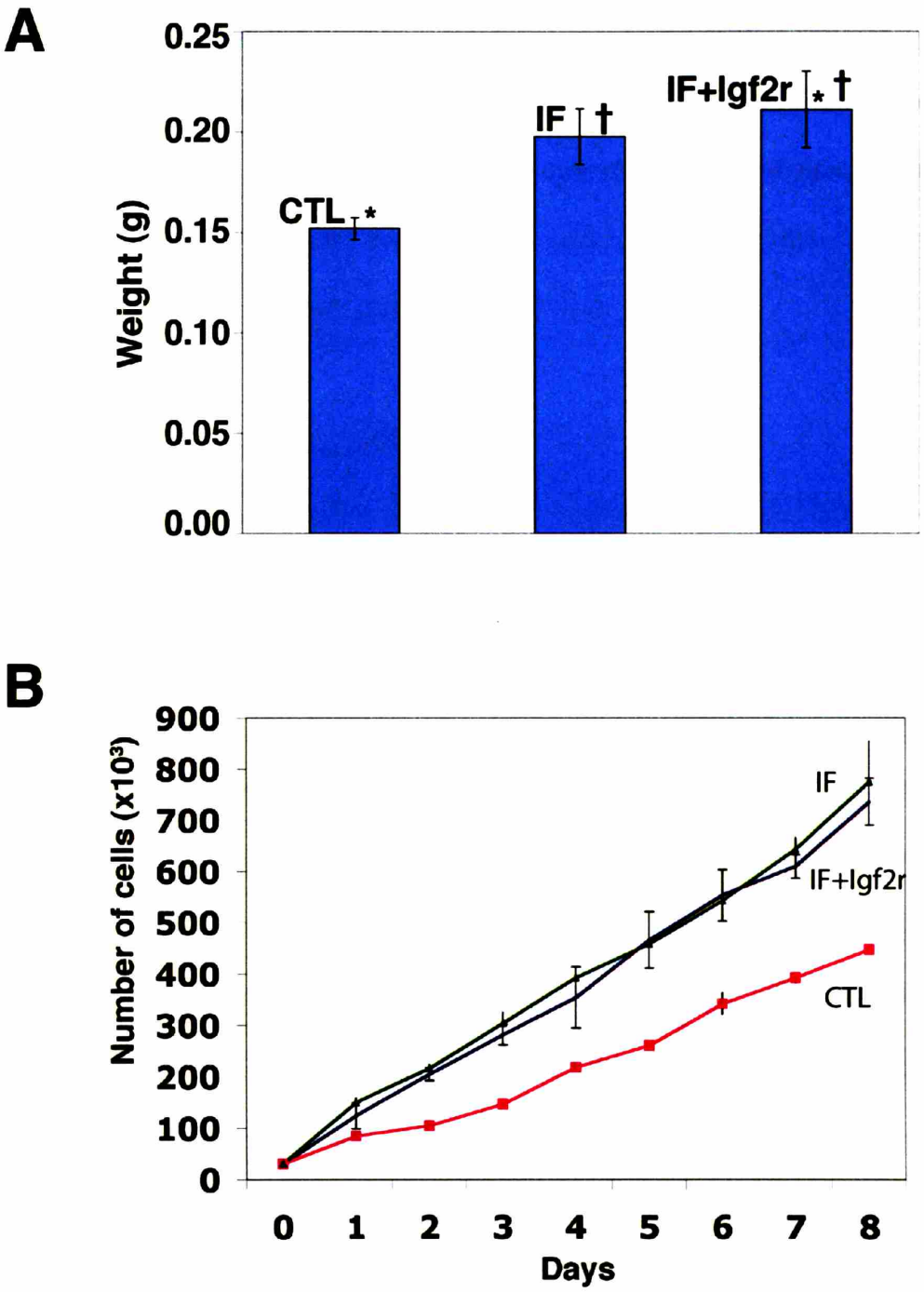

Figure 3.5 Growth characteristics of IF+Igf2r chimeric embryos and MEFs
(A) Increased body weight of E13.5 IF chimeric embryos is not altered by reactivation of *Igf2r* expression. P values: * = 8.0 x 10-3, † = not significantly different. (B) Growth rate of IF, IF+Igf2r and CTL murine embryonic fibroblasts.

each other, although these rates were markedly higher than that of control MEFs (**Fig 3.5B**). We conclude from these experiments that an imbalance between *Igf2* and *Igf2r* expression is not the primary cause of the overgrowth phenotype or the lethality observed in the IF-embryos. However, given that re-activation of *Igf2r* expression in IF-embryos extended their development from E11.5 to E12.5, it is likely that Igf2 signaling does contributes to the embryonic phenotype of IF-embryos.

3.4.5 Imprint free androgenetic embryos

If DNA methylation is the sole mechanism responsible for maintaining imprinting, then erasing the methyl marks associated with imprinting in AT-ES cells would be expected to extend embryonic development from E8.5 to E11.5. To test this, *Dnmt1* expression was sequentially inactivated and then re-activated in AT-ES cells, according to the strategy used to generate IF-ES cells. IFAT-embryos were then derived by tetraploid complementation, and timed dissections performed to determine the developmental stage reached by IFAT-embryos. Unlike AT-embryos, IFAT-embryos underwent turning and developed relatively normally to the E9.5-10.5 stage (**Fig 3.6A; Table 3.1**). However surprisingly, the IFAT-embryos failed to develop beyond this stage and were found necrotic and partially reabsorbed at later time points. This finding, contrary to expectations, suggests that a mechanism/s other than DNA methylation are responsible for the parent-of-origin specific patterns in imprinted gene expression. At present, the nature of such a mechanism is unclear.

