Chapter 6
Epigenetic Control of Genome Expression

6.1 Introduction

From the standpoint of evolution, diploidy is generally considered advantageous for two reasons. First, because diploid organisms possess twice as many genes as haploids and in these conditions twice as many favorable mutations arise per generation. This of course increases the genetic diversity in the population and, finally, contributes to the progress of adaptive evolution. Diploidy is also considered advantageous because, when a recessive mutation occurs in a given gene, there is always a backup copy of the original allele on the other chromosome, offering a chance for the population to assess, with no risk, which one of the two alleles is most advantageous for the future of the species in a given environmental context. In most cases, the new mutant allele is neutral and has no selective advantage; sometimes it is harmful and is more or less rapidly eliminated. On rare occasions, it is beneficial and can then gradually replace the original allele.

However, mammals are not perfectly diploid since the males have an X and a Y chromosome while females have two X chromosomes. This difference, which is associated with sex determination, requires that a mechanism of gene dosage compensation be developed to equilibrate the transcriptional activity of the X-linked genes between the two sexes. Understanding this mechanism and its determinism has elicited a great number of investigations over the last fifty years, and scientists have discovered that, in eutherian mammals, the females are functionally haploid for the major part of their X chromosome. As we will explain in this chapter, this functional haploidy is controlled by an epigenetic mechanism leading to the inactivation of one of the two X chromosomes.

In the same mammals, scientists have also discovered that some autosomal regions, sometimes reduced to one or a few genes, are also functionally haploid, exclusively expressing the alleles inherited from one of the two parents and not those inherited from the other, due to the intervention of similar epigenetic mechanisms. These discoveries have broken the dogma of the superiority of diploidy over haploidy and have revealed the existence of a new kind of control of the transcriptional activity of mammalian genes. Although the epigenetic mechanisms

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controlling the transcriptional activity of the X chromosome are not exactly the same as those at work for the autosomal regions, they nonetheless have so many similarities that we will describe them here, in the same chapter.

6.2 X-Chromosome Inactivation in Mammals

In mammals, the XX/XY sex-determination system is common, and only rare exceptions have been reported.[1] In the mouse, females have two large X chromosomes while males have an X and a Y on which the sex-determining region (*Sry*) is the master regulator of sex determination. In its absence, for example in mice with an XX or XO karyotype, the embryo develops as a normal, healthy and fertile female.[2]

The XX/XY system is both simple and robust, since relatively few anomalies in sex determination (intersexuality or sex ambiguities) have been reported, but the presence of two X chromosomes in the female versus a single one in males clearly raises a problem associated with gene dosage imbalance. For this reason, during their evolution mammals have found an efficient way to compensate (or more precisely to equilibrate) the transcription of X-linked genes between the two sexes.

6.2.1 In Female Mammals Only One X is Transcriptionally Active

The XX/XY sex-determination system exists in many diploid organisms, and different ways of solving the question of XX/XY dosage compensation have been retained. In the fruit fly *Drosophila melanogaster*, for example, the male-specific lethal (MSL) complex increases transcription of the single X chromosome to equalize expression of X-linked genes between the two sexes (Larschan et al. 2011). In *Cænorhabditis*

[1] At least two exceptions to the classical XX/XY mechanism of sex determination have been reported. The first one is found in wood lemmings (*Myopus schisticolor*), a species of Cricetidae rodent in which there are two types of X chromosomes (X and X*) and a Y chromosome. XX genotypes develop as females and XY develop as males, as in other mammals. However, both X*X and X*Y develop as females because the X* chromosome carries a mutation that inhibits the male-determining effect of the Y chromosome. The three categories of females (XX, X*X, and X*Y) are fertile, but X*Y females only produce X* ova. This sex determination system induces a strong distortion in the sex ratio (3/1 instead of the normal 1/1) and is considered an adaptation to the extreme seasonal reductions in population size that might otherwise threaten the survival of the species. Another remarkable exception is the mole vole *Ellobius lutescens*, another species of Cricetidae rodent in which both the male and female have the same odd number of chromosomes with a single X and no Y. In this species the sex-determination process is not yet completely understood.

[2] The development of testes as gonads also depends upon some other genes (*Foxl2, NrOb1, Sox9,* etc.).

elegans, dosage compensation is achieved by the female in which transcription from the two X chromosomes is simply halved (Kelly et al. 2002). In mammals, yet another solution has evolved with one of the two X chromosomes being inactivated in the female.

In 1961, the Harwell geneticist Mary F. Lyon suggested that, to ensure correct gene dosage compensation between male and female mammals, one out of the two X chromosomes was randomly and permanently inactivated during embryonic and adult life (Lyon 1961). This hypothesis, as reported by Lyon herself (Lyon 2002), was based on two main observations: (i) one X chromosome is sufficient for normal (female) mouse development; and (ii) mice heterozygous for some alleles at the X-linked coat color loci *mottled* (*Atp7a*^{Mo}) or *dappled* (*Atp7a*$^{Mo\text{-}dp}$) show a variegated effect in heterozygotes, with a pattern of mottling resembling that "seen in somatic mosaics". Lyon's hypothesis has been validated in a great number of mammalian species, and the mechanism of inactivation has been progressively elucidated at the molecular level.[3]

To explain how X-chromosome inactivation works, we have selected two examples: the first one is common and refers to tortoiseshell and calico female cats, while the second is historical and refers to glucose-6-phosphate dehydrogenase deficiency in women. The choice of these two examples may appear paradoxical in a book dedicated to the mouse, but it has the great advantage of being didactic.

6.2.1.1 Calico Cats and G6PD-Deficient Women

In the cat, the *Orange* locus is X-linked, it has two alleles: black O^b and orange O^o (or o), and no homolog on the Y chromosome. In male cats this locus determines two phenotypes: black (O^b/Y) or orange (O^o/Y), depending on the allele carried by the X chromosome. In females there are three genotypes: O^b/O^b, O^o/O^o, and O^b/O^o and also three phenotypes: black (O^b/O^b), orange (O^o/O^o), and a third phenotype called *tortoiseshell*, which is observed in the heterozygous O^b/O^o. This phenotype is clearly different from the uniform phenotype we would expect to get for a cat heterozygous for an autosomal gene involved in the determinism of coat color and exhibiting the classical dominant/recessive or semi-dominant allelic interactions. Here, in contrast, the phenotype suggests that the two alleles, O^o and O^b, are expressed independently and exclusively, rather than simultaneously in the pigment-forming cells (the melanocytes). In other words, the fur of each female cat appears as a mixture of hairs in which the individual melanocytes express either one or the other of two different alleles at the X-linked *Orange* locus. This is a clear-cut and classical example of the functional inactivation of one of the two X chromosomes in female mammals (Fig. 6.1).

[3] On July 22, 2011, at the occasion of the 50 Years of X-Inactivation Conference held in Oxford, the Lyon hypothesis became the Lyon law.

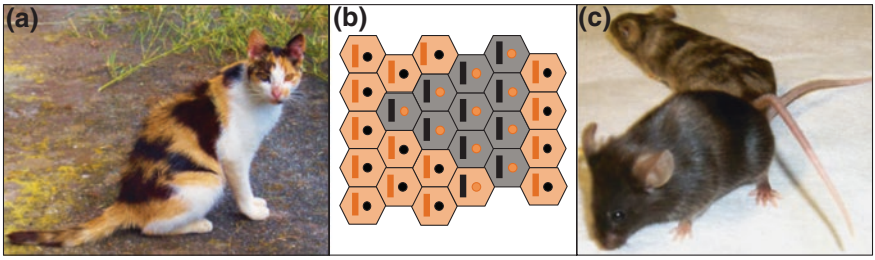


Fig. 6.1 *Calico cats and dappled mice*. **a** The figure represents a female cat with a typical "three-color" coat. Cats with such a coat color are called *calico* and are heterozygous for two different alleles at the X-linked *Orange* (*O*) locus: black O^b and orange O^o. The spots are either *black* or *orange* depending on the active X chromosome in the melanocytes. The *white* areas represent the unpigmented background and are due to a recessive autosomal spotting allele, called *piebald*. This allele, extremely common in the cat, makes the (*orange* or *black*) spots encoded by the O^b or O^o alleles even more visible (Courtesy of Dr. Abitbol, Alfort Veterinary School, France). **b** The diagram represents three contiguous clones of melanocytes, derived from independent stem cells in which a different X chromosome is inactivated. Since X inactivation occurs early in development and is irreversible, many of the observed spots in the adult cat represent a cluster of cells derived from the same stem cell. **c** The figure represents a female mouse heterozygous for the $Atp7a^{Mo\text{-}dp}$ (*dappled*) allele. Mutations at this X-linked locus are common and affect copper metabolism (Courtesy of Dr. Eppig, The Jackson Laboratory Bar Harbor, Maine, USA)

Looking at the fur of different female cats with a similar O^b/O^o genetic constitution, one may also note that the X chromosome that is inactivated in the melanocytes results from a random process because there is no specific pattern for the distribution of the orange or black pigment, while the proportion of orange/black fur remains close to 50 %. Also, it seems clear that once an X chromosome is inactivated, this status persists in the daughter cells, resulting in the appearance of a mosaic female made up of a mixture of cells, with one or the other X chromosome actively producing either one of the two alternative gene products at the *Orange* locus.[4] Since, as we shall discuss later, X inactivation occurs quite early in development, patches of cells with a similar pattern of X inactivation can become quite large and are easily seen on the female's coat. Some O^b/O^o female cats have an even more spectacular coat color pattern when, by chance, they also carry an autosomal spotting allele (for example *piebald*), because this allele makes the orange and black fur patches even more distinct on an otherwise white background. Female cats with this coat color pattern are called *calico*.

Another observation that illustrates well the consequences of X inactivation at the phenotypic level was published in 1962, by Ernst Beutler (Beutler et al. 1962),

[4] The term "mosaic" is appropriately used in this context (see Chap. 2) because all the cells in a female organism derive from the same egg and have the same genetic makeup at the *Orange* locus. The difference in gene (or allele) expression depends upon the active/inactive status of one of the two Xs. This results from an epigenetic mechanism, but not from a difference at the DNA or chromosome level.

a few months after the publication of Lyon's theory, and refers to the human genetic deficiency in glucose-6-phosphate dehydrogenase (G6PD). To explain their observation concerning the kinetics of dehydrogenation of glutathione (GSH) by the enzyme G6PD from the erythrocytes of heterozygous human females, Beutler and colleagues came to the conclusion that two populations of erythrocytes co-existed in females heterozygous for the X-linked deficiency (*G6PDX* gene) rather than a single one, as would have been the case for enzymes encoded by autosomal genes. Once more, the situation appeared to be the consequence of monoallelic and independent expression of G6PD in the individual red cells of the heterozygous patients.

Many more examples of mosaicism have been reported in female mammals, including humans, to illustrate this point. The so-called Barr body, which was observed and reported years ago, even before Lyon's hypothesis, as a darkly stained dot in the nucleus of cells prepared from oral swabs, represents a heteropycnotic X chromosome. Karyotypes with X-chromosome aneuploidy (monosomics or XO, trisomics XXX or XXY male patients) display a number of Barr bodies that always contain one less than the total number of X chromosomes in the karyotype, indicating that there is a biological mechanism that somehow "counts" the total number of X chromosomes in mammalian cells in addition to the mechanism inducing inactivation of all X chromosomes but one.

