CLONING FLIES:
NUCLEAR TRANSPLANTATION IN *Drosophila melanogaster*

by

Andrew J. Haigh

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
October 2005

Your file Votre référence
ISBN: 978-0-494-16714-4
Our file Notre référence
ISBN: 978-0-494-16714-4

DALHOUSIE UNIVERSITY

To comply with the Canadian Privacy Act the National Library of Canada has requested that the following pages be removed from this copy of the thesis:

Preliminary Pages

Examiners Signature Page (pii)

Dalhousie Library Copyright Agreement (piii)

Appendices

Copyright Releases (if applicable)

Table of Contents:

List of Figures:

List of Tables:

Abstract:

Nuclear transfer involves the transplantation of one or more nuclei from a donor cell to a functionally enucleated recipient embryo, creating a genetically identical cloned organism. Embryonic and somatic nuclear transplantation have been successful to varying degrees in amphibians, arthropods, and mammals. Since the early sixties, numerous attempts have been made to produce viable *Drosophila* by embryonic nuclear transplantation, though none have survived through the larval stages. I have successfully used embryonic nuclear transfer to create viable adult *Drosophila* clones. Embryonic H2A-GFP tagged donor nuclei were transplanted into fertilized functionally enucleated embryos. Nuclear transplant success rates are comparable to those observed in mammals. I extend the work to show that genomic imprinting associated with a mini-X chromosome is lost in *Drosophila melanogaster* clones. These individuals represent the first cloned adult *Drosophila*, which also, to our knowledge, constitute the first cloned insects. Also, I illustrate that the *Drosophila* White and Garnet proteins colocalize at the endosome membrane.

List of Abbreviations:

ICR	Imprint Control Region
SWI/SNF	Switch / Sucrose Non-Fermenter (yeast)
HDAC	Histone Deacetylase
DNMTase	DNA Methyltransferase
H	Histone
Lys or K	Lysine
LOS	Large Offspring Syndrome
CpG	Cystine methylation
CTCF	CCCTC Binding Factor
δ	delta
Su(var)	*Suppressor of variegation*
trx	*trithorax*
ms(3)K81	*male sterile (3) K81*
Igf2	*Insulin-like growth factor 2*
H19	*H19*
Igf2r	*Insulin-like growth factor 2 receptor*
Ins-2	*Insulin 2*
Dp(1:f)LJ9	*Duplication (Chromosome 1, free) La Jolla 9*
GFP	*Green Florescent Protein*
DsRed	*Discosoma sp. Red*
dp	*dumpy*
e	*eagle*
ci	*cinnabar*
brm	*brahma*
l(2)gl	*lethal (2) giant larvae*
Kr	*Kruppel*

Gene	*Drosophila*	Mammal
Su(var)205	*Su(var)205 / HP1*	*M31 / M32 / HP1^{Hsα}*
Su(var)3-9	*Su(var)3-9*	*Suv39h1 / Suv39h2*
HDAC	*HDAC1 / HDAC X*	*hHDAC1 / hHDAC2 / hHDAC3*
w	*white*	*ABCG1 (ABC8)*
g	*garnet*	*δ-AP3*
syt	*synaptotagmin*	*synaptotagmin*

Acknowledgements:

I thank Dr. Vett Lloyd, Dr. Ian Meinertzhagen, Dr. Tom MacRae, Dr. Thomas Grigliatti, Dr. Michael Hart, Dr. Robert Latta, Dr. Susan Mackenzie, Christine Beauchamp, Patricia Collins, William MacDonald, Lori MacEachern, Matt Anaka, Eva Barkova, Greg Handrigan, Adam MacNeil, Tara Edwards, Jennifer Warford, and my family.

I acknowledge the *Drosophila* stock center in Bloomington, Indiana for the fly strains used in this study.

Epigraph:

I am writing a novel about the future – on the horror of the Wellsian Utopia and a revolt against it. Very difficult. I have hardly enough imagination to deal with such a subject. But it is non the less interesting work.

Yours,

Aldous Huxley

(Excerpt from a letter written to Mrs. Kethevan Roberts, 18 May, 1931)

1. Introduction

1.1 1895 – 1966

There is little doubt that Huxley can be credited with introducing the concept of reproductive cloning to public consciousness. However, when *Brave New World* was first published in 1932, the science that it predicted was still in its absolute infancy and the term "clone" did not enter the scientific lexicon until approximately 30 years later. J.B.S. Haldane, a friend and contemporary of Huxley and considered one of the fathers of population genetics, mused on the "production of a clone from cells of persons of attested ability," yet warned, "On the general principal that men will make all possible mistakes, we shall no doubt clone the wrong people" (Haldane 1963).

The scientific foundation that led to the startling advances in nuclear transplant technology over the past ten years is widely credited to German embryologist and Nobel Prize winner Hans Spemann. In 1902, he used a hair from the head of his newborn son to split a salamander embryo to generate two identical offspring. This finding contested August Weismann's earlier assertion that the hereditary information contained within a cell decreases with each subsequent cell division (Weismann 1889). In 1938, Spemann published *Embryonic Development and Induction*, in which he describes nuclear transfer experiments conducted a decade earlier and first proposed his "somewhat fantastical" experiment of transplanting the nucleus of a differentiated cell to an enucleated egg – the theoretical foundation of modern cloning technology (Spemann 1938). However, upon closer examination of early experimental embryological texts, Beetschen and Fischer (2004) conclude that a French biologist, Yves Delage (1854-1920), conceived the same nuclear transfer experiment some 41 years prior to Spemann's seminal publication

(Spemann's text was originally published in German in 1936). Like Spemann, Delage also disagreed with Weismann's theory that hereditary information decreased with subsequent cell divisions, and in 1895 he wrote, "Every nucleus, at least at the beginning of ontogenesis, is a sex nucleus and if, without any deterioration, the egg nucleus could be replaced by the nucleus of an ordinary embryonic cell, we should probably see this egg developing without changes" (Delage 1895, translated in Beetschen and Fischer 2004).