Figure 3.6 Generation of AT and ATIF embryos

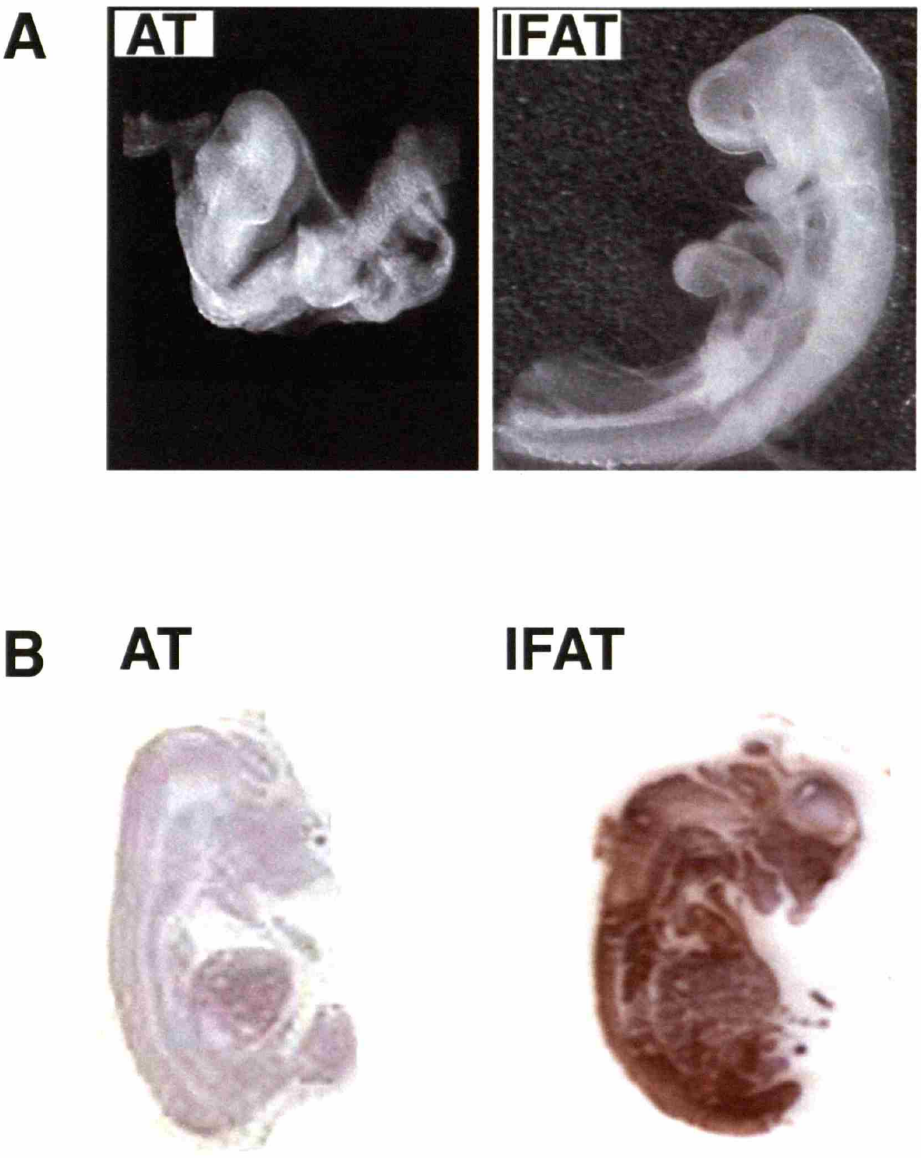

Figure 3.6
(A) Development of embryos derived from both AT and IFAT ES cells using tetraploid complementation. AT= day E8.5 and IFAT= day E9.5-10.5. (B) Restricted contribution of AT ES cells to chimeric day E13.5 embryos and unbiased contribution of IFAT ES cells to day E13.5 embryos.

It has previously been demonstrated that AT cells display a biased tissue contribution in chimeric embryos (Thomson and Solter 1988). We therefore examined whether IFAT-ES cells displayed a similar developmental bias. IFAT-ES cells, carrying the GFP reporter, were injected into wild-type blastocysts and tissue contribution was examined at E13.5 by immunohistochemical staining for GFP protein. Unlike AT-ES cells, which showed a bias towards striated muscle, skeletal elements, fetal liver and hypothalamus, IFAT cells contributed equally to all tissues in the E13.5 embryo (**Fig 3.6B; Table 3.1**). Thus, LOI in AT-embryos was sufficient to rescue AT-ES cell pluripotency, consistent with the imbalanced methylation state of AT cells being responsible for their developmental bias.

3.5 Discussion

In this study we have addressed whether the DNA methyl marks associated with maintaining imprinting are required for normal embryonic development. As comparisons, we also examined the developmental potential of three other altered methylation states represented by parthenogenotes, androgenotes and *Dnmt1* null embryos. Our analysis showed that global LOI resulted in a failure of the embryo to develop beyond E11.5 and induced an Igf2-independent overgrowth phenotype. However, IF-embryos were the most advanced developmentally compared with the uniparentally-derived embryos (AT and PT) and *Dnmt1*-null mutants (DN).

Understanding the functional significance of imprinting in mammals has been a central question to the field. To date, the 'parental conflict' hypothesis provides the best explanation for why imprinting evolved and predicts that paternally expressed genes should promote growth and maternally expressed genes should slow it down. The most striking examples of imprinted genes that support this hypothesis are the paternally expressed growth factor, *Igf2* and its negative regulator *Igf2r*, which is maternally expressed. Mouse embryos deficient in *Igf2* show a 40% reduction in growth, whereas *Igf2r*-null embryos exhibit an overgrowth phenotype and perinatal lethality (DeChiara, Efstratiadis et al. 1990; Lau, Stewart et al. 1994; Wang, Fung et al. 1994). Remarkably, the double mutant is normal-sized and viable (Ludwig, Eggenschwiler et al. 1996). Such results have led to the prediction that imprinting will be dispensable under conditions where the imprints on both parental genomes are erased (Moore and Haig 1991) (Jaenisch 1997). The results of this study show that IF-embryos are not viable.

However, caution must be exercised when interpreting these results, as a number of caveats exist. Firstly, at least one imprinted locus, *Igf2*, is known to undergo 'methylation drift' and can become re-activated following loss of the methylation that maintains imprinting (Tucker, Beard et al. 1996; Biniszkiewicz, Gribnau et al. 2002; Holm et al 2005). As mention above, an imbalance in the expression of *Igf2* and *Igf2r* leads to embryonic overgrowth and perinatal lethality. To examine the contribution that reactivated *Igf2* expression was playing in the LOI phenotype, we reactivated *Igf2r* expression in IF-embryos and demonstrated a slight increase in developmental potential from E11.5 to E12.5. However, restoring monoallelic expression of *Igf2r* failed to rescue the IF embryonic overgrowth phenotype. We conclude from this result, that the *Igf2* pathway makes only a small contribution to the phenotype of IF-embryos and other imprinted genes must be primarily responsible. Although we cannot completely rule-out that other imprinted loci do not become reactivated in the same way as *Igf2*, our previous microarray analysis of IF murine embryonic fibroblasts suggests that the phenomenon of methylation drift is unique to the *Igf2* locus (Holm et al 2005). Secondly, imprinting is an adaptation that probably arose fairly recently in mammalian evolution, as non-mammalian vertebrates possess most, if not all, of the orthologues found imprinted in mammals but they express them biallelically (Tilghman 1999). Therefore, it could be argued that an imprint-free state should be defined as biallelic expression at all imprinted loci, thus reflecting the 'ancestral' ground state of the mammalian genome. For this study, we have defined an imprint-free state as one in which the DNA methyl marks associated with imprinting are erased. However, due to the complexities of how different DMRs function, several imprinted genes, including the negative growth regulator *Grb10*