6.2.1.2 X Inactivation is a Two-Step Process that Occurs Early During Embryonic Life

The mechanism and precise timing of X-chromosome inactivation (XCI) were debated for a few years after the initial publications of Lyon's hypothesis. Nowadays it is established that, in the mouse, two different forms of X-chromosome inactivation occur successively during early female embryogenesis. The first is an *imprinted* or selective inactivation, which starts at the 4–8-cell (morula) stage and affects only the paternal X chromosome (X_p).[5] By embryonic day 6.5 (i.e., when gastrulation begins), the X_p is reactivated in the cells that will give rise to the embryo proper, then the classical X_p or X_m *random* inactivation ensues that will be retained as such for the rest of the organism's life (Morey and Avner 2011; Pollex and Heard 2012).[6] In contrast to the tissues of the embryo proper, the imprinted or selective X_p inactivation is maintained in the extra-embryonic tissues (placenta) for the rest of gestation.

[5] This X_p-specific inactivation is consistent with the observation that, at the pachytene step of male meiosis, the X_p is condensed with the Y chromosome in an inactive XY body while, at the same pachytene stage of female meiosis, the two X chromosomes are visible and form a normal bivalent.

[6] Unlike in eutherian mammals, the imprinted X_p inactivation persists in all cells of protherian mammals (marsupials) including in the cells of the embryo proper.

As we already mentioned, the randomness of X-inactivation in the embryonic and adult tissues is faithfully translated at the phenotypic level. For example, when looking at the external appearance of adult calico cats one can observe that in most instances approximately 50 % of their spots are orange (O^o/–) while the other 50 % are black (−/O^b). This randomness was also demonstrated more rigorously by Davidson and colleagues (Davidson et al. 1963), who derived clones of epithelial cells from female patients heterozygous for two different forms of the enzyme glucose-6-phosphate dehydrogenase ($G6PD^A$/$G6PD^B$). In their experiment, the authors found that of 14 clones of cells derived from the same heterozygous patient, seven showed the *A* form of the enzyme while the other seven showed the *B* form, and none contained both the *A* and *B* forms.

6.2.1.3 X-Inactivation Is Complete … or Nearly so

X inactivation is thought to be highly stable in somatic cells and does not revert in the cells of the developing embryos, after implantation or in the cells of adult females. However, it has been reported that a few genes on the inactivated X chromosome could reactivate at low levels during aging. This is obviously a consequence of some relapse in the X-inactivation process, but remains marginal and concerns only a minority of the X-inactivated genes.

The situation is rather different with another limited set of genes, which are on the mouse X chromosome and escape X inactivation completely. Most of these "escapees" map to the pseudo-autosomal region (PAR), which means that they have a homolog on the Y chromosome. The pseudo-autosomal regions on the X and Y chromosomes pair and recombine during meiosis, (almost) as if they were autosomal, and it makes sense to believe that this is probably the reason why they are not inactivated: after all, there is no reason to apply any form of dosage compensation to these genes. *Steroid sulfatase* (*Sts*) is the best known example of these genes mapping to the PAR; mice homozygous for a deficient allele (Sts^-/Sts^-) have been reported as a model for a common neurodevelopmental disorder in humans, *attention deficit hyperactivity disorder* (ADHD) (Trent et al. 2011). However, unexpectedly, the same Sts^-/Sts^- mice are not a model for the human X-linked recessive disease *ichthyosis,* although human patients appear to be affected on the orthologous gene *STS*.

Besides the genes mapping to the PAR, some other genes mapping to the X chromosome also escape inactivation and are found to be transcribed from the inactive X chromosome. Most of these genes have (or had) a homolog on the Y chromosome or elsewhere in the genome, but this homolog is no longer functional. They are orphan genes and probably do not encode any functional proteins. The reason why these genes escape inactivation is unclear, but this does not seem to be a problem since XO females appear to be normal though sub-fertile. In contrast, in humans, where many more genes escape X inactivation, XO females present a severe phenotype known as Turner syndrome, which is probably due to these escapee genes, both in the PAR and elsewhere on the X chromosome.

6.2.2 The Mechanisms Controlling X-Chromosome Inactivation

6.2.2.1 Characterization of an X-Inactivation Center (XIC)

Elucidating the mechanisms leading to X-chromosome inactivation consists of understanding how two genetically identical and transcriptionally active X chromosomes, that lie within the same nucleus, can be differentially treated in such a way that one of them remains active while the other is inactivated. The first important observation in this matter was that all inactivated genes are on the same chromosome, while all active alleles are on the other. To interpret this observation, scientists hypothesized that the inactivation was essentially a chromosomal issue and that a master switch, controlling inactivation, might exist somewhere on the X chromosome from which inactivation starts and spreads along the rest of the chromosome. The identification of this master switch or *inactivation center* (XIC) was achieved in several laboratories in the mid-1990s, using strategies that are common in genetics, consisting of the demonstration of the physical association of a short chromosomal segment with its potential to inactivate the flanking regions of a given chromosome. Studying the consequences of various reciprocal translocations and deletions involving the X chromosome and mouse autosomes allowed the demarcation of a region of chromosome X with these properties. Confirmation of these observations came from experiments of transgenesis with cloned DNAs of various size followed by analysis of the consequences of the transgene on the flanking regions.

The extent of the region enclosing the inactivation center has been defined by studying X-chromosome deletions and by performing transgenesis in embryonic stem cells. Both experiments have permitted the characterization and delimitation of a region spanning a few hundred kilobases. Female embryonic stem cells (ES cells) have been invaluable tools for studying the XIC because these cells have their two X chromosomes active when undifferentiated, while X inactivation proceeds, as in embryos, when they start to differentiate in vitro.

A second discovery was that the XIC contains a gene, called *Xist* (for *X-inactive-specific-transcript*) that is transcribed into a non-coding RNA expressed only from the inactive X chromosome (Brown 1991). The *Xist* RNA was found to coat the inactive X chromosome in *cis* and to correlate with the onset of X inactivation.

Although the properties and localization of *Xist* RNA strongly suggest that it should be a key element in the X-inactivation process, further experimental evidence was required to show that this locus is necessary for inactivation of the X chromosome. This was shown through the use of various deletions of the *Xist* gene that prevent production of full-sized *Xist* RNA (Penny et al. 1996; Marahrens et al. 1997). In these cases the chromosome bearing the deletion is not inactivated, indicating that a complete, intact *Xist* gene is required, in *cis*, for inactivation to take place.

Further support for a critical role of XIST comes from experiments in which the *Xist*-cDNA, under an inducible promoter, was inserted into an autosome in male ES cells. Induction of *Xist*-RNA provoked coating of the chromosome in *cis* and repression of gene transcription for this autosome. Although other factors are probably also involved, these experiments demonstrated that *Xist*-RNA is a key trigger for chromosome-wide silencing and that it may do so by binding to the chromosome from which it is expressed. These experiments also demonstrated, along with previous studies from X-autosome translocations, that specific X-linked sequences are not required for *Xist*-RNA to coat a chromosome.

The XIC candidate region harbors four non-protein-coding genes, *Xist*, *Tsix*, *Jpx,* and *Ftx*, which are involved in X-inactivation. The XIC also contains binding sites for both known and unknown regulatory proteins.

The *Xist* transcript has no significant open reading frame and the product remains in the nucleus, coating the inactive X chromosome. This suggests that *Xist* is among those loci that produce a functional RNA molecule that is never translated into a protein (a non-coding RNA—see Chap. 5). *Xist* expression is detected early in pre-implantation development, often from both X chromosomes, just prior to X inactivation at the 4–8-cell stage (Okamoto et al. 2004; Patrat et al. 2009). In the mouse, the paternal X chromosome is initially subject to X inactivation as a result of an imprint in the gametes that leads to the paternal nonrandom inactivation found in extra-embryonic cells. Later, in the inner cell mass, *Xist* is activated from one of the two X chromosomes in cells that will form the epiblast. Random X inactivation follows and *Xist* transcription on the active X chromosome is silenced. Recent evidence suggests that XIST regulation involves a combination of *cis*-elements including antisense transcription as well as *trans*-acting factors that are tightly integrated with the pluripotent and stem cell proteins (for a recent review, see Augui et al. 2011).

The inactive X chromosome has been associated with several putative epigenetic marks (or non-sequence-based heritable changes) including DNA methylation, histone modifications, and Polycomb group complexes. DNA methylation is probably the best studied to date. Methylation of the cytosine base occurs enzymatically after DNA synthesis, and in mammals is restricted to the dinucleotide 5′-C_pG-3′ (C_pG). About 7 % of C_pGs are present at relatively high density in clusters called C_pG islands, which are usually located at the 5′ ends of genes. The remaining C_pGs are dispersed throughout the genome, usually as singlets. Most C_pG islands are unmethylated, but those near inactivated genes on the X chromosome, and those near some imprinted genes on autosomes, are methylated. Methylated C_pG islands repress transcription, and most silent genes on the inactive X chromosome have such methylated C_pG islands in normal cells. It is believed that DNA methylation acts in a synergistic way with other chromatin modifications to lock in the inactive state in a highly stable fashion in somatic cells.

The mouse has played a fundamental role in our understanding of the mechanisms of gene regulation and expression underlying processes such as X inactivation, as it has rendered observations and experiments possible that were not possible in any other species up until quite recently.

6.2.2.2 X-Inactivation Skewing

Most women heterozygous for the X-linked mutation DMD (Duchenne muscular dystrophy—*DMD*$^{+}$/*DMD*^{mut}) remain completely asymptomatic during their life and are generally unaware that they are carriers until they give birth to an affected son. This situation is common to many other pathologies where females are heterozygous for a deleterious X-linked mutation. The lack of overt phenotype or only mild phenotype in females is generally explained by considering that around 50% of their cells express the normal allele from the active (transcribed) X chromosome, with the mutated allele being on the silent, inactive X chromosome. This explains why these carrier females are protected from the clinical effects of X-linked mutations such as in the case of the *DMD* gene.

However, such situations of intercellular complementation are far from being the rule, and after careful analysis of other X-linked human pathologies, it has been observed that X inactivation may occur randomly at first (i.e., 50 % X^{+}/50 % X^{mut}) but, with time, the cells in which the X chromosome carries an allele with deleterious effects (X^{mut}) are counter-selected more or less efficiently, depending on the case, giving the impression of X-inactivation skewing. This is the case in a form of X-linked mental retardation (XLMR), ATR-X syndrome, which is caused by mutations in a ubiquitously expressed, chromatin-associated protein and in which phenotypically normal female carriers have highly skewed X-chromosome inactivation of the X chromosome that carries the mutant allele. Interestingly, the homologous disease has been modeled in mice heterozygous for a null *Atrx* orthologous allele, and it has been observed that X-chromosome inactivation is balanced early in embryogenesis but becomes skewed over the course of development because of a strong selection favoring cells expressing the *Atrx* wild-type allele (Garrick et al. 2006).

Selection against the cell lineage that carries the mutant allele on the active X chromosome appears logical, especially if it is the price to pay for surviving in better conditions, but it is not the rule. For example, unfavorable skewing of X inactivation has been reported in young females suffering from hemophilia B where the paternal X chromosome, carrying a normal copy of the FIX gene, was predominantly the inactive one, leading to the phenotypic expression of hemophilia B in these young girls (Espinós et al. 2000).

X-inactivation skewing is sometimes influenced by chromosomal rearrangements. An excellent example of such skewed X inactivation is provided by the *T(X;16)16H* (or Searle's) reciprocal translocation in the mouse. In this translocation, a piece of the telomeric region of chromosome X is attached to the centromeric part of chromosome 16, and vice versa. As expected, the piece of X chromosome that carries the X-inactivation center is inactivated, but inactivation spreads over the breakpoint and concerns all the genes on the piece of chromosome 16 that is attached to the broken X, resulting in a deleterious functional haploidy. Conversely, all the X-linked genes on the non-inactivated piece of X chromosome are expressed, where they should not be. In fact, for the female mice heterozygous for Searle's translocation, the only way to survive is to

inactivate their normal X chromosome. It is likely that such a situation, which is extreme in the case of *T16H* mice, probably exists with other mutations, although sometimes with a less dramatic effect—leading to a less extreme skewing.