It was not until 1952 that Briggs and King (1952), studying the developmental constraints imposed on nuclei through progressive differentiation, generated the first cloned organisms via nuclear transplantation. They reasoned that in order to test the totipotency of a differentiated nucleus they could transplant it into an enucleated embryo and assess development. In order for this experiment to be successful, however, they first needed to demonstrate that viable embryos could develop from transplantation of an undifferentiated nucleus transplanted into an enucleated oocyte. They were able to generate *Rana pipiens* tadpoles from donor nuclei isolated from late blastula-stage cells (Briggs and King 1952). Following on this work, John Gurdon cloned ten *Xenopus laevis* tadpoles from the differentiated intestinal epithelium of feeding tadpoles (Gurdon 1962). He observed that cloning success was inversely proportional to the developmental age of the transplanted nucleus, a finding that has been documented many times since (Paterson and Wilmut 2002). In the following four years Gurdon would generate a number of adult cloned frogs, some of which were fertile, from tadpole intestinal epithelium, illustrating that the genes required to support development to adulthood were not lost on cellular differentiation (Gurdon and Uehlinger 1966). However, this conclusion would be

questioned based on the presence of less differentiated stem cell populations within the intestinal epithelium from which the viable offspring may have been inadvertently generated (Hochedlinger and Jaenisch 2002).

1.2 First attempts at invertebrate cloning

Procedures for transplantation of insect cleavage nuclei from one embryo to another were pioneered by E.J. DuPraw (DuPraw 1967). He would transplant nuclei from cleavage stage honeybee embryos into the anterior end of newly fertilized embryos, and then place a small ligature behind the site of nuclear injection. The purpose of this was two-fold – it would artificially heal the wound and it effectively isolated the recipient's genetic material, which resides at the anterior end, from the rest of the embryo. While the procedure appeared to be effective, and a small proportion of the injected embryos hatched as larvae, subsequent work could not confirm that the resulting embryos had been effectively enucleated by "tying off" the anterior end as recipient nuclei may have been able to pass through the ligature (DuPraw 1967).

In Munich, early in his career, Karl Illmensee carried out the first nuclear transplant experiments in *Drosophila*, generating a small number of larvae via transplant of nuclei from fertilized embryos into unfertilized eggs or into eggs fertilized with irradiated sperm (Illmensee 1968, Illmensee 1972). While he was unable to generate adult flies (the most advanced developed only to the third instar larval stage) he was able to demonstrate that transplanted nuclei were able to direct development of imaginal cuticular structures (Illmensee 1972, for review see Illmensee 1976) and thus concluded that the developmental potential of blastoderm stage nuclei is unrestricted. His work with

cytoplasmic transplantation would prove instrumental in elucidating the mechanisms of segmentation and pattern formation in *Drosophila*. However, he will likely be better remembered for his subsequent claims regarding mammalian cloning.

1.3 Karl Illmensee

Few in the contemporary history of biology have been as mired in public revelry and scientific controversy as Illmensee. The scandal that unfurled around him in the early 1980s played out on the pages of publications such as Cell, Nature, New Scientist, and The New York Times, leaving a notable blemish on his career and reputation. In *Clone: the Road to Dolly and the Path Ahead* Gina Kolata argues, quite convincingly, that Illmensee's actions at the time would push cloning research to the fringes of science for more than a decade and destroy a number of careers in their wake.

In 1981, he made headlines around the world after publishing a paper documenting the first successful cloning of three mice via nuclear transplant (Illmensee and Hoppe 1981). His technique involved the collection of blastocysts from donor mice, isolating the inner cell masses, followed by their subsequent dissociation into single cells. He then injected the nuclei of these single cells into newly fertilized zygotes, and with the same needle removed both the recipients' pronuclei. The resulting embryos were cultured *in vitro*, followed by implantation into foster uteri, where the mice were carried to term (Illmensee and Hoppe 1981).

Illmensee's announcement was well received by the scientific community, and his laboratory at the University of Geneva attracted graduate students, post-doctoral fellows, and research associates eager to learn his nuclear transplantation technique. However, he

apparently repeatedly avoided what should have been a routine demonstration, thus raising initial suspicions that the work had been fabricated. Illmensee's seemingly erratic behavior was monitored for some time, and after careful consideration he was confronted by his lab workers, eventually leading to a full scale investigation of his procedures (Kolata 1998). In 1983, Illmensee eventually conceded "having falsified ('faked') protocols including experiments that had not been carried out," and he would pull a sentence from a National Institutes of Health grant application claiming he had cloned mice from tumour cell nuclei (Budiansky 1984, Kolata 1998).

Among the most prominent and vociferous scientists who doubted the validity of Illmensee's claims was Davor Solter. Despite numerous attempts to repeat Illmensee's work, he and post-doctoral student James McGrath had been unable to recreate the mouse cloning experiments, and Illmensee repeatedly avoided demonstrating the technique to the two. In subsequent experiments, McGrath and Solter (1984) attempted to recreate the work of Illmensee and Hoppe (1981) by cloning mice through isolating nuclei from one-, two-, four-, and eight-cell embryos and inner cell mass stage cells and injecting them into fertilized zygotes. They found that while nuclei from one- or two-cell stage embryos would support development to blastocyst stage, inner cell mass nuclei would not, much less support development through adulthood, leading them to suggest "that the cloning of mammals by simple nuclear transfer is biologically impossible" (McGrath and Solter 1984). While their conclusion may have been drawn prematurely, to date nobody has successfully generated live offspring using Illmensee's cloning method.