and the cell cycle inhibitor $p57^{kip2}$, are silenced in response to LOI (Holm et al 2005). As a result, it is unlikely that the IF-embryos generated in this study exhibit biallelic expression at all imprinted loci and therefore do not represent the 'ancestral' ground state. The silencing of some imprinted genes will undoubtedly contribute to the LOI phenotype. For example, *Grb10* knockout mice display similar characteristics to IF-embryos, including an Igf2-independent overgrowth phenotype that is first detectable at E12.5 (Charalambous, Smith et al. 2003). Thirdly, the role of the placenta must be considered when interpreting the phenotype of IF-embryos. When using tetraploid complementation as a means of generating embryos, the IF-ES cells give rise solely to the embryo proper, whereas the normally imprinted tetraploid cells contribute to the placenta (Eggan, Rode et al. 2002). Because the transfer of nutrients from the placenta to the embryo is critical for normal growth, and given that imprinted genes are known to regulate placental size and function, it is possible that a regulatory imbalance between the normally imprinted placenta and the IF-embryo contributes to the IF phenotype. One strategy to overcome this in future studies would be to perform nuclear transfer experiments with IF-ES nuclei and generate a cloned embryo that would lack imprinting in both the embryo proper and extraembryonic tissues.

One of the most interesting results of this study is the observation that androgenetic embryos deficient in imprinted methyl marks are not developmentally equivalent to biparentally-derived IF-embryos. This finding raises the possibility that other mechanisms in addition to DNA methylation exist to maintain parent-specific expression patterns. The recent observation that Dnmt3L/Dnmt3a complexes can interact

with polycomb- and thrithorax-like proteins (Burgers, Fuks et al. 2002), suggests that one such mechanism may involve stable changes in chromatin organization.

In summary, the results of this study indicate that although removing the DNA methyl marks associated with imprinting confers the greatest developmental potential to the embryo compared with the imbalanced uniparental states of androgenotes and parthenogenotes, it is not sufficient for viability. Whether this is due to a failure to restore biallelic expression at every imprinted locus, or whether it is caused by the existence of other non-DNA methylation mechanisms for maintaining imprinting is not clear at present and requires further study.

3.6 Experimental Procedures

3.6.1 Generation of imprint free and control ES cells.

The 2-Frt Flanked *Dnmt1* Inactive Allele was constructed as described in Holm et al 2005. Imprint free (IF) and Control (CTL) embryonic stem (ES) cells were generated as previously described (Holm et al 2005).

3.6.2 Reactivation of monoalleleic *Igf2r* expression in IF-ES cells.

Monoallelic expression was restored to IF-ES by targeted mutation of the DMR within intron2 of the *Igf2r* gene as described (Wutz, Theussl et al. 2001). Briefly IF-ES cells were cultured as previously described (Li, Bestor et al. 1992) and $10x10^6$ cells were electroporated with 30 mg *Not*I linearized R2DNT targeting vector using a Genepulser (Biorad) set to 960 mF and 200 V, and selected in Hygromycin (Sigma) for 8 days. Targeting of R2DNT was screened by Southern blot analysis. Subsequently, the selection cassette was removed by transient transfection with 30 mg Cre expression plasmid (Gu, Marth et al. 1994) and selection in medium containing 2 mM gancyclovir.

3.6.3 Generation of imprint free androgenetic and androgenetic ES cells.

All experiments on live vertebrates were preformed in accordance with relevant institutional and national guidelines and regulations. Massachusetts Institute of Technology's committee on animal care has approved the experiments and has confirmed that all experiments conform to the relevant regulatory standards. The most recent review and approval received on November 4th 2004.

Male mice that were either heterozygous for the inactive 2Frt *Dnmt1* allele or homozygous for the conditional 2LoxP *Dnmt1* allele (Jackson-Grusby, Beard et al. 2001) were crossed to wild-type C57B6 female mice. Fertilized embryos were dissected at E0.5, and cultured until pronuclear formation. The male pronuclear was removed from embryos derived from the mating of female C57B6 mice to male mice heterozygous for the 2Frt *Dnmt1* allele. The male pronucleus was placed under the zona of embryos generated from crossing female wild-type C57B6 mice to male mice homozygous for the conditional 2LoxP *Dnmt1* allele, in which the female pronucealus had already been removed. Electric current was passed across the embryo and male pronuclus causing membrane fusion. Reconstructed androgenetic embryos were cultured until the blastocyst stage, and explanted for ES cell line derivation as described (Hochedlinger and Jaenisch 2002). ES cells were cultured as previously described (Li, Bestor et al. 1992). The androgenetic ES cell line tested positive for both the 2Frt and the 2LoxP *Dnmt1* alleles. These cells were exposed to Cre-recombinase and Flpe-recombinase through lipofectamine mediated, transient transfection as previously described.

3.6.4 Derivation of parthenogenetic ES cells.

Metaphase II oocytes were isolated as previously described (Hochedlinger and Jaenisch 2002). Briefly, oocytes were collected from the oviducts of superovulated B6D2F1 female mice (Taconic) and stored in KSOM embryo culture media (Specialty media). Embryos were activated for 6 hours in calcium-free MCZB medium in the presence of 10 mM Sr2+ and 5 μg/mL of cytochalasin B before replacement by KSOM. Parthenogenetic embryos were cultured until the blastocyst stage, and explanted for ES

cell line derivation as described (Hochedlinger and Jaenisch 2002). ES cells were cultured as previously described (Li, Bestor et al. 1992).

3.6.5 Derivation of primary embryonic fibroblast cells from ES cells.

MEFS were derived from day 13.5 embryos obtained by injecting ES cells into wild-type BDF1 blastocysts and implanted into foster mothers. MEFs derived from chimeric embryos were selected for two passages with the appropriate antibiotics. MEFs were frozen at passage 2 or 3 and with the exception of long-term culture were used for all experiments before passage 6. Only pure populations were used as assayed by genomic southern or PCR. MEFs and 293T ecotrophic packaging cell line were grown in DMEM supplemented with 10% FBC, 5mM Glutamine and penicillin/streptomycin. All the cells used tested negative for mycoplasma.