At this point, it is interesting to note that, 40 years ago, Cattanach had already reported skewed X inactivation in F1 hybrid mice. He observed that, depending on the cross and strains involved, the percentage of inactivated X chromosomes was different for X chromosomes of different genetic origins. Cattanach quantified his observations by defining four alleles at a locus that he designated as the X-inactivation controlling element (symbol *Xce*) with four alleles: $Xce^a < Xce^b < Xce^c < Xce^d$ in order of the tendency of the X chromosome to remain active (Simmler et al. 1993; Thorvaldsen et al. 2012). As of today, the identity of the *Xce* locus remains unknown, although its genetic localization has been much refined, indicating that multiple elements on the X chromosome contribute to the *Xce* and that some of these may lie within the X-inactivation center (see below).

6.3 Parental Imprinting of Autosomal Genes

As discussed above, X-chromosome inactivation is an original and sophisticated method which has evolved to equilibrate the transcriptional activity of the genes on this chromosome between male and female mammals. Being epigenetic by nature, X-chromosome inactivation does not alter the DNA sequence and is completely erased when the primary germ cells enter gametogenesis. However, the X chromosome is not the only segment of the mammalian genome that can be modified epigenetically, as we will now discuss.

6.3.1 Evidence of Genomic Imprinting in the Mouse

6.3.1.1 The Unusual Behavior of the Hairpin-Tail Allele at the *T*-Locus

The *T*-locus of the mouse (brachyury *T*-Chr 17) has several mutant alleles; some are dominant while others, mostly found in wild mice, are recessive. Dominant alleles have an effect on the notochord derivatives and are characterized, when heterozygous, by a shortened tail with extensive variation in expressivity. *T/T* homozygotes die during embryonic development, at about mid-gestation.

Hairpin-tail (T^{hp}) is unique in the allelic series at the *T* locus, in the sense that the phenotype of the heterozygote offspring depends upon the origin of the mutant allele. When T^{hp} is inherited from a $T^{hp}/+$ male mated to a wild-type female (+/+), the offspring are all viable and about 50 % of them have a shortened tail, as expected. However, when the cross is set up the other way around (i.e., between a

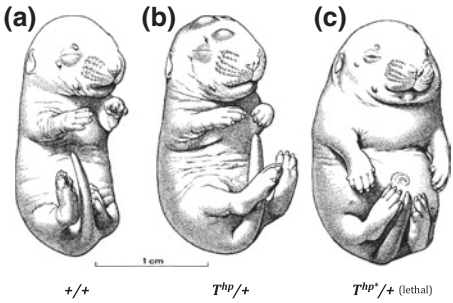


Fig. 6.2 *Inheritance of the hairpin-tail mutation*. 17-day-old embryos collected in the uterus of a T^{hp*}/+ mother mated with a T^{hp}/+ male: +/+; T^{hp}/+; T^{hp*}/+. Embryo (**a**) is normal. In embryo (**b**), the T^{hp} allele is of paternal origin, while it is of maternal origin* in embryo (**c**). Offspring with a genotype $T^{hp}*/T^{hp}$ die shortly after implantation and are not represented in the figure. The tail shortening effect of T^{hp} is not obvious in this figure, especially in embryo (**b**), probably for lack of expressivity (from Johnson 1974a). T^{hp} has been characterized as a large deletion on chromosome 17, which includes the (imprinted) gene encoding IGF2R

wild type +/+ male and a T^{hp}/+ mutant female), the progenies are reduced (nearly halved) and no mutant phenotypes are observed: they all die in utero at a relatively late stage of gestation.[7] This peculiarity of the hairpin-tail allele, which was first reported in 1974 as "*a case of post-reductional gene action in the mouse egg*" (Johnson 1974a, b), is not a simple maternal effect, since T^{hp}/+ × T^{hp}/+ matings produce two types of T^{hp}/+ heterozygous embryos: one is viable ab utero with a short tail, while the other is unviable (Fig. 6.2).

Nowadays, we know that the T^{hp} allele is associated with a deletion in the centromeric region of chromosome 17 (T-associated maternal effect—*Tme*). We will later discuss the molecular nature of this structural change and its consequences, but at this stage and from a historical point of view, it is important to note that the identification of this allele at the *T* locus was quite fortunate. If, by chance, the original T^{hp} mutant allele had occurred in a female germ cell it would have been lost and the discovery of a "post-reductional gene action" would have been delayed.

6.3.1.2 The Fate of Embryos Resulting from the in Vitro Re-Association of Pronuclei

For many years, and for technical reasons, it was impossible to grow mouse eggs in vitro, from the one-cell stage up to the stage of expanded blastocyst. Once this difficulty was overcome, one of the first experiments undertaken by embryologists was to try and reconstruct artificially diploid embryos by re-associating pronuclei

[7] Some exceptions have been reported, but they are extremely rare and fall well below the expected 50 %.

from embryos at the one-cell stage in different combinations. The rationale for undertaking this sort of experiment was to check whether a given haploid genome could merge with any other haploid genome to result in a viable mouse organism. Such experiments were completed in the early 1980s, in particular in England and in the USA, and led to the unambiguous conclusion that the development to term of reconstructed pseudo-diploid embryos requires the association of a maternally derived and a paternally derived pronucleus. Any other association (i.e., two male pronuclei or two female pronuclei) appeared lethal a few days after implantation (Barton et al. 1984; McGrath and Solter 1984; Surani et al. 1984) (Fig. 6.3).

The result of these experiments suggested that the haploid genome in a pronucleus was marked in a specific manner according to its parental origin, and that the male and female contributions were not functionally equivalent. This mark has become known as the *parental genomic imprint* or simply *genomic imprinting*.

Other experiments, focusing on the study of the developmental potentialities of cells derived from either gynogenetic embryos (resulting from the association of two female pronuclei) or androgenetic embryos (resulting from the association of two male pronuclei), merged together or associated independently with cells of a normal embryo in a single chimeric organism, indicated that androgenetic cells preferentially contribute to the formation of extra-embryonic tissues while gynogenetic cells, in contrast, preferentially contribute to the formation of embryonic

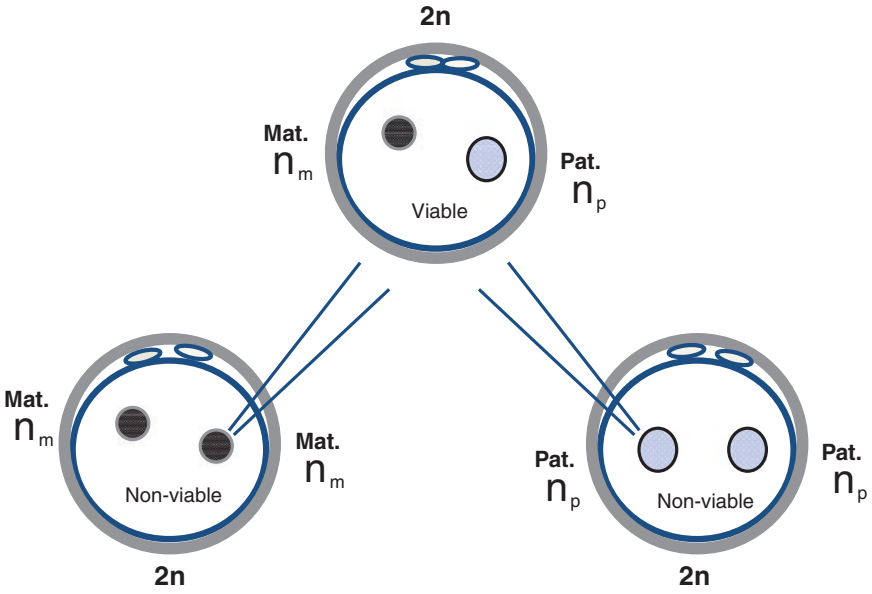


Fig. 6.3 *The fate of reconstructed, pseudo-diploid embryos*. The development to term of reconstructed, pseudo-diploid embryos requires the association of maternally derived and paternally-derived pronuclei. Reconstructed embryos with either two maternal or two paternal haploid sets are unviable

tissues. Another conclusion that can be drawn from these experiments is that parthenogenetic development is strongly hindered in the mouse although it occurs, occasionally, in other vertebrate species (it is common in fish and some reptiles, and has also been reported in birds).

6.3.1.3 The Fate of Embryos Resulting from Uniparental Disomies

The conclusions of the experiments reported above have been confirmed and refined by another totally different kind of experiments, achieved mostly in England, in the mid-80 s at the Harwell MRC Laboratory, by B.M. Cattanach, C.V. Beechey, J. Peters, and A.G. Searle. These experiments made use of two types of chromosomal rearrangements (Robertsonian translocations and reciprocal translocations) that were available in the large genetic repository at Harwell.

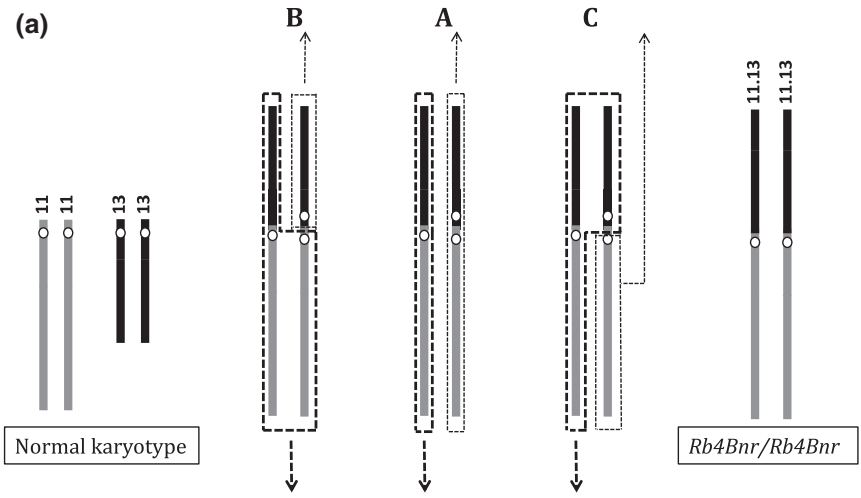
As described in Chap. 3, devoted to cytogenetics, mice whose genetic constitution consists of a single Robertsonian translocation plus the two acrocentric chromosomes whose arms are homologous to the arms of the Robertsonian translocation are perfectly normal since they have a balanced karyotype although reduced by one centromere. Such mice, however, often produce a high percentage of unbalanced gametes—i.e., gametes with either one extra (acrocentric) chromosome or, reciprocally, with one missing (acrocentric) chromosome. As we already discussed, these unbalanced gametes, resulting from meiotic non-disjunction, yield trisomic or monosomic embryos when merging with a normal gamete (Fig. 6.4).