1.4 Mammalian twinning and embryonic cloning

The events that would eventually lead to the first successful nuclear transplantation in mammals stemmed from a different series of experiments, however they were also directed at determining the developmental capacity of each cell in an early mammalian cleavage embryo (for review see Wilson and Stern 1975). Blastomeres or groups of blastomeres at various cell stages were isolated and implanted back in to the uterus of the host in order to evaluate the developmental potential of the cells. While varying degrees of success were reported in rats (Nicholas and Hall 1942), rabbits (Seidel 1952), and mice (Tarkowski and Wroblewska 1967), it was not until 1979 that sheep embryos could be effectively manipulated to produce twinned offspring from individual blastomeres (Willadsen 1979). By isolating blastomeres from two-celled cleavage stage sheep embryos, Willadsen was able to generate monozygotic twins by introducing each blastomere into its own empty zona pellucida, followed by subsequent encapsulation in agar. The reconstructed embryos were then cultured in the sheep oviduct for several days, at which point they were retrieved for examination, and those deemed to be developing normally were extracted from the agar and transferred to uteri for gestation to generate monozygotic twins. Three such sets of twins were produced by splitting two celled embryos (Willadsen 1979).

In order to investigate the developmental capacity of 4- and 8-cell stage embryos Willadsen went on to either split the respective embryos in two or break them down into their individual blastomeres prior to reconstruction and embedding. He found that there were no differences with respect to developmental capacity between single blastomeres derived from 2-cell stage embryos, paired blastomeres from 4-cell stage embryos, or quartets of blastomeres from 8-cell stage embryos. From each half-embryo he was able to

generate live lambs (often twinned) at approximately the same rate (Willadsen 1980). In contrast, while he was able to generate live offspring from single blastomeres from 4- and 8-cell embryos, those derived from 8-cell embryos showed reduced viability (Willadsen 1981).

In order to further investigate the developmental capacity of blastomeres at the 8- and 16-cell stage, Willadsen successfully pioneered a cloning technique that ultimately resulted in the birth of viable sheep generated by nuclear transplantation. He isolated cells of 8- and 16-cell stage embryos and combined them with enucleated unfertilized eggs, followed by embedding in agar and embryo culture as before (Willadsen 1986). Cell fusion was either Sendai virus-mediated, similar to the technique previously attempted by McGrath and Solter (1984), or accomplished via electrofusion. Through this method Willadsen (1986) was able to generate apparently normal foetuses from 16-cell blastomeres, and the first live mammalian offspring via embryonic nuclear transplant from 8-cell blastomeres.

1.5 Beyond the mid-blastula transition

Zygotic genome activation in cattle and sheep commences at the 8- to 16-cell stage (Campbell 1999), and so it could not be definitively concluded that nuclear reprogramming was necessary to support development when initially cloning sheep.

Working almost simultaneously with Willadsen, in 1987, the First laboratory announced the generation of cloned cattle via embryonic nuclear transplantation (Robl *et al.* 1987). They initially used a method similar to that of Willadsen, and went on to confirm the totipotency of 9- to 15-cell stage blastomere nuclei (Prather *et al.* 1987).

Later, cloned cattle would be generated from both 48- and 64-cell stage blastomeres, thus moving closer to generating cloned offspring from differentiated cells (Bondioli *et al.* 1990).

Two years later, Smith and Wilmut (1989) derived cloned lambs from 16-cell and inner cell mass (152-cell) stage nuclei, illustrating that cloned offspring could be derived from zygotically active nuclei. They went on to conclude that "stem cells would provide an ideal source of nuclei for transfer, and nuclear transfer would be the most efficient means of producing nonchimeric offspring from transformed embryonic stem cells" (Smith and Wilmut 1989). Five years later, Sims and First (1994) derived cloned calves from inner cell mass derived cell cultures. Embryonic stem cells from cultures would be fused to enucleated oocytes, and through this method four apparently normal calves were born (Sims and First 1994). In each case the stem cell culture from which each calf was derived was less than 28 days old, suggesting that the totipotency of the stem cell lines was limited by each successive passage (Sims and First 1994). The subsequent advance occurred when Campbell and colleagues (1996) cloned sheep from embryonic cell cultures displaying the morphology and genetic signature of differentiated epithelial cell lines, illustrating that cloned mammals could be generated from early stage differentiated cell nuclei. This result raised the prospect of altering the genetic makeup of cells in culture prior to nuclear transplantation, thus facilitating the generation of genetically modified livestock (Solter 1996). The most profound demonstration of the totipotency of mammalian cell somatic nuclei would follow shortly thereafter.

In 1996 Wilmut and colleagues cloned a sheep from a cell population established from the mammary gland of an adult, and thus definitively illustrated the totipotency of

somatic cells (Wilmut *et al.* 1997). The groundbreaking paper was both the result of over 100 years of theoretical speculation into the inherent potential of a cell's genetic material and the consequence of a series of logical progressions in nuclear transplantation research. The birth of Dolly (so named for the "impressive mammaries" from which she was derived) would propel cloning from Huxley's dystopian fantasy to the forefront of public consciousness, and in its wake spark heated ethical debate on the very nature of individuality. The work further realized the potential of therapeutic cloning, the derivation of embryonic tissue cultures customized to be an identical genetic match for the nuclear donor, which could radically alter the practice of medicine.