3.6.6 Growth curves.

All experiments were repeated at least 3 times using cells from a minimum of 5 different fetuses for each genotype. For growth curves 3 x 10^4 cells were plated into 12 well dishes and fed every 2ndday (Sage, Mulligan et al. 2000).

3.6.7 Production of mice.

For tetraploid blastocyst injections, two-cell embryos derived from B6D2F1 females were first electro-fused in 0.3 M mannitol/0.3% BSA using the LF-101 cell fusion instrument (Protech International). For diploid blastocyst injections, fertilized zygotes were isolated from the oviducts of day 0.5 pregnant B6D2F1 allowed to develop

to the blastocyst stage in culture. Between five and 15 ES cells were injected per blastocyst. These were transferred into day 2.5 pseudo-pregnant B6D2F1 recipient females. C-sections were performed at timed intervals for embryos derived from tetraploid blastocyst. Chimeric animals were sacrificed at day E13.5 and embryos were removed at necropsy and either fixed in formalin for sectioning and GFP immunihistochemistry or stained for the presence of the β-galactosidase gene.

3.6.8 Teratoma tumor formation.

To test pluripotency, exponentially growing ES cells were resuspended in PBS and 1 x 10^6 cells were injected subcutaneously into SCID mice. Tumor development was monitored for 14 days and the tumor removed at necropsy and fixed in formalin.

3.6.9 Histology.

Normal and tumor tissue samples were fixed in 10% buffered formalin for 24 h and embedded in paraffin. Sections (5 μm) were used for immunohistochemical analysis. Immunostaining was performed using an avidin-biotin immunoperoxidase assay. Primary antibody anti-GFAP (1:500; Dako). Sections were incubated with primary antibodies for 24 h and subsequently with biotinylated secondary antibodies (Vector Laboratories) for 30 min, followed by incubation with avidin-coupled peroxidase (Vector Laboratories) for 30 min.

3.6.10 β-Galactosidase detection.

Dissected whole embryos were fixed in 0.5% glutaraldehyde and 4% paraformaldehyde in PBS for 12 hours and incubated with the substrate 5-bromo-4-

chloro-3-indolyl-ß-D-galactoside (Xgal) for 4 hours. The presense of the β-galactosidase is denoted by a blue ferrocyanide precipitation (Holt and O'Sullivan 1958).

Chapter 4:

Perspectives and Conclusions

The goal of this study was to generate imprint-free cells by sequentially inactivating and then reactivating *Dnmt1*. While our data to date are consistent with the generation of an imprint-free state, there are a number of important caveats to consider. Firstly, the ES cells carrying the conditional alleles of *Dnmt1* were heterozygous for the expression of *Dnmt1*. Thus, we cannot rule-out that some of the effects observed with IF cells were not the result of haploinsufficiency for *Dnmt1*. However arguing against this, numerous studies both published and unpublished have shown that *Dnmt1* heterozygotes are normal, healthy animals that are equivalent to wild-types (Li et al 1992; 1993). Secondly, we cannot be completely sure that the restoration of global DNA methylation following reactivation of *Dnmt1* did not inadvertently affect the expression status of non-imprinted genes. Such perturbations may have contributed to the embryonic lethality and cancer phenotype observed in the IF mice and may have also been 're-set' following passage through the germline. While our microarray analysis of IF-MEFs did not identify any abnormalities in non-imprinted gene expression, we cannot rule-out that altered gene expression might have been present in other cell types or embryonic stages. Thirdly, we detected abnormal expression at the *Igf2/H19* locus due to *de novo* remethylation. At present, we do not know whether there are other imprinted genes that undergo a similar 'methylation drift'. Therefore, it is possible that the IF phenotype may be influenced by imbalanced imprinting at critical imprinted loci that control the cell cycle or embryonic growth.

Despite these caveats, if we assume that the phenotypes observed, e.g. embryonic lethality, altered growth characteristics, resistance to TGF-β, immortality, and a propensity to form tumors in the adult are caused by a *bona fide* LOI, then the key question that remains is: What are the imprinted genes responsible for these effects? While the answer to this question can only be obtained from further research, it is possible to speculate about potential candidate genes.

4.1 LOI and growth inhibition by TGFβ

LOI induced MEFs to become refractory to the cytostatic effects of TGFβ. A failure to undergo cell cycle arrest in response to TGFβ is a hallmark of cancer cells, however, the exact mechanism by which this is achieved is still not fully understood. In primary epithelial cells, TGFβ triggers cell cycle arrest by up-regulating cytostatic proteins such as the cyclin dependent kinase (CDK) inhibitors, $p15^{Ink4b}$ and $p21^{Cip1}$, while down-regulating growth promoting transcription factors such as c-Myc and Id family members (Chen, Kang et al. 2002; Kang, Chen et al. 2003; Kowanetz, Valcourt et al. 2004). More recently, embryonic studies have suggested that p53 may participate in the TGFβpathway and a direct interaction between p53 and Smad2 and Smad3, two downstream effectors of TGFβ signaling, has been demonstrated (Cordenonsi, Dupont et al. 2003). Furthermore, p53-null MEFs display a defective cytostatic response to TGFβ and a concomitant inability to induce $p21^{Cip1}$ expression, a known p53 target (Cordenonsi, Dupont et al. 2003). Theses observations provide an attractive explanation for the loss of TGFβ-induced growth inhibition seen with IF-MEFs, given that these cells have abnormally low levels of p53 protein. Wild-type levels of $p21^{Cip1}$ protein were detected

in the IF-MEFs by Western blot analysis. However, these cells were not treated with TGFβ, therefore it is not known whether they show an attenuated ability to induce $p21^{Cip1}$ in response to TGFβ exposure. Further studies of IF-MEFs and their cellular response to TGFβ are needed to investigate this issue. For example, it should be possible to restore *p53* expression to IF-MEFs by viral transfection and then examine their response to TGFβ treatment.