In the mouse, most trisomic and all monosomic embryos die in utero at a stage of development that varies with the chromosome involved.[8] However, when by chance an unbalanced gamete with, for example, one missing acrocentric chromosome combines with a gamete with one extra chromosome of the same pair, this results in an embryo with a $[(n-1)+(n+1)] = 2n$ (euploid) chromosome complement, regardless of whether the two chromosomes of the pair in question were contributed by one and the same parent or not. Such embryos, with the two chromosomes of a given pair originating from the same parent, are said to result from uniparental disomies (UpDi).[9]

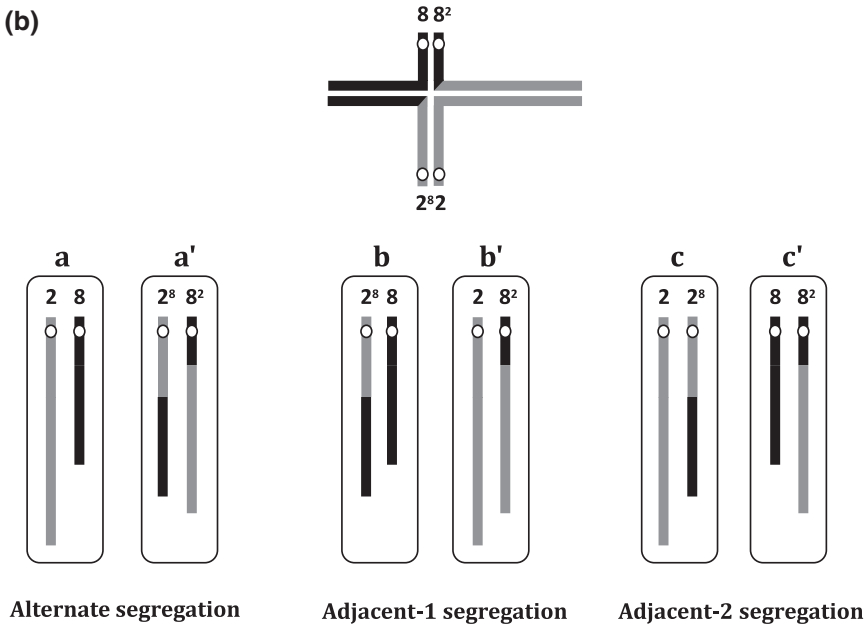
The observations by Cattanach and colleagues, made on the progenies of mice with a variety of different chromosomal translocations, were that viable and normal embryos resulting from complementary double non-disjunctions (UpDis) were (i) rather rare and (ii) very much dependent on the chromosome pair involved. In fact, in many instances, dramatic effects on development, including enhanced or retarded growth and sometimes lethality in utero, could be observed in the progenies (Cattanach and Kirk 1985; Cattanach 1986). Cattanach demonstrated that only a few chromosomes could be inherited as uniparental disomies, still leading

[8] *Ts19* is the only trisomy viable ab utero but only a few mice survive after 10 days.

[9] Uniparental disomies can be of maternal (MatUpDi) or paternal (PatUpDi) origin.



Meiosis in a Mouse heterozygous for a Robertsonian translocation



Meiosis in a Mouse heterozygous for a reciprocal translocation

Fig. 6.4 *Double non-disjunction in mice heterozygous for translocations*. In mice heterozygous for translocation the meiotic process often results in the production of a high percentage of aneuploid gametes due to the abnormal segregation of the chromosomes. **a** Represents the disjunction of chromosomes 11 or 13 in mice heterozygous for the Robertsonian translocation *Rb(11.13)4Bnr*. When a gamete with an extra chromosome arm merges with a normal gamete, this results in a trisomic embryo (see Chap. 3 for explanations). However, when the same aneuploid gamete merges with a complementary unbalanced aneuploid gamete missing the same chromosome arm, this recreate a normal (2n) karyotype with the exception that, in this case, the same parent provides the two chromosomes of a given pair and the other parent none of the gamete of the pair in question. In this case, the embryo is said to result from uniparental disomy (UpDi). Such embryos are viable only when the two elements of a chromosome pair involved in the UpDi are not imprinted. In the original experiments by Cattanach and colleagues (see text), identification of the parental origin of the chromosomes was done by using the phenotypic genetic markers vestigial tail (*vt*) for chromosome 13 and dominant, wavy coat (Re^{wc}) for chromosome 11. Nowadays, molecular markers like SNPs or microsatellites would rapidly distinguish the origin of the different chromosomes in such a cross. In the cross represented here, Cattanach and colleagues observed that the offspring resulting from MatUpDi11 (maternal uniparental disomy of chromosome 11) were smaller than their normal sibs while the offspring resulting from PatUpDi11 were bigger. This was a demonstration of the parental imprinting of (at least a segment of) the chromosome 11. Doted lines show the three different segregations of chromosome 11 including non-disjunctions. (Adapted from Cattanach's original drawings). **b** Represents the disjunction of chromosomes 2 and 8 in mice heterozygous for the reciprocal translocation *T(2;8)26H*. These mice produce a variety of gametes (**a–a′**, **b–b′**, **c–c′**) with a variety of chromosomal segment association, depending on the type of segregation (see Chap. 3 for explanations). Some of these gametes carry duplicated segments (for example, **b′** and **c** for Chr 2; **b** and **c′** for Chr 8) while some others carry segmental deletions (for example, **b** and **c′** for Chr 2; **b′** and **c** for Chr 8). The gametes with either a segmental deletion or a segmental duplication (**b–b′** and **c–c′** on the picture) produce unviable offspring when they merge with a normal gamete—only gametes of the **a–a′** type produce embryos with a balanced (viable) karyotype. When the cross is between two progenitors heterozygous for the same reciprocal translocation, there are rare cases where two gametes resulting from complementary non-disjunctions fuse together, restoring a balanced karyotype (i.e., when the duplications complement the deficiencies). These offspring, resulting from complementary uniparental partial disomies, are rare but they can be identified if genetic (or molecular) markers segregate in the cross, labeling the various chromosome arms. A major impediment to this kind of experiment is that many reciprocal translocations, when heterozygous, are sterile in one sex or the other. The production of neonates resulting from complementary double non-disjunction is also laborious because, unlike for Robertsonian translocations, mice heterozygous for reciprocal translocations produce small-sized progenies due to embryonic lethality (semi-sterility; see Chap. 3 for explanations). (*Adapted from Cattanach's original drawings*)

to normal healthy offspring. In all other cases, anomalies were observed, generally associated with difference in body size.

The general conclusions of these experiments are that normal development to term of a mouse embryo requires that some specific chromosomes be inherited from the mother or from the father, and sometimes from both the father and the mother (for example, chromosomes 7 or 11). This again suggested that a parent-of-origin-specific expression exists, at least for some genes, and for one and/or the other of the two parental chromosome homologs.

In addition to this series of experiments (made with mice heterozygous for Robertsonian translocations and concerning intact, complete acrocentric chromosomes), scientists at Harwell used another approach to screen the whole mouse

genome for specific imprinted regions. The strategy made use of an assortment of reciprocal translocations, a very common type of chromosomal rearrangement, resulting from the reciprocal exchange of chromosome arms between two non-homologous chromosomes. Here again, mice heterozygous for reciprocal translocations produce a variety of aneuploid gametes and, by inter-crossing such mice, it is possible to obtain normal, 2n embryos whose genomes result from the fusion of complementary unbalanced gametes. These experiments were arduous and required many crosses because, as we explained in Chap. 3, the progenies of mice heterozygous for a reciprocal translocation are much reduced in number. After carefully screening hundreds of progenies, the scientists at Harwell could observe the presence (or suspect the absence) of conceptuses resulting from uniparental duplication/deficiency for a particular chromosomal region and, finally, they could summarize their observations by drawing a chromosomal map indicating the maternally or paternally imprinted chromosomal regions (See Fig. 6.5).

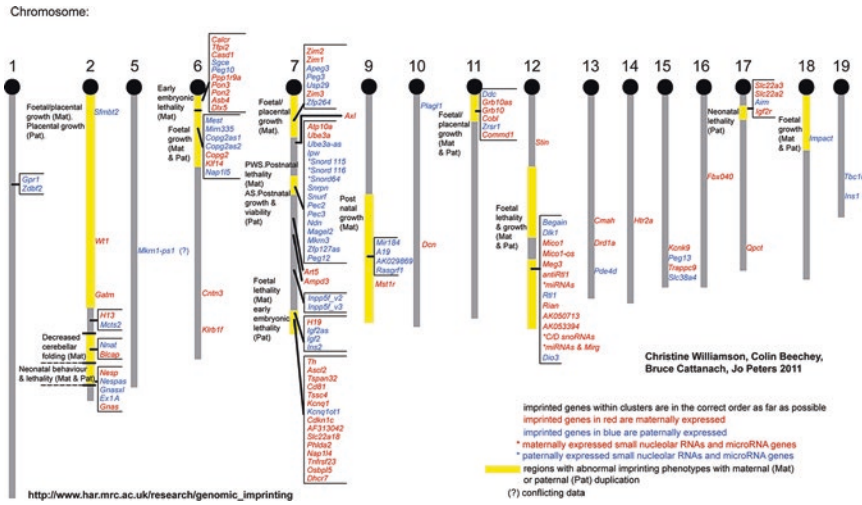


Fig. 6.5 *The Harwell map of mouse imprinted genes and regions*. Some chromosomal segments (outlined on the map) must be inherited from the male parent or from the female parent or, sometimes, simultaneously from both the male and the female parents. This is a consequence of genomic imprinting, which occurs during the process of gamete formation, and results in the functional inactivation of some specific genes encoding proteins or RNAs. The size of the imprinted segments has been estimated based on experimental data (see references), and in most instances it is excessively large compared to the actual size of the cluster of imprinted genes (1 Mb on average). Most (although not all) imprinted genes in the mouse are also imprinted in human and rat species. The establishment of this map has required an enormous investment in terms of crosses, and was possible only in a few laboratories (like MRC Harwell) where a large repository of translocations of all kinds existed. This map is now being progressively refined by direct analysis of the transcripts

6.3.2 Characterization of the Imprinted Regions in the Mouse

6.3.2.1 Imprinted Regions Harbor Genes that are Transcribed Exclusively from One Allele

The first imprinted region that was (partially) characterized at the molecular level was precisely the one that was discovered first and is associated with the "hairpin-tail phenotype". The characterization of the region in question was achieved by making a fine genetic map of the chromosome 17 proximal segment and performing a quantitative assessment of the transcription products of the genes mapping to that region. Providentially, another allele at the same *T/t* locus (t^{Lub2}) was discovered, which is recessive and associated with similar developmental defects as T^{hp}. When the chromosome carrying the t^{Lub2} mutation is inherited from the mother, embryos heterozygous for this mutation are severely affected by edema and death generally occurs between days 15–17 of gestation, just as for $T^{hp}/+$ mice born to a $T^{hp}/+$ mother (Winking and Silver 1984). Genetic and molecular analyses indicated that T^{hp} and t^{Lub2} were overlapping deletions of chromosome 17, with T^{hp} spanning a distance of about ~7 Mb and t^{Lub2} only ~0.8 Mb.

The t^{Lub2} haplotype has been characterized in detail, and several genes (Chr 17 cen—*Plg*, *Igf2r*, *Tcp1*, *Sod2*) have been identified within the deleted region. Remarkably, among all these genes *Igf2r*, the gene encoding the insulin-like growth factor type-2 receptor (IGF2R) appeared to be transcribed exclusively from the maternal allele, while the other genes were transcribed from both the paternal and maternal alleles.

Considered together, these observations explain all the observed phenotypes; in short, since *Igf2r* is deleted in the T^{hp} and t^{Lub2} chromosomes, and given that *Igf2r* is not transcribed from the paternal allele, any embryo with a $T^{hpM}/+\ ^{P}$ or $t^{Lub2M}/+\ ^{P}$ constitution has no functional IGF2R and accordingly cannot survive to birth. Embryos with the reciprocal genotype (i.e., $T^{hpP}/+\ ^{M}$ or $t^{Lub2P}/+\ ^{M}$) are normal since the maternal copy is intact and transcribed, exactly as in normal embryos. For all other genes, hemizygous embryos survive normally as they generally do with most other autosomal genes (Barlow et al. 1991).

Igf2r encodes a trans-membrane receptor protein whose function is to transport mannose-6-phosphate tagged proteins and insulin-like growth factor 2 (IGF2) to lysosomes; it is an essential protein for the completion of a normal gestation. The conclusions drawn from these observations have been validated by studying, independently, the fate of embryos inheriting a non-functional copy (i.e., a knockout allele—see Chap. 8) of the *Igf2r* gene from their mother or from their father.