1.6 Subsequent advances in nuclear transplantation

In the years prior to the advent of somatic cell cloning, successful nuclear transplant techniques similar to those developed by Willadsen (1986) and First (Prather *et al.* 1987) have been used in mice (Tsunoda *et al.* 1987) and rabbits (Collas and Robl 1990). In the years since, clones derived from embryonic or somatic nuclei have been reported in rhesus monkeys (Meng *et al.* 1997), mice (Wakayama *et al.* 1998), cows (Cibelli *et al.* 1998), goats (Baguisi *et al.* 1999), pigs (Polejaeva *et al.* 2000), rabbits (Chesne *et al.* 2002), cats (Shin *et al.* 2002), zebrafish (Lee *et al.* 2002), mules (Woods *et al.* 2003), horses (Galli *et al.* 2003), rats (Zhou *et al.* 2003), fruit flies (Haigh *et al.* 2005), and dogs (Lee *et al.* 2005). In each case the methods vary somewhat, but the underlying procedures share many common fundamentals, and many of the cloned offspring share the same subtle genetic abnormalities.

Mice enter the mid-blastula transition, and therefore commence zygotic gene expression, earlier in development relative to sheep and cows (Campbell 1999). As a result, a technique for the consistent generation of cloned laboratory mice was developed some time after nuclear transplant techniques had been successfully established in sheep and cows. As the primary mammalian model organism, cloning the laboratory mouse was considered an important step towards developing and testing stem cell therapies prior to their introduction to human patients. In 1997, mice developed from somatic (cumulus) cell nuclei transferred to enucleated oocytes were born, further illustrating that differentiated adult cells could support clonal development (Wakayama *et al.* 1998). Wakayama and Yanagimachi (1999a) would go on to report the development of cloned males derived from tail-tip cells, late-passage embryonic stem cell lines (Wakayama *et al.* 1999), and would successively clone six generations of mice, each generation cloned from the previous (Wakayama *et al.* 2000a). Eggan and colleagues (2004) generated viable mouse clones from olfactory sense neurons, demonstrating that clones can be derived from terminally differentiated nuclei. In each of the above cases the respective nuclei were transferred into enucleated oocytes, allowing for the generation of live offspring.

1.7 Illmensee revisited

Tsunoda and Kato's (1998) cloning of mice from inner cell mass nuclei prompted a call from Illmensee for a re-examination of the rejection of his earlier work. He claimed that "with these positive data on the cloning of mice, the time has come for the correct evaluation of our earlier results on the first cloning of a mammal" (Illmensee 1999).

However, Tsunoda and Kato transplanted nuclei into enucleated oocytes rather than enucleated zygotes as Illmensee and Hoppe (1981) had done. This prompted Solter's response that "one could equally well argue that the success of the Apollo missions confirms Jules Verne's or Cyrano de Bergerac's descriptions of voyages to the Moon" (Solter 1999). Wakayama and colleagues (2000b), in reviewing these experiments, concluded that "zygotes are inappropriate nucleus recipients for the cloning of mice," concurring with Solter that "there is no firm reason for *Hineininterpretierung* of the claim of mouse cloning using zygotes." As such, Illmensee's claim to have cloned the first mammal is still widely questioned.

The fallout over his disputed claim to have cloned mice would cause many colleagues and co-workers to be suspicious of Illmensee's earlier work. In addition to his work with *Drosophila*, he claimed to have created both anderogenetic and parthenogenetic mice, and that he had created mosaic mice from embryonic cells mixed with cancer cells (Hoppe and Illmensee 1977, Illmensee *et al.* 1978). The following work, documenting the first successful generation of adult *Drosophila* clones, demonstrates that blastoderm stage nuclei remain totipotent. This supports Illmensee's earlier assertion that the developmental potential of blastoderm stage nuclei is not limited in *Drosophila* (Illmensee 1972).

1.8 Cloning fruit flies

In October of 2004, it was announced that we had successfully generated the first adult fruit fly clones, which also, to our knowledge, constituted the first cloned insect (Haigh *et al.* 2005). The clones were derived from blastula stage nuclei injected into

fertilized yet genetically sterile eggs. Thus, the work more closely parallels the mammalian cloning technique employed by Willadsen (1986) rather than Wilmut and colleagues (1997). The following section clearly outlines the procedures employed to produce cloned *Drosophila*, followed by the original report published in *Genetics*. I then go on to examine the genomic imprint in cloned flies using a well-established assay system. In light of these promising results, I discuss *Drosophila* as a model organism for cloning research. The following chapter documents the localization of the *Drosophila white* protein – work that relates to the cloning research in technique rather than theory, and as such will be considered separately. Finally, the cloning work presented will be critically evaluated and future directions of this work will be considered. This study will conclude with a concise summation of the major findings presented herein.

2.1 Linking Paragraph

The following describes the method by which I cloned *Drosophila melanogaster*. Certain aspects of the process, such as the nuclear donor or recipient strains used, may vary, and are considered in later chapters. I found that deviations in environmental factors such as ambient room temperature or the grade of oil used can adversely affect cloning success, and therefore the guidelines that follow should be strictly adhered to.

"I simply have qualities and abilities which I've picked up over the years that not every scientist has."

Karl Illmensee, as quoted in
Bild der Wissenschaft August 1983

At Dalhousie University in Halifax, Canada, biologists have succeeded in cloning insects for the first time. Researchers (in the laboratory) of Vett Lloyd transferred nuclei from fruit fly embryos into fertilized eggs. Over 800 cloning trails resulting in only five adult animals.