An alternative possibility is that the growth-inhibiting effects of TGFβ are defective in IF-MEFs because of an imprinted gene that is no longer expressed in these cells. One such candidate is $p57^{Kip2}$, which like $p21^{Cip1}$, belongs to the Cip/Kip family of cyclin-dependent kinase inhibitors (CDKIs) (Hatada and Mukai 1995). $p57^{Kip2}$ is a maternally transcribed gene that is regulated by an imprint control region that needs to be methylated in order for the locus to be expressed (Diaz-Meyer, Day et al. 2003). Thus, in cells deficient in imprinting, it is predicted that $p57^{Kip2}$ transcription would be silenced. Consistent with this, the microarray data of IF-MEFs showed an absence of $p57^{Kip2}$ expression. In the hematopoietic system, the cytostatic effects of TGFβ are mediated by $p57^{Kip2}$ (Scandura, Boccuni et al. 2004) and in humans, mutations in $p57^{Kip2}$ have been found in patients with Wiedemann-Beckwith syndrome, a human overgrowth disorder that is accompanied by an increased risk of embryonal tumors (Hatada, Inazawa et al. 1996) (Thompson, Reese et al. 1996). Given that $p57^{Kip2}$ plays a role in both the growth-suppressive pathway downstream of TGFβ and causes an embryonic overgrowth phenotype, two features also associated with LOI, it is tempting to speculate that loss of $p57^{Kip2}$ expression may be major contributor to the LOI phenotype.

4.2 The role of *Grb10* in regulating mitogenic growth.

The *Grb10* gene, which is maternally expressed in mice, is another likely candidate for contributing to the LOI phenotype. *Grb10* encodes a cytoplasmic adapter protein that modulates coupling to a number of receptor tyrosine kinases (Riedel 2004). In addition, Grb10 has also been found to interact with non-receptor tyrosine kinases such as Tec and Bcr-Abl, and other cellular signaling molecules such as Raf-1 and the mitogen activated protein (MAP) kinase kinase, MEK (Lim, Riedel et al. 2004). Disruption of the active maternal allele results in an embryonic overgrowth phenotype in which the mutant mice are 30% larger than normal (Charalambous, Smith et al. 2003). These results established Grb10 as a potent growth inhibitor. Although Grb10 is capable of interacting with, and suppressing, Igf-stimulated cell signaling and DNA synthesis, genetic experiments *in vivo* demonstrated that the overgrowth phenotype of Grb10 null mice was independent of Igf2 (Dufresne and Smith 2005) (Charalambous, Smith et al. 2003). These findings suggest that loss of Grb10 function leads to an activation of fetal growth pathways. The microarray analysis of IF-MEFs revealed a marked downregulation of *Grb10* expression. As found for $p57^{Kip2}$ above, the DMR controlling *Grb10* expression shows a maternal methylation mark that is likely needed for transcription. Thus, in an IF state, neither maternal nor paternal alleles of *Grb10* are expressed, and IF cells would be expected to behave like *Grb10* null cells. The finding that IF-chimeric embryos were 40-50% larger than control chimeras is consistent with loss of Grb10 contributing to the LOI overgrowth phenotype. The importance of Grb10 in the LOI phenotype could be directly tested in future studies by restoring *Grb10* expression either *in vitro* (eg MEFs) or *in vivo*.

4.3 Role of Igf signaling and mitogenic growth

Signaling of Igf1 and Igf2 through the Igf1r is an important pathway for fetal growth, as highlighted by mouse knockout studies that have shown dwarfing phenotypes following loss of each locus (DeChiara, Efstratiadis et al. 1990), (Liu, Baker et al. 1993), (Baker, Liu et al. 1993)). In addition, stimulation of the Igf pathway has been implicated in the formation of numerous cancers (Feinberg and Tycko 2004). Thus, increased Igf signaling in response to LOI is an obvious candidate for contributing to the abnormal growth characteristics of IF cells. In IF-embryos however, reactivation of monoallelic *Igf2r* expression had only a subtle effect on developmental potential and failed to rescue the overgrowth phenotype. In addition, the re-activation of *Igf2r* in IF-MEFs had no effect on growth rate. It is possible that the level of restored *Igf2r* expression in the IF cells is just not sufficient to balance out the amount of *Igf2* being generated. In which case, a better test of the involvement of the Igf pathway in the IF phenotype would be to target the *Igf2* locus directly. Alternatively, the Igf pathway may not be a major player in the IF phenotype at the developmental time points examined. Evidence in support of this comes from the finding that the overgrowth phenotype of *Igf2r* mutant embryos does not become manifest until after E13 (Ludwig, Eggenschwiler et al. 1996), whereas in this study IF-embryos were qualitatively larger at E11.5. However, we cannot rule out that an imbalance between *Igf2* and *Igf2r* expression in IF cells is a significant contributor to the formation of tumors in adult chimeras. In support of this, IF-MEFs in which *Igf2r* expression is restored show a slight, but statistically significant, decrease in the number of colonies that form in the foci formation assay (data not shown). To explore this

further, it will be interesting to examine the tumorigenic potential of IF+Igf2r-ES cells in adult chimeras.

4.4 The role of imprinting in the regulation of the tumor suppressor genes *p19^{Arf}* and *p53*

When normal primary cells are continuously passaged, they exhibit progressive increases in the protein level of p16^{Ink4a} and undergo replicative senescence (Sherr 2001). p16^{Ink4a} is a cell cycle kinase inhibitor that targets the CyclinD-cdk4/6 complex, thereby preventing phosphorylation of Rb-E2F and inhibiting progression into S phase. In IF-MEFs, p16^{Ink4a} levels showed the expected increase in protein level as passage number increased. However, despite the presence of high p16^{Ink4a} levels, IF-MEFs failed to senesce and instead were immortal. Previous studies have shown that p16^{Ink4a} is unable to prevent Rb phosphorylation in the absence of p53 (Sherr 2001). Consistent with this, IF-MEFs were found to have decreased levels of p53 at early passages and an absence of protein by mid-passage. The altered protein profile of p53 in IF-MEFs correlated with that of p21^{Cip1}, a known downstream target of p53 and a cell cycle inhibitor of cyclinE-cdk2 complexes (Waldman, Kinzler et al. 1995; Gartel and Tyner 1999). While low levels of p21^{Cip1} persist in IF-MEFs after continuous passage, they must not be sufficient to induce cell cycle arrest. These data suggest that abnormal levels of p53 are responsible for the failure of IF-MEFs to undergo replicative senescence.

p19^{Arf} is a key player in the stabilization of p53 protein that acts by binding the p53 ubiquitin ligase, Mdm2, and relocalizing it to the nucleolus (Sherr and McCormick 2002; Vousden 2002). In the absence of p19^{Arf}, p53 is rapidly degraded and has a protein

half-life of only 9-20 minutes (Harris and Levine 2005). In normal cells approaching replicative senescence, $p19^{Arf}$ levels are found to increase, thus resulting in p53 stabilization and activation of downstream targets. In contrast, IF-MEFs display a failure to upregulate $p19^{Arf}$ protein, thus raising the possibility that the abnormal levels of p53 in IF-MEFs are the result of defects in $p19^{Arf}$ regulation.