In a series of experiments performed two years later, i.e., once the detailed mechanisms generating imprinting were unraveled, scientists created a non-imprinted allele of *Igf2r* (designated *R2Delta*) by deleting an essential element repressing the paternal allele in mouse ES cells (actually the ICE—see below). Maternal inheritance of this *R2Delta* allele had no phenotype, as expected. However, paternal inheritance resulted in biallelic expression of *Igf2r*. In this case,

embryos were affected by a 20 % reduction in body weight late in embryonic development that persisted to adulthood. Paternal inheritance of the functional *R2Delta* allele rescued the lethality of a maternally inherited *Igf2r* null allele and a maternally inherited *Tme* (T-associated maternal effect) mutation. These data suggested that one of the biological reasons for imprinting *Igf2r* is probably to trigger an increase in body weight at birth. These data confirmed the importance of the *Igf2r* gene in the *Tme* deletion phenotype (Wutz et al. 2001).

The second region that was recognized as imprinted, and characterized at the molecular level, was the telomeric region of chromosome 7. This region was identified by studying the progeny of an intercross between mice heterozygous for the reciprocal translocation *T(7;18)50H*. Embryos with the maternal duplication and paternal deficiency of distal Chr 7 (*MatDp7/PatDf7*) are growth-retarded and die around day 16 of gestation; the reciprocal maternal deficiency and paternal duplication embryos (*MatDf7/PatDp7*) die at an unidentified but much earlier stage. The imprinted region harbors, among others, the gene encoding insulin-like growth factor 2 gene (*Igf2*), a gene functionally and physiologically related to the gene encoding its receptor *Igf2r* (DeChiara et al. 1991).

IGF2 is a growth-promoting hormone acting during gestation and sharing structural similarities with insulin. *Igf2* is imprinted differently from *Igf2r* since it is transcribed exclusively from the paternal allele. The observations relative to the growth retardation of the embryos resulting from chromosome 7 uniparental disomies have been confirmed by studying the mice carrying a null (knockout) allele of *Igf2*. As expected, non-complementation of the Igf^- allele by the normal *Igf2* allele was observed when the wild-type allele was inherited from the mother.

6.3.2.2 Making the Inventory of Imprinted Genes in the Mouse

Many genes have been progressively discovered in the various imprinted regions identified by Harwell's scientists, and a good proportion of these regions have now been characterized at the molecular level. As indicated on the map (Fig. 6.5), there are at least 15 and probably up to 25 imprinted regions spread over 16 different autosomes and these regions are apparently distributed randomly, i.e., with no specific pattern. They are either telomeric or centromeric and harbor clusters of genes (from 3 to 11) rather than single independent genes. Some geneticists think that this clustering of the imprinted genes is probably not by chance, and may reflect subordination to a common mechanism of inactivation. This conclusion, however, should be reconsidered when a greater number of imprinted genes or regions are identified in different mammalian species.

The genes mapping to the same imprinted cluster do not appear to be functionally related. Even more surprisingly, some genes in a given cluster are maternally expressed while others are paternally expressed (for example, *Igf2* and *H19* on distal Chr 7). This is in good agreement with the original observation at Harwell that,

at least for some pairs of chromosomes, one element must be inherited from the father and the other from the mother.

As we mentioned, the function of the genes mapping to the imprinted clusters is not always fully characterized, and for some of them it may take some time before we precisely determine all their functions. This is particularly true if we consider that some of these genes, for example *H19*, do not encode proteins but non-coding RNAs instead.

The analysis of the transmission of knockout (null) alleles, produced by in vitro gene targeting in the mouse, will be of great help for the future identification of imprinted regions or genes. It seems, however, that genes of this category represent only a minority of the genes because if the wild-type alleles of the genes that have been knocked out were imprinted, their uniparental transmission to the progeny would be impossible or associated with some pathology, and this would almost certainly have already been noticed by researchers. The analysis of the transmission patterns of knockout alleles is indeed an efficient way to screen for genomic imprinting in the mouse, and the occurrence of any phenotypic alterations exclusively transmitted by one sex and not by the other should trigger curiosity and call for further investigation. Similarly, identification of a new imprinted gene in humans (or any other mammalian species) should be considered as an indication for a candidate in the homologous region in the mouse.

As of today, the number of imprinted genes reported in the mouse is around 140. Studies of the total number of imprinted genes are currently being refined by other methods (Yu et al. 2012). Sequencing the whole transcriptomes of interspecific mouse hybrids resulting from crosses in both directions (for example, a female of a laboratory inbred strain × a *Mus m. musculus* male or vice versa), and looking for tissue/cellular distribution of species-specific SNPs is a promising way of achieving the complete inventory of imprinted genes in the mouse (Fig. 6.6).

Of the 140 genes that have been reported as being imprinted in the mouse, a quite large proportion has also been found to be imprinted in humans, but exceptions exist. *Igf2,* for example, has been found to be imprinted in the human, rat, and mouse species but the gene encoding the receptor for this molecule, *Igf2r*, is imprinted in the rat and mouse species but not in humans (Weidman et al. 2006). In addition to this observation, it is worth noting that, from interspecific comparisons that have been made, it seems that the degree of homology in terms of imprinted genes parallels the phylogenetic distances. This is not so surprising and, with a better knowledge of the imprinted genes across mammalian species, it should be possible to learn more about their function. Already, by comparing the known functions of the imprinted genes in the three above-mentioned species (human, mouse, and rat), it is obvious that most of these genes code for growth factors expressed during embryonic life either in the fetal membranes, the placenta or in the embryo proper.

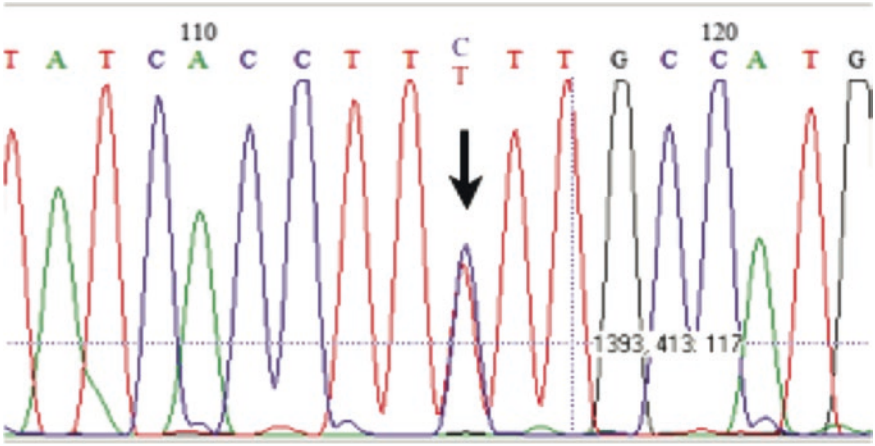


Fig. 6.6 *Molecular identification of imprinted genes using SNPs in the cDNAs*. One can easily check if the two alleles at a given locus are co-expressed in embryonic or adult tissues by analyzing the SNP pattern of the transcribed RNAs. The figure represents part of the sequence of the transcripts of the gene encoding β-hemoglobin (HBB) in the bone marrow cells of F1 mice heterozygous for a single, untranslated nucleotide polymorphism (a silent mutation) in exon 2 of the gene. The figure shows that both alleles are transcribed, since one can recognize the profile of a C/T SNP (*arrow*) in the sequence of the corresponding cDNA. If the gene encoding β-hemoglobin chain (*Hbb*) was among the genes undergoing genomic imprinting, one would have found a single transcript (either from the C or from the T allele) depending on the direction of the cross. Sequencing the whole transcriptome of interspecifc F1 mice is an efficient way of making the inventory of imprinted genes in a given species or in a given tissue

6.3.3 What are the Molecular Mechanisms that Control Genomic Imprinting?

6.3.3.1 DNA Methylation Modifies Transcriptional Activity

Understanding the biological mechanisms involved in the establishment and maintenance of genomic imprinting has motivated a large number of experiments carried out mainly in the mouse and using the most sophisticated techniques. The results obtained have much clarified the situation, even if some aspects require a closer look. We will summarize the state of knowledge as it stands now. However, before doing this, it is important to note that the molecular mechanisms in question had to comply a priori with some basic constraints. First, imprinting may interact with the transcription process but in no way may it alter the DNA sequence of the imprinted regions. Imprinting, as we discussed, is strictly epigenetic, which means that the information in the DNA sequence is not altered. Second, the imprinted regions must be transmitted unchanged to the daughter cells during the development of the embryo and in the adult to ensure the continuation of imprinting, at least for some time, in the different cell lineages. Third, the

epigenetic alteration(s) must be initiated in the paternally or maternally inherited chromosomes independently, and at a time when they are not in the same nucleus; that is, during gametogenesis or immediately after fertilization, before the fusion of the pronuclei. Finally, the parental imprint must be erasable (or reversible) in order to be set differently when the allele goes into a gamete of the opposite sex (Ferguson-Smith 2011).

One of the imprinted regions that has been the most extensively studied is, again, the one that maps to the distal part of mouse chromosome 7. This region, in fact, contains two contiguous clusters: one with four genes (cen–… *H19–Igf2–Igf2as–Ins2*), encompassing around 1 Mb, and another one, more distal, harboring around 15 genes (around the *Kcnq1* locus). Both clusters have a homolog in human and rat, and the genes in question are equally imprinted in these two species.

H19 encodes a 2.3-kb ncRNA that is highly preserved across mammalian species, indicating that it presumably has an important function. Embryos heterozygous for a maternally inherited knockout allele or homozygous for the *H19* knockout allele exhibit increased placenta and body weight (Gabory et al. 2009).

Igf2 encodes a hormone that has similarity with insulin and is probably a major fetal growth factor. Mice heterozygous for an *Igf2* knockout allele ($Igf2^{-}$), transmitted through the male, exhibit pre- and post-natal growth retardation. In contrast, when the disrupted (null) allele is transmitted maternally, the heterozygous offspring are phenotypically normal. Both *H19* and *Igf2* are widely expressed during embryonic development, and then they are down-regulated in most adult tissues.

Shortly after the characterization of the *H19–Igf2* cluster and its complete sequencing, it was demonstrated that imprinting of these two genes is concomitant with the methylation of an *imprinting control region* (ICR) or *differentially methylated region* (DMR), which is 2 kb long and inserted between the two genes. Proper imprinting of *H19* and *Igf2* requires the ICR integrity because, when this region is altered or deleted by genetic engineering, imprinting is abolished. Similarly, proper imprinting of the *H19–Igf2* cluster requires that the ICR be methylated on the paternal allele and unmethylated on the maternal allele.

As already discussed concerning the mechanisms at work in the case of X inactivation, DNA methylation is a biochemical process that consists of the addition of a methyl (CH_3) group at the C-5 position of cytosine, at specific sites known as 5′-C_pG-3′ dinucleotides or C_pG islands. When methylation occurs in the 5′ regulatory regions of many genes, this generally results in transcriptional silencing of these genes. Experiments performed in the early 1990s demonstrated that DNA methylation is probably a crucial step in determining imprinting in mammals, since deficiency in DNA methyltransferase activity (for example, as a consequence of a targeted null mutation at the *Dnmt1* gene) impedes normal imprinting and the homozygote mutant embryos die around day E9.5 (Li et al. 1993). Methylation is stable and can be inherited through mitotic cell division in the differentiated tissues. Methylation alters the spatial conformation of the DNA, making it more compact and accordingly less accessible to DNA-binding proteins, but it does not

alter the sequence proper. Methylation is also reversible and accordingly complies with the constraints mentioned above.[10]

The *H19–Igf2* imprinted region of mouse chromosome 7 and its human homolog on chromosome 11p15.5 have been extensively studied with the aim of elucidating the mechanisms at work for imprinting establishment and maintenance in mammals. Most of the results gathered in the mouse have been cross-validated in humans, and vice versa. As mentioned, these results have revealed the existence of ICRs and DMRs, as regulatory elements for imprinting of the gene cluster, and have underlined the role of methylation of the C_pG islands as previously observed in plants. Methylation of these regions results in silencing or activation of the cluster, depending on the initial status of the genes concerned (Ferguson-Smith et al. 1993; Constância et al. 1998; Reik et al. 2001; Reik and Walter 2001).