Bild der Wissenschaft February 2005

2. *Methods*

2.3 Fly stocks

All *Drosophila* strains were maintained at room temperature (22°C +/- 3°C). In some instances stocks were incubated at 18°C in order to slow development. Flies were raised on an agarose, molasses, and cornmeal medium supplemented with 10% tegosept in order to inhibit mould formation (Appendix, http://fly.bio.indiana.edu/molasses-food.htm).

Virgin females were mated to males expressing a male-sterile mutation in order to generate fertilized yet inviable recipient embryos. Various stocks were used to generate donor embryos. Stocks and crosses used in each experiment are listed in their respective chapters.

2.4 Cloning

2.2.1 Preparation

- Nitex mesh wash baskets were constructed by removing the bottom 2/3 of a 50 ml Falcon tube and carving a large opening in the flask lid. A square piece of 10μm nitex mesh (Sefar America) was placed on the cut tube and the lid was secured (Figure 3.1a).
- Embryo glue was prepared by placing 20 cm of double-sided 3M Scotch tape in 20 ml of heptane in a 25 ml scintillation vial, followed shaking. This process was repeated 3 times. Slides were prepared by repeatedly (5-6 times) applying a 5mm widthwise strip of embryo glue across the slide with a paintbrush and allowing it to dry. Slides were stored in a slide box until use.

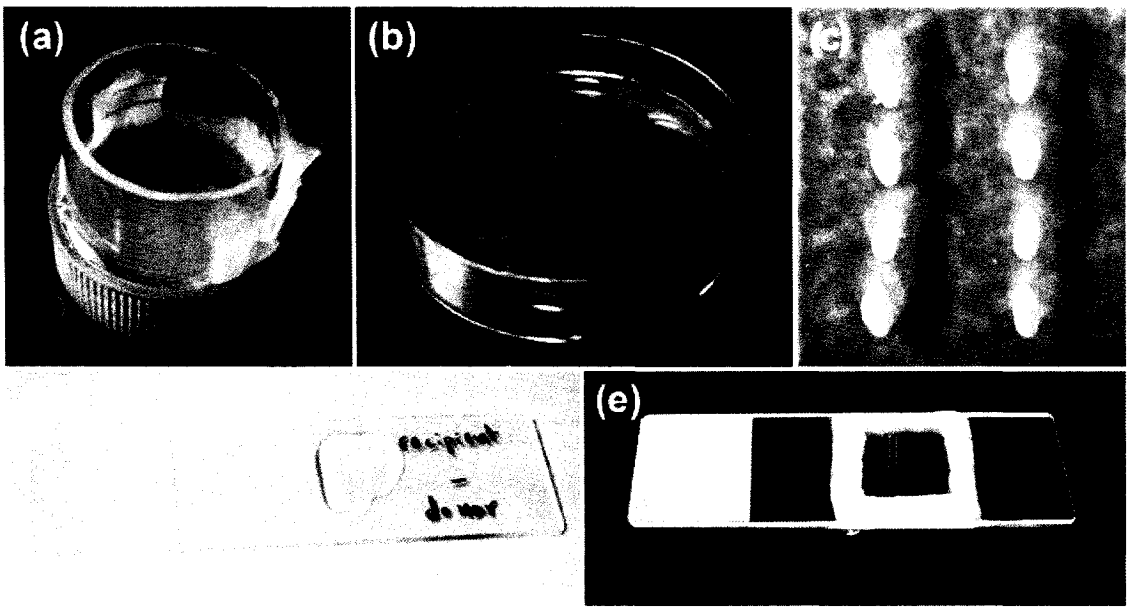

Figure 2.1: Cloning materials and methods. (a) Nitex wash basket. (b) Embryos arranged down the center of grape juice agar plate with (c) ventral faces opposed. (d) Recipient and donor embryos arranged on slide prior to injection, and (d) recipient embryos post-injection.

- Microinjection needles were prepared by pulling 10 cm length borosilicate glass capillaries (1.2 mm O.D., 0.90 mm I. D., cat # B120-90-10, Sutter Instrument Company) on a 700C David Kopf Instruments vertical pipette puller (graciously provided by Dr. Steve Shaw, Department of Psychology, Dalhousie University). Pulled needles were mounted on a Precision Micromanipulator (Fine Scientific Tools). The sealed tip of the needle was broken against the side of a standard coverslip mounted on a slide, such that the needle retained a sharp point but cytoplasm could still be effectively aspirated from the embryo.

2.2.2 Embryo collection

- Unless otherwise indicated, the following procedures were carried out at 22°C.
- Approximately 200 adult *Drosophila* from each of the donor and sterile recipient strains were placed in separate 250ml glass jars inverted over a grape juice agar plates (Appendix) streaked with yeast paste (Appendix). The flies were allowed to lay eggs continually for two hours until the donor strain plate was replaced. Eggs laid in the 30 minutes following were collected as donors for nuclear transplantation and aged for a further 30 minutes.
- Grape juice agar plates streaked with yeast paste were then replaced for both donor and recipient strains and flies were allowed to lay for 30 minutes.
- During the laying of recipient and donor eggs, aged donor embryos were dechorionated and prepared. Yeast paste and embryos were suspended in distilled and deionized water (ddH_2O via milliQ water filtration system) using a soft paintbrush and collected by filtering them through the Nitex wash baskets

(described in 2.2.1). Embryos were dechorionated by washing in 0.3% $NaHCO_3$ (50% bleach) for 50 seconds. $NaHCO_3$ solution was prepared fresh each day. Immediately following dechorionation, embryos were washed five times by transferring Nitex wash basket to different wells of a six-well culture plate and submerging the embryos in ddH_2O. Donor embryos were then transferred to a grape juice agar plate (Appendix) to prevent desiccation. A 22 mm^2 area was cut from the center of the agar to facilitate transfer of the embryos to the slide.