Regulation of $p19^{Arf}$ locus is not fully understood. It is known that $p19^{Arf}$ responds to stress signals but the mechanisms regulating transcription as well as post-translational control of $p19^{Arf}$ protein are currently the subject of much debate. At the transcriptional level, there is evidence to suggest that the $p19^{Arf}$ promoter is regulated by the transcription factors DMPF1, E2F, and the AP-1 members, c-Jun and Fra-1 (Inoue, Roussel et al. 1999; Inoue, Wen et al. 2000; Rowland, Denissov et al. 2002; Ameyar-Zazoua, Wisniewska et al. 2005). At the post-translational level, there is the possibility that $p19^{Arf}$ may be regulated by protein degradation as both the mouse and human proteins undergo N-terminal polyubiquitination (Kuo, den Besten et al. 2004). Stability of $p19^{Arf}$ depends on the nucleolar protein nucleophosmin/B23, which binds to $p19^{Arf}$ with high stoichiometry and retards its turnover. $p19^{Arf}$ mutants that do not efficiently associate with nucleophosmin/B23 are unstable and functionally impaired (Kuo, den Besten et al. 2004). The absence of $p19^{Arf}$ induction in IF-MEFs may reflect a defect in either $p19^{Arf}$ transcriptional upregulation or enhanced protein turn-over. At present, the identity of an imprinted gene that could be responsible for this defect is not known.

In addition to an effect on $p19^{Arf}$, LOI may also impact on the activity of Mdm2 and thereby affect the level of p53. Stabilization of Mdm2 can occur via phosphorylation mediated by the mitogen-induced kinase, Akt. Phosphorylation of Mdm2 inhibits its auto-ubiquitination and subsequent degradation (Mayo and Donner 2001; Zhou, Liao et

al. 2001). A large number of mitogenic pathways are transduced through Akt (Song, Ouyang et al. 2005) and imprinted genes, many of which are involved in cell growth, are likely to influence Akt activation. The best example is Grb10, which acts as a negative regulator of the Akt pathway by preventing the interaction of insulin receptor substrate proteins with tyrosine kinase receptors (including Igf1r; (Lim, Riedel et al. 2004)). Given that *Grb10* is most likely silenced in IF-MEFs, it is not unreasonable to expect Akt activation to be enhanced resulting in greater stabilization of Mdm2 protein. Akt activation is also likely to be upregulated by the imbalance in *Igf2* and *Igf2r* expression in IF-MEFs, which lead to increased signaling through the Igf1r. Increased Mdm2 stabilization resulting from upregulated mitogenic signaling may be sufficient to alter the rate of p53 degradation, and thereby contribute to the decreased levels of p53 in IF-MEFs.

Taking all of these data in consideration, a model can be proposed to explain the effects of LOI on cell growth characteristics (**Fig 4.1**). Based on this model, imprinted gene products are proposed to interfere with the p53-p19^{Arf} pathway in three major ways. Firstly, an as-of-yet unidentified imprinted gene is required for inducing $p19^{Arf}$ expression or stabilizing its protein levels. In the absence of high levels of p19^{Arf}, Mdm2 is not inhibited, thus resulting in increased p53 ubiquitination and degradation. Secondly, Mdm2 activity is further enhanced by increased stimulation of mitogenic pathways resulting from an imbalance in *Igf2* and *Igfr2,* and a loss of *Grb10* expression. The loss of p53 in IF-MEFs is then expected to make these cells refractory to the growth-suppressive effects of TGFβ, as induction of $p21^{Cip1}$ is dependent on p53.