The DMRs are the main signature of imprinted genes. Some are called primary or germline DMRs (such as the *H19–Igf2* ICR or the *Igf2r* ICE), because they acquire their differentially methylated status in the germline, and others are called secondary or somatic DMRs and acquire their methylation after fertilization. In the case of the *H19–Igf2* locus, the insulator protein, called CTCF, binds only to the unmethylated ICR and produces a boundary. This results in the interaction of downstream enhancers with the *H19* promoter but not with the *Igf2* promoter on the maternal allele. This was defined as the *enhancer competition model* and explains the monoallelic expression of these genes.

6.3.3.2 Other Mechanisms Involved in the Control of Imprinting

The Role of ncRNAs

Analysis of several imprinted regions also revealed that some specific ncRNAs are probably essential intermediate molecules for the establishment (and maintenance) of imprinting. This assumption was validated by observations made on the imprinted *Igf2r* cluster on mouse chromosome 17. In this cluster, the ICE (imprinting control element) acts as a promoter for a long ncRNA named *Airn* (for antisense of *Igf2r* RNA non-coding) from the unmethylated paternal allele. When *Airn* expression is abolished, the *Igf2r* imprint is removed, suggesting a mechanism of transcription interference (Latos et al. 2012). This mechanism, however, does not exist in humans where *Igf2r* is not imprinted.

[10] Several assays have been designed to assess the methylation status of the genomic DNA. One of the most popular consists of the initial treatment of DNA with sodium bisulfite, which converts cytosine residues into uracil (U) or thymidine (T), but leaves 5-methylcytosine residues unaffected. Once treated with bisulfite the DNA can then be directly sequenced or digested with restriction enzymes (like *Bst*UI), which only cleave sites that were originally methylated (CGCG) but not those that were originally unmethylated (TGTG). Combined bisulfite restriction analysis (or COBRA) is a widespread technique allowing quantification of DNA methylation. It has been extensively used in cancer research and epigenetics studies.

A recent report indicated that within each cluster all imprinted genes show concordant parent-of-origin-specific gene expression except for the ncRNAs that show expression from the opposite parental allele. Such strict reciprocal parent-specific expression seen between mRNAs and imprinted macro ncRNAs strongly indicates that ncRNAs regulate imprinting in such clusters (Saxena and Carninci 2011). This has also been shown for the *Kcnq1* locus, in which the *Kcnq1ot1* long ncRNA is required to maintain DNA methylation and transcriptional gene silencing of the adjacent imprinted genes (Mohammad et al. 2012).

The Role of Histones

Histone modifications have also been considered as an important mechanism in establishing the imprint either directly or indirectly, and in many cases the alleles that display DNA methylation also carry histone marks associated with inactivity. Many points still remain to be clarified concerning the mechanisms of establishment and maintenance of imprinting in mammals (Chen and Dent 2014).

6.3.3.3 Marks of Imprinting are (in General) Cleared Between Generations and Reset During Gametogenesis

The sex-specific marks on DNA, which result in (or lead to) genomic imprinting, and consequently to functional haploidy of the non-imprinted alleles, persist in general from conception throughout all embryonic stages and up to the adult state in most somatic cells. These marks, however, have to be completely erased at a certain critical period of the life cycle since they are likely to be set differently at each generation.

Experiments and observations have demonstrated that epigenetic marks (histone modifications and DNA methylation) on most of the genome start to become erased in primordial germ cells of both sexes at around day 11.5 of gestation, upon entry of the germ cells into the gonads. Genes then acquire new sex-specific DNA methylation marks during fetal development in males and a little later, during the growing oocyte phase, in the early neonatal period in females. The mechanisms involved during the clearing out of the imprinting marks (active or passive DNA demethylation) have not been completely unraveled (Ferguson-Smith 2011).

More importantly, acquired methylation of the ICRs or DMRs of imprinted genes needs to be preserved during the massive wave of demethylation that occurs in the embryo after fertilization. It is now known that imprinted genes display hexanucleotide motifs that are methylated and recognized by several proteins (such as Zfp57, TRIM 28, or Stella). The complex formed between the hexanucleotide motif and these proteins protects the ICRs from being demethylated at these early stages of development and is a signature of the imprinted genes. These observations reveal that both genetic and epigenetic signals are required to establish and maintain the imprinted status of a gene.

When discussing X-chromosome inactivation we mentioned that the inactive X chromosome could sometimes reactivate in somatic cells, especially when the animals age. The situation is similar and even more common with autosomal imprinting, and cases of tissue-specific variations have been reported in the mouse. For example, in the developing embryos only the paternal allele at the *Igf2* locus is expressed, while the maternal allele is silent. However, in the choroid plexus and leptomeninges the situation is different and both alleles are transcriptionally active (DeChiara et al. 1991). Another example of tissue-specific imprinting is provided by the *Cdh15* gene. The germline DMR of this gene is protected from erasure of methylation during the first steps of embryogenesis but becomes methylated after implantation. This led to the proposal of the existence of both *bona fide* imprinted germline DMRs and *transient* germline DMRs (Proudhon et al. 2012).

Another interesting situation is provided by the viable yellow allele at the *agouti* locus (A^{vy}-Chr 2). This mutation is transmitted as a dominant allele; it is viable when homozygous (unlike the classical yellow allele A^{y}, which is homozygous lethal), but the coat color of affected mice exhibits variation, ranging from pure homogeneous yellow, through mottling with dark patches, to an agouti-like coat (pseudo-agouti) similar to the wild-type allele *A*. Homozygous (A^{vy}/A^{vy}) and heterozygous (A^{vy}/a) mice tend to become obese and diabetic, and the degree of obesity is correlated with the coat color, yellow mice being more affected than agouti ones (Morgan et al. 1999).

The A^{vy} mutation is the result of the insertion of an intra-cisternal A-particle (IAP or retrotransposon) into a non-coding exon 5′ of the agouti gene. Functional analysis revealed that the expression of the mutant allele is controlled by the long terminal repeat (LTR) of the IAP. When the LTR in question is hypomethylated, the A^{vy} allele is transcribed, the coat is yellow, and the mouse is bigger than normal. When the viral LTR is methylated (and accordingly inactivated), the coat is agouti. Variegation of coat color in $A^{vy}/+$ mice (which is sometimes also observed in $A^{y}/+$ mice) is very likely the consequence of some mosaicism at the somatic cell level.

When $A^{vy}/+$ males are mated with *a/a* (black non-agouti) females, there is no significant difference in the proportions of yellow, mottled or pseudo-agouti phenotypes in the progenies, and this occurs independently of the coat color (yellow, mottled or agouti) of the male. The situation is different when the cross is set up the other way, i.e., between an *a/a* (non-agouti) male and $A^{vy}/+$ female. In this case there is some sort of *transgenerational epigenetic inheritance* in the sense that the distribution of phenotypes in the progenies is related to the phenotype of the dam and not of the sire—for example, yellow mothers produce more yellow offspring than agouti mothers. Clearly, it appears that imprinting marks are not erased when transmitted through the female, while they are erased when transmitted through the male. Several laboratories have confirmed these observations and it has been demonstrated that selection of a certain phenotype (for example, the percentage of pseudo-agouti offspring in the progeny) could increase the prevalence

of the trait in successive progenies (Blewitt et al. 2006; Cropley et al. 2012). The behavior of the A^{vy} allele, which is quite uncommon in mouse genetics, may appear anecdotal but similar situations might be common if we consider the abundance of IAP in the mammalian genomes (Morgan et al. 1999).

6.3.4 Genomic Imprinting Across Mammalian Species

To date, the differential expression of alleles according to their parental origin has been reported and documented only in flowering plants (Nowack et al. 2007) and in mammals. In mammals, it seems to be an exclusive characteristic of the eutherians and metatherians[11] (marsupials), while prototherians (for example the platypus, *Ornithorhynchus anatinus*) do not exhibit genomic imprinting. In other words, genomic imprinting seems to correlate with gestation of the embryo inside the uterus and placentation (viviparity) but not with egg laying (oviparity). Genomic imprinting has never been reported in fish, amphibians, reptiles or birds (Dünzinger et al. 2005).

In mammals, the imprinted regions are in general relatively well preserved across the different species and for each of the imprinted regions in the mouse, for example, there is in many instances a homologous region in the rat and in humans—with, however, a few remarkable exceptions. From these phylogenetic observations one may conclude that genomic imprinting probably appeared concomitantly with the viviparous mode of reproduction (i.e., ~180 Myr ago). One may also observe that the more closely related are any two species, the greater are the homologies between the different imprinted regions. However, after careful observation it is sometimes discovered that rare but noticeable differences exist between closely related species, as if the process of genomic imprinting was still in evolution in that class of vertebrates.

As we discussed in a previous chapter, some morphological differences between inter-specific hybrids have been reported which depend upon the way the cross that produced these hybrids was set up. Even in the *Mus* genus, in which so many species have been identified including *Mus m. musculus* and *Mus m. domesticus*, some morphological and anatomical differences have been noted that could be attributed to point differences in terms of genomic imprinting. For example, female mice of the *Mus spretus* species do not (or very rarely) produce viable offspring when crossed with laboratory mouse males, while the reverse is not true. The placental hypertrophy of some of these rare F1 hybrids or backcross offspring has been attributed to an X-linked locus (*Ihpd* for interspecific hybrid placental dysplasia) with several alleles, but could also be interpreted as differential imprinting due to differential X inactivation.

[11] In marsupials, the number of imprinted genes is much lower than in eutherian mammals.

6.3.5 The Origin and Evolution of the Imprinting Mechanisms in Mammals

The existence of genomic imprinting raises a number of issues that can be summarized in the following question: what advantage can justify, for a mammalian embryo, having a number of its genes maintained in a functionally haploid status, while diploidy is generally considered more advantageous with regards to evolution? The answer to this basic question is not yet definitively known, and several hypotheses have been developed over the last decade (Wood and Oakey 2006).

One of the first explanations that came to mind was the consideration that imprinting emerged during evolution as a mechanism to clear the genome of spontaneously occurring mutations with lethal or deleterious effects, for the simple reason that such mutations, when they occur within an imprinted region, are eliminated when the region in question becomes functionally haploid. This hypothesis unfortunately has several weaknesses, and in particular it does not explain why such a clever mechanism appeared so late in evolution and has remained an exclusive privilege of mammals.

A more consistent explanation is that genomic imprinting is a very efficient way of inhibiting parthenogenetic (gynogenetic or androgenetic) development in mammals. Indeed, and as explained above, the development of a normal mouse embryo from two female (or two male) pronuclei (i.e., from only one parent or from two parents of the same sex) is strongly repressed. This is a direct consequence of genomic imprinting at the *H19–Igf2* and *Dlk1–Gtl2* loci, as demonstrated by Japanese scientists who succeeded in producing bi-maternal mice after artificially erasing (i.e., by genetic engineering) the imprinting at these loci (Kono et al. 2004; Kawahara et al. 2007; Kawahara and Kono 2012). Although more likely than the previous one, the hypothesis stating that genomic imprinting exists only to impede parthenogenesis in mammals is not entirely convincing and is definitely not sufficient. In fact, the possibility that parthenogenetic development could occur in mammals cannot, a priori, be regarded as a disadvantage, since that sort of development exists occasionally in some classes of vertebrates as an exceptional and alternative way of reproduction, for example to escape a reproductive dead end. From this point of view, the possibility of the mammals using parthenogenesis for one or two generations would also appear advantageous.