- After recipient and donor eggs were layed, the second batch of donor embryos was set aside to age. Recipient embryos were collected and dechorionated as above. Separate Nitex wash baskets, six-well culture plates, 0.3% $NaHCO_3$, and ddH_2O were used to prevent embryo contamination.
- Recipient embryos were transferred to a separate grape juice agar plate from which a 22 mm^2 area had been cut. Embryos were arranged "head to tail", ventral faces opposed, in two lines down the center of the square cut from the agar (Figure 3.1b, Figure 3.1c). Embryo manipulation was done with fine forceps and a dissecting microscope.
- Recipient embryos were transferred to a prepared microscope slide by removing the square block of agar with the aligned embryos from the Petri plate. The slide was then inverted and gently lowered onto the agar block such that the embryos were transferred to the upper half of the strip of embryo glue.
- Recipient embryos were desiccated by placing the slide, embryo side up, in a jar of drierite (W. A. Hammond Drierite Company Ltd) for approximately 6 min. Desiccation time was determined empirically.

- While recipient embryos were desiccated, donor embryos were aligned in a similar fashion.
- Following recipient embryo desiccation, donor embryos were transferred to the lower half of the strip of glue on the slide and the embryos were covered in a thin layer of Halocarbon 200 oil (Halocarbon Products Corporation). Both Halocarbon 27 or 700 grade oils are not appropriate for this work. Areas of recipient and donor embryos were labeled prior to nuclear transplantation (Figure 3.1d).
- In order to inject large numbers of embryos per day, donor and recipient flies were rotated on and off grape juice agar plates streaked with yeast paste at 30 minutes intervals such that every hour a fresh batch of recipient embryos coincided with donor embryos aged 60-90 minutes. During the intervals the flies were not on grape juice agar/yeast plates, the 250 ml glass jars were covered with empty Petri plates and sealed with Parafilm.

2.2.3 Nuclear transplantation

- Slides were mounted on a compound microscope (10X) for injection (Leitz Biomed).
- Approximately 80% of the donor embryo's cytoplasm (approximately 256-2048 nuclei) was aspirated into the needle and injected into 1-5 "sterile" recipient embryos. In some cases, transplanted nuclei and cytoplasm leaked from recipient embryos so that only half of the volume transplanted was contained within the embryo. Therefore, approximately 25-250 donor nuclei were transplanted to each recipient. Any excess cytoplasm was aspirated from the puncture site.

- Following transplantation, all donor embryos were removed from the slide using fine forceps under a dissecting microscope in order to prevent contamination.

2.2.4 Embryo culture

- A 3–4 mm high layer of Vaseline was placed around the injected embryos (Figure 3.1e). They were then covered with a 2–3 mm layer of Halocarbon 200 oil and incubated in a sealed, moist container for 36-48 hours at 18^0C.
- As larvae hatched, they were picked from the slide using a metal probe and placed in an 8 dram Schnell vial containing standard cornmeal-molasses *Drosophila* medium and raised at room temperature until they hatched as adults or died.

2.5 Experimental manipulation

- Adults and embryos were subjected to experimental analysis to confirm they were in fact clones and to assess the status of the genomic imprint as described in the following chapters.

3.1 Linking Paragraph

An advance online copy of the following chapter was made available to the public in October of 2004, and it was published as a note in Genetics in February of 2005. The announcement that we had generated the first fruit fly, and to our knowledge, the first insect, via nuclear transplantation, was well received by the media and the scientific community. The work leading up to this announcement took place during the summer and autumn of 2003.

[Date]

[Name of Publication]
[Address of Publication]

I am preparing my [degree type] thesis for submission to the Faculty of Graduate Studies at Dalhousie University, Halifax, Nova Scotia, Canada. I am seeking your permission to include a manuscript version of the following paper(s) as a chapter in the thesis:

[List title of paper, authors, journal or book, volume number, page numbers, year of publication.]

Canadian graduate theses are reproduced by the Library and Archives of Canada (formerly National Library of Canada) through a non-exclusive, world-wide license to reproduce, loan, distribute, or sell theses. I am also seeking your permission for the material described above to be reproduced and distributed by the LAC(NLC). Further details about the LAC(NLC) thesis program are available on the LAC(NLC) website (www.nlc-bnc.ca).

Full publication details and a copy of this permission letter will be included in the thesis.

Yours sincerely,

[Name]

Permission is granted for:

a) the inclusion of the material described above in your thesis.

b) for the material described above to be included in the copy of your thesis that is sent to the Library and Archives of Canada (formerly National Library of Canada) for reproduction and distribution.

Name: _____ Title: _____

Signature: _____ Date: _____

The Generation of Cloned *Drosophila melanogaster*

Andrew J. Haigh, William A. MacDonald and Vett K. Lloyd

Department of Biology, Dalhousie University, Halifax, Nova Scotia B4H 4J1, Canada

Manuscript received August 18, 2004
Accepted for publication October 25, 2004

3.4 *Abstract*

We report here the first successful use of embryonic nuclear transfer to create viable adult *Drosophila melanogaster* clones. Given the generation time, cost effectiveness, and relative ease of embryonic nuclear transplant in *Drosophila*, this method can provide an opportunity to further study the constraints on development imposed by transplanting determined or differentiated nuclei.