Figure 4.1 Model of imprinted gene regulation in the cell cycle

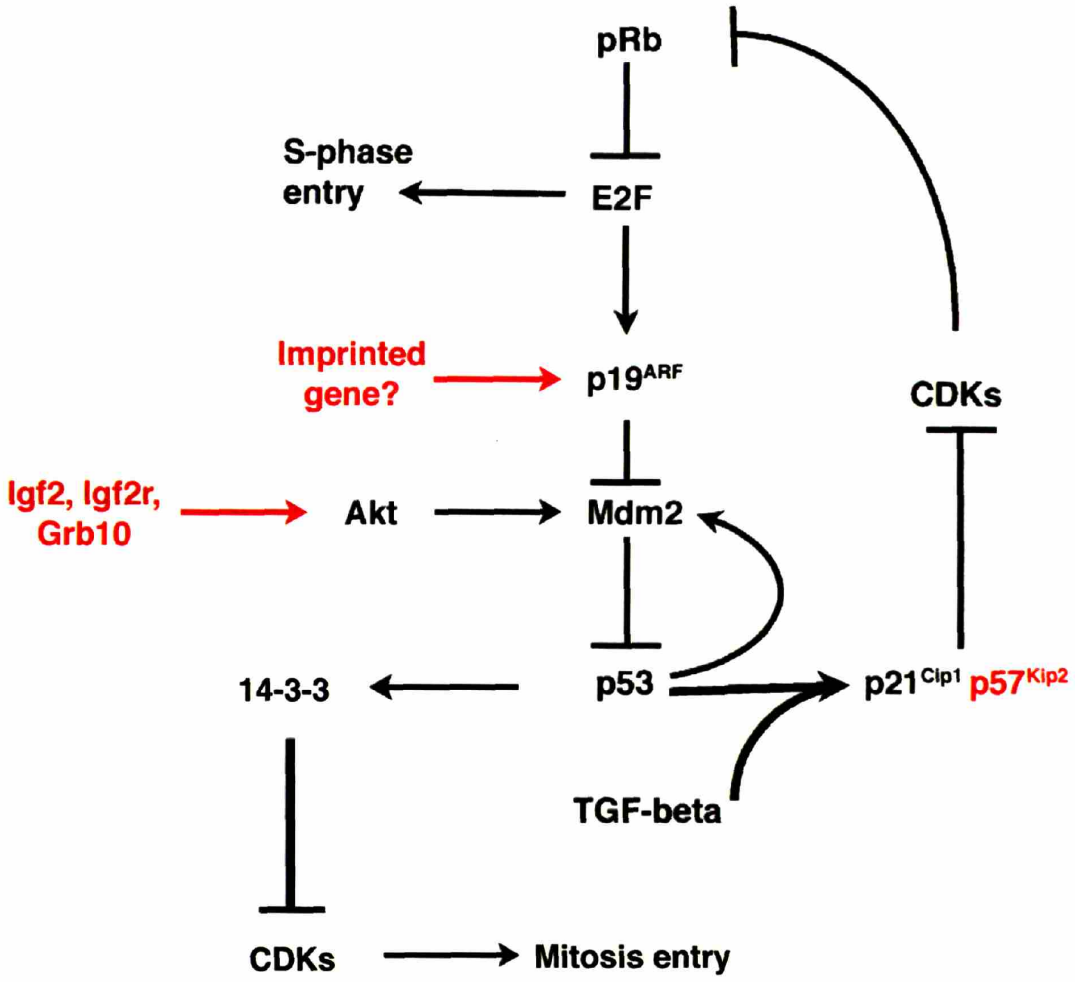

Figure 4.1 Model for imprinted gene regulation in the cell cycle.
Simiplified model illustrating the regulatory interactions between proteins involved in the G1/S and G2/M phase transition. The proposed involvement of imprinted gene products are shown in red and are postulated to influence the control of the cell cycle in the following way: (1) an as of yet unidentified imprinted gene product is involved in the upregulation of $p19^{Arf}$ expression or protein stability, (2) Igf2, Igf2r and Grb10 regulate mitogenic signaling through Akt, which is involved in stabilizing Mdm2, (3) $p57^{Kip2}$ is a member of the Cip/Kip family of cyclin-dependent kinase inhibitors and is proposed to play a role in TGFβ induced senescence. Adapted from Dupont et al 2004.

The loss of p53 in IF-MEFs is then expected to make these cells refractory to the growth-suppressive effects of TGFβ, as induction of $p21^{Cip1}$ is dependent on p53. Similarly, loss of expression of the related cell cycle inhibitor, $p57^{Kip2}$, may also contribute to the failure of IF-MEFs to undergo cell cycle arrest and provides the third involvement of an imprinted gene in the IF phenotype.

4.5 Imprinting and embryonic development.

In this study we have shown that removal of the methylation that maintains imprinting in ES cells leads to an inability to support embryonic development in tetraploid complementation experiments. One predication from the Haig theory is that the elimination of imprinting from both the paternal and the maternal genomes would not be deleterious to the developing embryo, as the resulting effects would cancel each other out (Moore and Haig 1991; Jaenisch 1997). We have assumed that because differential methylation maintains imprinted gene expression, selective removal of this methylation would generate an imprint-free state, thus permitting a direct test of the Haig hypothesis. So, does the failure to generate live IF mice argue against the Haig hypothesis? Likely not because a number of caveats need to be considered when interpreting our results. First, we did not measure the methylation status of all imprinted genes, therefore we cannot rule-out that some loci escaped the erasure of DNA methyl marks by our conditional *Dnmt1* strategy. Second, it is possible that the embryonic lethality of IF-embryos is the result of a 'bystander effect'— this refers to the notion that some imprinted genes may have evolved an additional function, downstream target, or protein

interaction that plays no part in the parent-offspring conflict but results in embryonic lethality when disrupted following LOI. Third, DNA methylation may not be the only mechanism that maintains imprinting. Our observation that androgenetic embryos stripped of their imprinted methyl marks were not developmentally equivalent to biparental IF-embryos, suggests that other mechanisms exist to control imprinted gene expression. One speculative possibility is that imprinting is also regulated by changes of chromatin structure. In support of this, Dnmt3L/Dnmt3a complexes are known to interact with proteins such as polycomb- and thrithorax-like factors, which are involved in heritable changes in chromatin structure (Burgers, Fuks et al. 2002). Fourth, the methylation mechanisms controlling expression at imprinted loci are often complex and in some cases DNA methylation is actually a necessary prerequisite for expression. In the cases of *Grb10* and $p57^{Kip2}$, expression of these genes is silenced from both parental alleles in the absence of imprinting, rather than reverting to biallelic expression. Thus, what we have designated as an IF state is likely a combination of biallelic expression at some imprinted loci and no expression at others. Fifth, the IF-embryos we generated were produced by tetraploid complementation. Therefore, the placentas of these embryos were derived from the tetraploid cells that were correctly imprinted. The embryonic lethality seen following LOI may be related to the fact that an imprinted placenta was nourishing an imprint free embryo. A future experiment that could be performed to address the importance of the placenta in the survival of IF-embryos would be to use nuclear transfer of an IF-ES cell nuclei to generate a cloned IF-embryo. Cells from this embryo would give rise to the embryo proper as well as contribute to the placenta, and therefore address the effect of LOI in the placenta on IF-embryo viability.

4.6 Future directions and perspectives on imprinting

As a result of the caveats mentioned above, our experiments cannot definitively prove the prediction that removing imprinting will have no deleterious effects to the embryo. It seems likely that we are a long way from achieving this goal, given that all the caveats will need to be adequately addressed. However, the next step should be to use nuclear transfer techniques to examine the role of the placenta in the IF phenotype. Furthermore, the phenotype of IF androgenetic embryos needs to be more closely examined. For instance, are they same size as androgenotes? How does their expression profile compare with that of androgenotes and biparental IF-embryos? Can the IF androgenetic phenotype be rescued by passaging through the germ line?

Does the definition of imprinting need to be revised? Would it be more accurate to define an imprint-free state based solely on expression level and not on the mechanism? For instance, should imprint-free mean biallellic expression at all imprinted loci? With regard to the *Igf2* and *Igf2r* genes, one could easily envisage that biallelic expression from both these loci would have little effect on development, as the increase in Igf2 levels would be balanced by increased clearance by Igf2r. Orthologues of genes imprinted in mammals are found in other non-eutherian vertebrates, including birds, fish, reptiles and amphibians, where they are biallelically expressed. Thus, it appears likely that the ancestor of mammals displayed biallelic expression of the genes that would later become imprinted. How imprinting evolved is not known, but it is probable that it was co-opted from a more ancient mechanism of DNA methylation (Jaenisch 1997). Indeed, even the most primitive vertebrates, the Hagfish and lamprey, have methylated genomes

but do not employ imprinting (Tweedie, Charlton et al. 1997). Given these observations, perhaps an imprint-free state should be defined as a return to a more 'ancestral state' in which the orthologues of all imprinted genes are expressed biallelically.