A third hypothesis on the origin of genomic imprinting is that it has no advantages at all and exists only by chance. According to this hypothesis imprinting is a mere artifact, a “red herring” so to speak, which results from the uncontrolled expansion to the neighboring regions of a defense mechanism used by mammals to control or neutralize the possible invasion of their genome by self-replicating parasitic DNAs such as retroviruses or retro-transposons (see Chap. 5). Just like the previous two, this hypothesis has some weaknesses and, in particular, it does not explain why imprinting exists only in mammals—while birds have to compete with so many retroviruses and retro-transposons invading their genomes. In the same way, it does not fit with the fact that imprinting is reversible.

If we summarize the information gathered from the observations made in humans (see below) and those collected from the many experiments that have been performed in the mouse species, we can establish correlations and draw some conclusions about the essence of imprinting in mammals and finally come to more coherent hypotheses. An important one is based on the observation that most—not to say all—of the genes that are imprinted have been found to play a role in the control of embryonic growth and development, in most instances through the development of the placenta. Based on this observation, a widely accepted hypothesis to explain the origin and evolution of genomic imprinting is the "*parental conflict hypothesis*", which is also known as the "*tug-of-war hypothesis*". The hypothesis states that the differences between parental genomes due to imprinting are the result of the divergent interests of each parent (or sex) concerning the evolutionary fitness of their genes (Haig 1997; Sha 2008). Since males can have a virtually unlimited number of offspring, the father's genes gain greater fitness through the vigor of the offspring, eventually at the expense of the mother, and this explains why paternally expressed genes tend to be growth-promoting for the embryo. The mother's interest, on the other hand, is to preserve nutrients and resources for her own use, to get rid of the offspring that are in her uterus as soon as possible, and thus be able to produce another litter as rapidly as possible. This would be in agreement with the observation that maternally expressed genes tend to be growth-limiting. Indeed, unlike other vertebrate embryos, mammals could theoretically stay in utero for an unlimited period of time, surviving at the expense of the mother's nutrients, unless a mechanism regulating gestation length intervenes. Genomic imprinting, indirectly controlling the embryo's growth, appears a good way to limit the duration of gestation. This hypothesis has the great advantage of justifying the existence of imprinting and its existence exclusively in mammals and, for this reason, it has been accepted for a good ten years. Nowadays, however, our understanding of the molecular mechanisms at work in genomic imprinting has revealed some inconsistencies, and the parental conflict hypothesis would probably need to be revisited. Recent observations have suggested co-adaptation between the mother and the conceptus at fetal stages (involving placental exchanges) and at post-natal stages with metabolic and behavior exchanges (Keverne 2013).

6.3.6 The Pathological Aspects Associated with Genomic Imprinting

6.3.6.1 Epigenetics and Human Diseases

The same year (1974a) when Johnson reported his observations concerning the phenotypic differences associated with the parental origin of the hairpin-tail (T^{hp}) mutant allele in the mouse (see above), Lubinsky and colleagues reported a similar parental effect in a family transmitting a syndrome now known as

Beckwith–Wiedemann syndrome (BWS) (Lubinsky et al. 1974). In fact, these two observations independently inaugurated the studies relating to the effect of genomic imprinting on gene expression in the mouse and human species, respectively. Nearly forty years after these publications, a lot has been learned concerning genomic imprinting and its importance in some human pathologies.

Beckwith–Wiedemann syndrome (OMIM 130650) is a rare disorder with an incidence of approximately one in 14,000 childbirths. It is characterized by the association of traits like macroglossia, greater than normal birth weight and size, neonatal hypoglycemia, and some other visceral defects (of the adrenal gland in particular). In most cases the BWS is sporadic, but around 15 % of the cases are familial and in many of these familial cases, mutations or deletions of genes within a region spanning approximately 1 Mb of human chromosome 11p15.5 have been reported (the mouse homologous region is on distal chromosome 7). Imprinting defects of genes in the same region have also been described in a very high proportion of BWS patients having a biallelic (rather than paternal monoallelic) expression of the *IGF2* gene. In these cases, the maternal copy of the gene *IGF2* is transcribed where it is normally inactivated in healthy babies. Finally, some babies affected by the BWS have been found to be the consequence of a paternal uniparental disomy (PatUpDi) of chromosome 11, and in these rare cases the two regions of chromosome 11, having escaped maternal imprinting, are both transcribed. Other patients exhibit loss of imprinting of a gene encoding a long ncRNA transcript, called KCNQ1OT1, which is also known to be imprinted in the mouse.

Another rare human syndrome, Russell–Silver syndrome (RSS-OMIM 180860—one in 70,000 childbirths), has also been found to be associated with an imprinting defect. In a recent survey concerning this disease, 10 % of all the cases were found to be associated with a maternal uniparental disomy (MatUpDi) of chromosome 7. In some other cases, the same 11p15.5 region of human chromosome 11 harboring the *H19* and *IGF2* genes appeared to be involved. The defect in this case is characterized by a suppression of IGF2 growth factor activity that explains the concomitant growth reduction observed in RSS patients. In these cases, where the same 11p15.5 region is concerned, the pathological features of RSS logically appear to be the opposite of those described for BWS (Butler 2009).

Prader–Willi (PWS) and Angelman (AS) syndromes are the two most studied cases of human diseases commonly related to defective genomic imprinting. Unlike BWS and RSS, which are often compatible with an almost normal adult life, PWS and AS are always severe and do not improve with aging. PWS and AS are caused by mutations, deletions, uniparental disomy or by abnormal imprinting of one or several different members of a gene cluster in the q11-q13 region of human chromosome 15 (Horsthemke and Wagstaff 2008).

Prader–Willi syndrome (OMIM 176270) occurs in one in 15,000 individuals and is characterized at a young age by hypotonia, short stature, mental deficiency, behavioral problems, and feeding difficulties. In a second phase, from the age of 3 years, developmental delay and psychomotor retardation are even more obvious but obesity becomes a life-threatening issue requiring strict dietary restrictions.

Angelman syndrome (OMIM 105830) is characterized by severe mental retardation with seizures, ataxia, uncoordinated movements, hypopigmentation, inappropriate hilarity, lack of speech, etc. In the late 1980s geneticists observed that PWS and AS were caused by deletions in bands 15q11-q13, and they reported that the observed phenotypic differences between the two syndromes in fact depended upon the parental origin of the deletion. Deletions occurring on paternal chromosome 15 generally resulted in PWS, while similar deletions occurring on maternal chromosome 15 resulted in AS. For this reason, PWS and AS were, and still are, considered as sister syndromes—which fits rather well with the symptomatology.

Nowadays, the situation has been much clarified, and by and large it is concluded that PWS is a consequence of the lack of the paternal copy of one or a few genes in the 15q11-q13 region, while AS is a consequence of the lack of a functional maternal copy of the *UBE3A* gene encoding ubiquitin protein ligase 3A (Moncla et al. 1999; Horsthemke and Wagstaff 2008).

In addition to the four syndromes described above, which are relatively well documented, a few other human diseases and pathological conditions, including certain forms of cancers, have been described as the very likely consequence of abnormal imprinting because of a clear effect of the parental inheritance. In most instances, however, the situation was reported as complex and difficult to analyze because of the interference of environmental factors and/or epistatic interactions with elements of the genetic background. It is likely that, with the rapid progress in sequencing technology and the development of quantitative analysis of RNA transcription, these diseases or syndromes will be clarified in the near future. This will definitely allow a better understanding of the role of epigenetic regulation in gene expression.

6.3.6.2 Epigenetic Manifestations in Some Animal Crosses

At several points in this book we have mentioned that some interspecific mouse hybrids exhibit a variety of pathological features depending on the direction of the cross. For example, crosses between male mice of the *Mus spretus* species and females of the *Mus m. domesticus* species produce viable hybrids but the sex ratio in the offspring progeny is much biased in favor of the female, and the male F1s are always sterile. This difference is in compliance with the so-called Haldane's rule and has been observed in several other cases of interspecific crosses (for example, between different *Drosophila* species, between *Bos taurus* and *Bison bison*, and between *Chrysolophus pictus* and *Gallus g. domesticus*).[12] In the case of mouse crosses, it has been established that the sterility of hybrids is controlled by a few genes, some of which have been localized on the genetic map. In contrast, the reasons for the shortage of males are still conjectural.

[12] Haldane's rule states "when in the offspring of two different animal races one sex is absent, rare, or sterile, that sex is the heterozygous [heterogametic] sex."

More interesting is the observation that crosses in the other direction (between *Mus m. domesticus* males and *Mus spretus* females) result in stillbirths in most cases, with a marked enlargement of the placenta.[13] A similar situation was reported for crosses between two other species of rodents of the genus *Peromyscus*, with strong parent-of-origin effects involving placental growth. Female *P. maniculatus* crossed with male *P. polionotus* produce neonates smaller than either parental strain, with placentas half the parental size. In contrast, female *P. polionotus* crossed with male *P. maniculatus* produce dysmorphic overgrown embryos whose placentas average up to 2.5 times the mass of the parental strains (Vrana 2007).

Such biases are difficult to explain in terms of Mendelian genetics if we consider that the genetic makeup of the above-mentioned reciprocal F1s are virtually the same, with one allele of each parental species in both cases. However, a possible (and likely?) explanation would be to guess that the parental alleles of some homologous genes are imprinted differently in the two F1s. This would explain all the observed phenotypes.

A similar observation has been made concerning the offspring of crosses made in zoological gardens between two species of the *Panthera* genus: *Panthera leo*, the African lion, and *Panthera tigris*, the Bengal tiger. The *liger*, a hybrid between a male lion and a tigress, is an enormous animal, with a total length reaching 3–3.5 m and a weight of up to 380 kg (~800 lb), while the reciprocal hybrid, the *tigon* (much less common), is slightly undersized compared to its parents. Here again, the explanations for these size differences are still somewhat speculative but, given that the imprinted genes often play a role in issues of hybrid growth, it is tempting to guess that this applies in the case of these two interspecific hybrids (Morison et al. 2001, 2005).

Finally, another interesting case is the *Callipyge* phenotype in sheep (abbr. *CPLG*—from the Greek "beautiful buttocks"). This mutation was first discovered in the USA segregating in a flock in Oklahoma. It causes lambs to develop large and muscular rumps, and for this important economical value it has been extensively studied by animal geneticists (Georges et al. 2013). It has then been demonstrated that the phenotype is fully expressed only in heterozygous individuals who receive the *CLPG* mutant allele from their father. When inherited from the mother, it is not expressed. This situation is known as *polar overdominance* and is another example of phenotypic alteration due to imprinting. The *CLPG* mutation is a single nucleotide substitution in what is probably a long-range control element (LRCE—see Chap. 5) within the *DLK1–GTL2* imprinted domain of several species of mammals. The mutation also exists in humans and in cattle, and has been created by genetic engineering in the mouse. It is a very interesting model for these sorts of phenotypic observations.

[13] Only some exceptional viable offspring have been bred from such a cross.

6.4 Conclusions

Initially discovered in the form of anecdotal observations (coat color of calico cats and the unexpected inheritance of the hairpin-tail mutation), X inactivation and genomic imprinting appear to be two important ways of regulating genomic expression. Diploidy, as we said, was generally considered as advantageous with regards to evolution because, having a backup copy for each and every gene, diploid organisms were more protected against the deleterious effects of mutations. After the discovery of genomic imprinting, this analysis must be seriously reconsidered. Indeed, if a gene mutates, the back-up (normal) copy of this gene may not be available for replacement if it is in an imprinted region and accordingly epigenetically inactivated. What then is the evolutionary advantage of imprinting for mammals? A close association has been established with viviparity, at least with the development of the embryo in utero, but this association by definition does not exist in flowering plants where the imprinting phenomenon has also been described. Nowadays, a theory is emerging suggesting that genomic imprinting might play an important role as a mechanism of reproductive isolation generating diversity. Many of these investigations are conducted in mammals (in particular, laboratory rodents), and it is likely that the evolutionary advantages of genomic imprinting will be established in the relatively near future.