3.5 *Note*

Cloning at the organismal level refers to the creation of a genetically identical individual from an existing individual, generally through nuclear transfer. Embryonic and somatic nuclear transplantation has been successful to varying degrees in amphibians (Gurdon and Uehlinger 1966), arthropods (Illmensee 1968), fish (Lee *et al.*. 2002), and mammals (Wilmut *et al.* 2002). This technology can be exploited to create stem cells for use in therapeutic cloning and is being used to increase the production of transgenic mammals producing pharmacologically important compounds. In many cases, the technology is constrained by a lack of fundamental understanding of the nuclear reprogramming events that occur following transplantation, resulting in a high frequency of developmental defects in the cloned offspring (Shi *et al.* 2003). We have successfully used embryonic nuclear transfer to create viable adult *Drosophila* clones. Embryos that hatch but fail to develop to adulthood exhibit characteristic developmental defects; hence we can potentially use this system to identify gene mutations or conditions that encourage complete nuclear reprogramming. The developmental programming of nuclei is a fundamental epigenetic process based, in part, on histone modification and packaging so

the events involved in nuclear reprogramming in *Drosophila* are likely conserved across taxa. The method outlined herein provides a straightforward, cost-effective means of studying the effects of epigenetic interactions on nuclear transplants.

Host embryos laid by white-eyed w^{1118} females were fertilized by homozygous *ms(3)K81* males. These males generate sperm incapable of participating in pronuclear fusion and thus the resulting embryos are unable to complete embryogenesis under control of their own DNA (Yasuda *et al.* 1995). Embyros donating nuclei possessed green-fluorescent-protein-labeled histone 2AvD (H2AvDGFP; Clarkson and Saint 1999) so donor nuclei were easily distinguishable from those of the host. Less than 2 µl of cytoplasm was aspirated from preblastoderm stage embryos 70–100 min after egg laying. Nuclei were drawn laterally from the ventral face of the embryo and 5–15 nuclei were transplanted to the ventral area of a 10- to 30-min-old recipient embryo. Nuclei drawn from a single donor embryo were injected into one to six recipients, potentially allowing for the generation of more than one clone from a single donor embryo. In the trial reported here, two of the five adult clones, both females, originated from adjacent embryos, suggesting that they may have been derived from one donor. Recipient embryos were incubated at 18°C until the completion of embryogenesis at which point larvae were raised on standard *Drosophila* culture medium.

Of the 820 w^{1118} host embryos into which *H2AvDGFP* nuclei were injected, 61 (7.4%) expressed *H2AvDGFP* from the donor nuclei, 14 (1.7%) of those hatched as larvae, and 5 (0.6%) eclosed as fertile adults expressing fluorescence from the

H2AvDGFP marker transgene (Figure 1). These individuals represent the first cloned adult *Drosophila*.

Evidence that these individuals represent animals derived from the injected embryonic nuclei stems from analysis of the mitochondrial and nuclear DNA of the clones. The *Drosophila* mitochondrial genome is highly variable in size (Lewis *et al.* 1994), and the length of the A + T-rich region differs between the w^{1118} and *H2AvDGFP* strains. This difference was detectable using PCR. Cloned animals possess nuclear DNA derived from the donor embryo but mitochondrial DNA from the host egg. Reciprocal transplantations (w^{1118} nuclei injected into *H2AvDGFP* host embryos) exhibited a reciprocal pattern of nuclear and mitochondrial DNA (Figure 2). DNA analysis of adult clones derived from *H2AvDGFP* donor nuclei yielded identical results.

The failure of the majority (98.3%) of the cloned embryos to develop normally is likely a consequence of multiple factors. To determine the percentage of embryos rendered inviable from mechanical damage intrinsic to the nuclear transplant procedure, viable w^{1118} nuclei were injected into diploid w^{1118} host embryos. Of 202 embryos injected, 21.8% (44) hatched, compared with 1.7% of cloned embryos, suggesting that ~ 80% of transplant-recipient embryos die from mechanical damage. Of the remaining 20%, in some cases nonuniform concentrations of GFP expression suggest the failure of donor nuclei to replicate and/or distribute themselves throughout the embryo. Characteristic defects in those expressing GFP, which die shortly after hatching, such as the absence of mouth hooks, defects in the tracheal system, and disorganized or absent

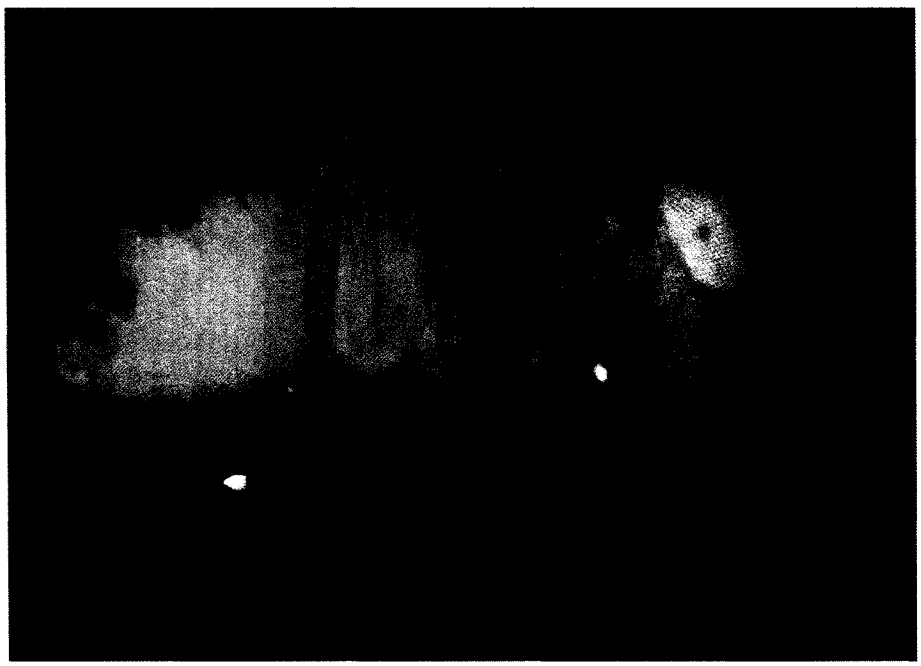

Figure 3.1: *Drosophila* adult derived from embryonic nuclear transplant expressing *H2AvDGFP*.