4.7 General Conclusions

In summary, it is clear that the regulation of imprinting in mammals is a complex process that is still not fully understood. This study has attempted to address the role that imprinting plays in development and cancer formation by specifically removing the methylation marks that maintain imprinted gene expression in ES cells. The analysis of embryos and adult chimeras derived from these cells demonstrated that loss of imprinting, as defined by a loss of the methyl marks that regulate imprinted gene expression, results in altered cell growth characteristics and a predisposition to develop cancer. A number of imprinted genes may be responsible for this phenotype, including $p57^{Kip2}$, *Grb10*, *Igf2* and *Igf2r*. However, the most significant proteins affected by LOI were p53 and $p19^{Arf}$, two critical mediators of cell cycle arrest, senescence, and of tumor prevention. Interestingly, neither protein is encoded by an imprinted gene. This raises the exciting possibility that an unknown imprinted gene acts as a critical regulator of the p53- $p19^{Arf}$ pathway. Elucidating the identity of this putative gene will be the challenge for future studies and will undoubtedly provide further insights into the regulation of cell growth and tumorigenesis.

Chapter 5:

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5.0 Appendix I:

Imprinted genes in the mammalian genome.

Table 5.1

Imprinted Loci	Chromosome	Expressed parental allele; Maternal/Paternal	Name
Gatm	*2*	M	L-arginine: Glucine amidino-transferase
Nnat	*2*	P	Neuronatin
Gnas	*2*	M	Guanine nucleotide binding protein, alpha stimulating
Gnasxl	*2*	P	Guanine nucleotide binding protein, alpha stimulating, extra large
Nesp	*2*	M	Neuroendocrine secretory protein
Nespas	*2*	P	Neuroendocrine secretory protein antisense
Dlx5	*6*	M	Distal-less homeobox 5
Calcr	*6*	M	Calcitorin receptor
Sgce	*6*	P	Sarcoglycan, epsilon
Peg 10	*6*	P	Paternally expressed gene 10
Neurabin	*6*	M	Neurabin
Pon 3	*6*	M	Paroxonase 3
Pon 2	*6*	M	Paroxonase 2
Asb4	*6*	M	Ankyrin repeat and suppressor of cytokine signaling
Peg1/Mest	*6*	P	Mesoderm specific transcript
Copg2	*6*	M	Coatomer protein complex subunit gamma 2
Copg2as	*6*	P	Antisense to Copg2
Mit1/lb9	*6*	P	Mest linked imprinted transcript 1
Nap1l5	*6*	P	Nucleosome assemble protein 1, like 5.
Zim2	*7*	M	Imprinted zinc-finger gene 2
Zim1	*7*	M	Imprinted zinc-finger gene 1
Peg3/Pw1	*7*	P	Paternally expressed gene 3
Usp29	*7*	P	Ubiquitin specific processing protease 29
Zim3	*7*	M	Zinc Finger Gene 3 from Imprinted domain
Zfp264	*7*	P	Zinc Finger gene 264
Snrpn	*7*	P	Small nuclear ribonucleoprotein polypeptide N
Snurf	*7*	P	Snrpn upstream reading frame

Pwcr1	*7*	P	Prader-Willi chromosome region 1
Magel2	*7*	P	Magel2
Ndn	*7*	P	Necdin
Zfp127/Mkrn3	*7*	P	Ring zinc-finger encoding gene
Zfp127as/Mkrn3as	*7*	P	Ring zinc-finger encoding gene antisense
Frat3	*7*	P	Frequently rearranged in advanced T-cell lymphomas.
Ipw	*7*	P	Imprinted in Prader-Willi Syndrome
Atp10c/Atp10a	*7*	M	Aminophospholipid translocasi
Ube3a	*7*	M	E6-AP ubiquitin protein ligase 3A
Ube3aas	*7*	P	Ube3a antisense
Inpp5f_v2	*7*	P	Inositol polyphosphate-5-phosphatase, varient 2
Napl14/Nap2	*7*	M	Nucleosome assembly protein 1 - like 4
H19	*7*	M	A cDNA clone isolated from a fetal hepatic library
Igf2	*7*	P	Insulin-like growth factor type 2
Igf2as	*7*	P	Insulin-like growth factor type 2, antisense
Ins2	*7*	P	Insulin 2
Mash2	*7*	M	Mus musculus achaete-scute homologue 2
Kvlqt1	*7*	M	Potassium voltage - gated channel subfamily KQT member 1
Kvlqt1as/Lit1/Kcnqlot1	*7*	P	Kvlqt1 antisense
Tapa1/Cd81	*7*	M	Cd 81 antigen
p57KIP2 / Cdkn1c	*7*	M	Cyclin-dependent kinase inhibitor 1C
Msuit	*7*	M	Mouse specific ubiquitously expressed imprinted transcript 1
Slc22l1	*7*	M	Solute carrier family 22 (organic cation transporter memter-1 like).
Ipl/Tssc3/Phlda2	*7*	M	Pleckstrin homology-like domain, Family A, member 2
Tssc4	*7*	M	Tumour suppressing subchromosomal transferable fragment 4
Obph1	*7*	M	Oxysterol-binding protein 1
A19	*9*	P	
Rasgrf1	*9*	P	Ras protein specific guanine nucleotide-releasing factor 1
Zac1	*10*	P	Zinc finger DNA binding protein
Dcn	*10*	M	Decorin
Meg1/Grb10	*11*	M	Growth factor receptor bound protein 10
U2af1- rs1	*11*	P	U2 small nuclear ribonucleoprotein auxiliary

			factor (U2AF), 35kDa, related sequence 1
Murr1	***11***	**M**	**U2af1-rs1 region 1**
Dlk/Pref1	***12***	**P**	**Delta like 1**
Meg3/Gtl2	***12***	**M**	**Gene trap locus 2**
Dio3	***12***	**P**	**Deiodinase Iodothyronine Type 3**
Rian	**12**	**M**	**RNA imprinted and accumulated in the nucleus.**
Rtl1/Peg11	**12**	**P**	**Retrotransposone-like gene 1**
Rtl1as/antiPeg11	**12**	**M**	**Antisense to Rtl1/Peg11**
Mirg	**12**	**M**	**MicroRNA containing gene**
Htr2a	**14**	**M**	**5-hydroxytryptamine (serotonin) receptor 2 A**
Slc38a4/Ata3	**15**	**P**	**Solute carrier family 38, member 4/Amino acid transport system A3**
Peg13	**15**	**P**	**Paternally expressed gene 13**
Slc22a2	**17**	**M**	**Membrane spanning transporter protein**
Slc22a3	**17**	**M**	**Membrane spanning transporter protein**
Igf2r	**17**	**M**	**Insulin-like growth factor type 2 receptor**
Igf2ras/Air	**17**	**P**	**Insulin-like growth factor type 2 receptor antisense RNA**
Impact	**18**	**P**	**Homology with yeast & bacterial protein family YCR59c/yigZ**