Unraveling the intimate molecular mechanisms at work in the establishment and maintenance of imprinting might be laborious, but it is a very important issue and there is no doubt that, in this matter more than in any other, the mouse will be an invaluable model.

References

Augui S, Nora EP, Heard E (2011) Regulation of X-chromosome inactivation by the X-inactivation centre. Nat Rev Genet 12:429–442

Barlow DP, Stöger R, Herrmann BG, Saito K, Schweifer N (1991) The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the Tme locus. Nature 349:84–87

Barton SC, Surani MAH, Norris ML (1984) Role of paternal and maternal genomes in mouse development. Nature 311:374–376

Beutler E, Yeh M, Fairbanks VF (1962) The normal human female as a mosaic of X-chromosome activity: studies using the gene for G-6-PD deficiency as a marker. Proc Natl Acad Sci USA 48:9–16

Blewitt ME, Vickaryous NK, Paldi A, Koseki H, Whitelaw E (2006) Dynamic reprogramming of DNA methylation at an epigenetically sensitive allele in mice. PLoS Genet 2(4):e49

Brown SD (1991) XIST and the mapping of the X chromosome inactivation centre. BioEssays 13:607–612

Butler MG (2009) Genomic imprinting disorders in humans: a mini-review. J Assist Reprod Genet 26:477–486

Cattanach BM (1986) Parental origin effects in mice. J Embryol Exp Morphol 97(Suppl):137–150

Cattanach BM, Kirk M (1985) Differential activity of maternally and paternally derived chromosome regions in mice. Nature 315:496–498

Chen T, Dent SY (2014) Chromatin modifiers and remodellers: regulators of cellular differentiation. Nat Rev Genet 15:93–106
Constância M, Pickard B, Kelsey G, Reik W (1998) Imprinting mechanisms. Genome Res 8:881–900
Cropley JE, Dang TH, Martin DI, Suter CM (2012) The penetrance of an epigenetic trait in mice is progressively yet reversibly increased by selection and environment. Proc Biol Sci B 279:2347–2353
Davidson RG, Nitowsky HM, Childs B (1963) Demonstration of two populations of cells in the human female heterozygous for glucose-6-phosphate dehydrogenase variants. Proc Nat Acad Sci USA 50:481–485
DeChiara TM, Robertson EJ, Efstratiadis A (1991) Parental imprinting of the mouse insulin-like growth factor II gene. Cell 64:849–859
Dünzinger U, Nanda I, Schmid M, Haaf T, Zechner U (2005) Chicken orthologues of mammalian imprinted genes are clustered on macrochromosomes and replicate asynchronously. Trends Genet 21:488–492
Espinós C, Lorenzo JI, Casaña P, Martínez F, Aznar JA (2000) Haemophilia B in a female caused by skewed inactivation of the normal X-chromosome. Haematologica 85:1092–1095
Ferguson-Smith AC (2011) Genomic imprinting: the emergence of an epigenetic paradigm. Nat Rev Genet 12:565–575
Ferguson-Smith AC, Sasaki H, Cattanach BM, Surani MA (1993) Parental-origin-specific epigenetic modification of the mouse H19 gene. Nature 362:751–755
Gabory A, Ripoche MA, Le Digarcher A, Watrin F, Ziyyat A, Forné T, Jammes H, Ainscough JF, Surani MA, Journot L, Dandolo L (2009) H19 acts as a trans regulator of the imprinted gene network controlling growth in mice. Development 136:3413–3421
Garrick D, Sharpe JA, Arkell R, Dobbie L, Smith AJH et al (2006) Loss of *Atrx* affects trophoblast development and the pattern of X-inactivation in extraembryonic tissues. PLoS Genet 2(4):e58
Georges M, Charlier C, Cockett N (2013) The callipyge locus: evidence for the trans interaction of reciprocally imprinted genes. Trends Genetics (in press)
Haig D (1997) Parental antagonism, relatedness asymmetries, and genomic imprinting. Proc Roy Soc Lond Ser B-Biol Sci 264:1657–1662
Horsthemke B, Wagstaff J (2008) Mechanisms of imprinting of the Prader-Willi/Angelman region. Am J Med Genet A 146:2041–2052
Johnson DR (1974a) Further observations on the haipin-tail (T^{hp}) mutation in the mouse. Genet Res 24:207–213
Johnson DR (1974b) Hairpin-tail: a case of post-reductional gene action in the mouse egg. Genetics 76:795–805
Kawahara M, Kono T (2012) Roles of genes regulated by two paternally methylated imprinted regions on chromosomes 7 and 12 in mouse ontogeny. J Reprod Dev 58:175–179
Kawahara M, Wu Q, Takahashi N, Morita S, Yamada K, Ito M, Ferguson-Smith AC, Kono T (2007) High-frequency generation of viable mice from engineered bi-maternal embryos. Nat Biotechnol 25:1045–1050
Kelly WG, Schaner CE, Demburg AF, Lee MH, Kim SK, Villeneuve AM, Reinke V (2002) X-chromosome silencing in the germline of *C. elegans*. Development 129:479–492
Keverne EB (2013) Importance of the matriline for genomic imprinting, brain development and behaviour. Philos Trans R Soc Lond B Biol Sci 368:20110327. doi:10.1098/rstb.2011.0327
Kono T, Obata Y, Wu Q, Niwa K, Ono Y, Yamamoto Y, Park ES, Seo JS, Ogawa H (2004) Birth of parthenogenetic mice that can develop to adulthood. Nature 428:860–864
Larschan E, Bishop EP, Kharchenko PV, Core LJ, Lis JT, Park PJ, Kuroda MI (2011) X chromosome dosage compensation via enhanced transcriptional elongation in Drosophila. Nature 471:115–118
Latos PA, Pauler FM, Koerner MV, Şenergin HB, Hudson QJ, Stocsits RR, Allhoff W, Stricker SH, Klement RM, Warczok KE, Aumayr K, Pasierbek P, Barlow DP (2012) Airn transcriptional overlap, but not its lncRNA products, induces imprinted Igf2r silencing. Science 338:1469–1472

Li E, Beard C, Jaenisch R (1993) Role for DNA methylation in genomic imprinting. Nature 366:362–365
Lubinsky M, Herrmann J, Kosseff A, Opitz JM (1974) Autosomal-dominant sex-dependent transmission of the Beckwith-Wiedemann syndrome. Lancet 1:932
Lyon MF (1961) Gene action in the X-chromosome of the mouse (*Mus musculus L.*). Nature 190:372–373
Lyon MF (2002) A Personal History of the Mouse Genome. Annu Rev Genomics Hum Genet 3:1–16
Marahrens Y, Panning B, Dausman J, Strauss W, Jaenisch R (1997) Xist-deficient mice are defective in dosage compensation but not spermatogenesis. Genes Dev 11:156–166
McGrath J, Solter D (1984) Completion of mouse embryogenesis requires both the maternal and paternal genomes. Cell 37:179–183
Mohammad F, Pandey GK, Mondal T, Enroth S, Redrup L, Gyllensten U, Kanduri C (2012) Long noncoding RNA-mediated maintenance of DNA methylation and transcriptional gene silencing. Development 139:2792–2803
Moncla A, Malzac P, Livet MO, Voelckel MA, Mancini J, Delaroziere JC, Philip N, Mattei JF (1999) Angelman syndrome resulting from UBE3A mutations in 14 patients from eight families: clinical manifestations and genetic counseling. J Med Genet 36:554–560
Morey C, Avner P (2011) The demoiselle of X-inactivation: 50 years old and as trendy and mesmerising as ever. PLoS Genet 7:e1002212
Morgan HD, Sutherland HG, Martin DI, Whitelaw E (1999) Epigenetic inheritance at the agouti locus in the mouse. Nat Genet 23:314–318
Morison IM, Paton CJ, Cleverley SD (2001) The imprinted gene and parent-of-origin effect database. Nucleic Acids Res 29:275–276
Morison IM, Ramsay JP, Spencer HG (2005) A census of mammalian imprinting. Trends Genet 21:457–465
Nowack MK, Shirzadi R, Dissmeyer N, Dolf A, Endl E, Grini PE, Schnittger A (2007) By-passing genomic imprinting allows seed development. Nature 447:312–315
Okamoto I, Otte AP, Allis CD, Reinberg D, Heard E (2004) Epigenetic dynamics of imprinted X inactivation during early mouse development. Science 303:644–649
Patrat C, Okamoto I, Diabangouaya P, Vialon V, Le Baccon P, Chow J, Heard E (2009) Dynamic changes in paternal X-chromosome activity during imprinted X-chromosome inactivation in mice. Proc Natl Acad Sci USA 106:5198–5203
Penny GD, Kay GF, Sheardown SA, Rastan S, Brockdorff N (1996) Requirement for Xist in X chromosome inactivation. Nature 379:131–137
Pollex T, Heard E (2012) Recent advances in X-chromosome inactivation research. Curr Opin Cell Biol. http://dx.doi.org/10.1016/j.ceb.2012.10.007
Proudhon C, Duffié R, Ajjan S, Cowley M, Iranzo J, Carbajosa G, Saadeh H, Holland ML, Oakey RJ, Rakyan VK, Schulz R, Bourc'his D (2012) Protection against de novo methylation is instrumental in maintaining parent-of-origin methylation inherited from the gametes. Mol Cell 47:909–920
Reik W, Dean W, Walter J (2001) Epigenetic reprogramming in mammalian development. Science 293:1089–1093
Reik W, Walter J (2001) Evolution of imprinting mechanisms: the battle of the sexes begins in the zygote. Nat Genet 27:255–256
Saxena A, Carninci P (2011) Whole transcriptome analysis: what are we still missing? Wiley Interdiscip Rev Syst Biol Med 3:527–543
Sha K (2008) A mechanistic view of genomic imprinting. Annu Rev Genomics Hum Genet 9:197–216
Simmler MC, Cattanach BM, Rasberry C, Rougeulle C, Avner P (1993) Mapping the murine Xce locus with $(CA)_n$ repeats. Mamm Genome 4:523–530
Surani MAH, Barton SC, Norris ML (1984) Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. Nature 308:548–550

Thorvaldsen JL, Krapp C, Willard HF, Bartolomei MS (2012) Nonrandom X chromosome inactivation is influenced by multiple regions on the murine x chromosome. Genetics 192:1095–1107

Trent S, Dennehy A, Richardson H, Ojarikre OA, Burgoyne PS, Humby T, Davies W (2011) Steroid sulfatase-deficient mice exhibit endophenotypes relevant to attention deficit hyperactivity disorder. Psychoneuroendocrinology 37:221–229

Vrana PB (2007) Genomic imprinting as a mechanism of reproductive isolation in mammals. J Mammal 88:5–23

Weidman JR, Dolinoy DC, Maloney KA, Cheng JF, Jirtle RL (2006) Imprinting of opossum *Igf2r* in the absence of differential methylation and Air. Epigenetics 1:49–54

Winking H, Silver LM (1984) Characterization of a recombinant mouse T haplotype that expresses a dominant lethal maternal effect. Genetics 108:1013–1020

Wood AJ, Oakey RJ (2006) Genomic imprinting in mammals: emerging themes and established theories. PLoS Genet 2:e147

Wutz A, Theussl HC, Dausman J, Jaenisch R, Barlow DP, Wagner EF (2001) Non-imprinted *Igf2r* expression decreases growth and rescues the *Tme* mutation in mice. Development 128:1881–1887

Yu M, Hon GC, Szulwach KE, Song CX, Zhang L, Kim A, Li X, Dai Q, Shen Y, Park B, Min JH, Jin P, Ren B, He C (2012) Base-resolution analysis of 5-hydroxymethylcytosine in the mammalian genome. Cell 149:1368–1380