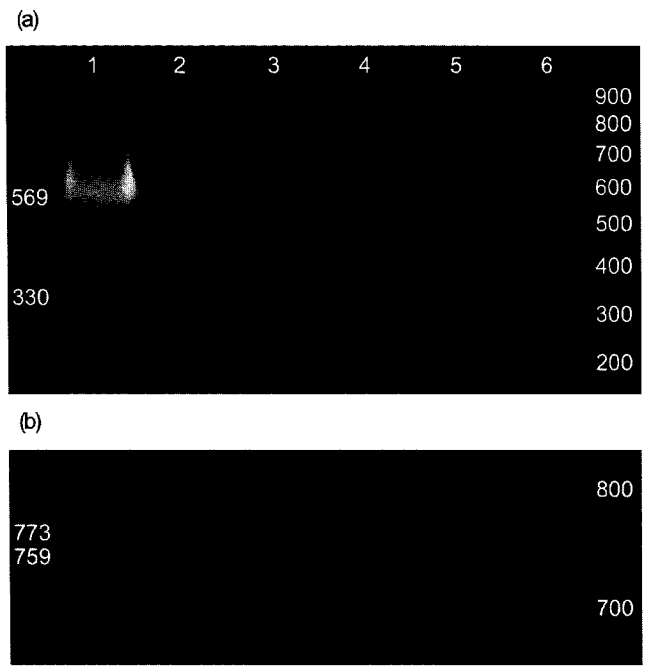

Figure 3.2: PCR analysis of nuclear and mitochondrial DNA from cloned *Drosophila*. (a) Analysis of nuclear DNA: primers 5'-ACTGTTTATTGCCCCCTC-3' and 5'-GTCGTCGAACAAAAGGTG-3' amplified a 330-bp fragment of *white* exon 6 present in both w^{1118} and the CaSpeR-4 *P*-element transformation vector used to create the *H2AvDGFP* strain. Primers 5'-ACATCAAATTGTCTGCGG-3' and 5'-CGCTCGTTGCAGAATAGT-3' amplified a 569-bp fragment of the CaSpeR-4 *P*-element transformation vector present in *H2AvDGFP* but absent in w^{1118} due to a deletion from ~+2100 to +11,000 relative to the *w* start codon. PCR allowed for the molecular detection of the CaSpeR-4-based transgene in nuclear transplant recipients. Genomic multiplex PCR at the *white* locus (1.5% agarose gel) is shown: lane 1, *H2AvDGFP* genomic control DNA exhibits a 569-bp band from the CaSper-4-derived *H2AvDGFP P*-element transformation vector and a 330-bp band from *white* exon 6; lane 2, w^{1118} genomic control DNA exhibits the 330-bp band but not the 569-bp Casper-4 band; lane 3, w^{1118} nuclei injected into the *H2AvDGFP* host embryo. The 569-bp CaSper-4-derived fragment is absent while the 330-bp w^{1118} fragment is present, indicating only w^{1118} nuclei in the cloned embryo; lane 4, *H2AvDGFP* nuclei injected into the w^{1118} host embryo. The 569-bp CaSpeR-4-derived fragment and the 330-bp w^{1118} fragment are present, indicating *H2AvDGFP* nuclei in the cloned embryo; lane 5, DNA negative control; lane 6, 100-bp ladder (MBI Fermentas). DNA was extracted from cloned late-stage embryos and adults using a technique modified from Hatton and O'Hare (http://www.bio.ic.ac.uk/research/ohare/t01816.htm). (b) Analysis of mitochondrial DNA: primers 5'-AATAACAAATTTTTAAGCC-3' and 5'-GAATAGGGGGAATAAATT-3' amplified a variable region of the mitochondrial genome ~759 bp in w^{1118} and 773 bp in *H2AvDGFP*, distinguishing host from donor mitochondria. PCR was performed across a variable region of the mitochondrial genome

Figure 3.2 (continued): (4% acrylamide gel): lane 1, the *H2AvDGFP* control amplifies a 773-bp fragment; lane 2, the w^{1118} control amplifies a 759-bp fragment; lane 3, w^{1118} nuclei injected into the *H2AvDGFP* host exhibits the 773-bp fragment from the *H2AvDGFP* host embryo mitochondria; lane 4, *H2AvDGFP* nuclei injected into the w^{1118} host exhibits the 759-bp fragment from the w^{1118} host embryo mitochondria; lane 5, DNA negative control; lane 6, 100-bp ladder (MBI Fermentas).

spiracles, could potentially be due to epigenetic constraints on reprogramming of donor nuclei.

The first attempts to clone *Drosophila* by embryonic nuclear transplantation produced ~1% of embryos able to complete embryogenesis and only one developed as far as the third instar larval stage (Illmensee 1968, 1972). The failure of these cloned *Drosophila* to survive to adulthood likely resulted from the failure to activate the unfertilized egg. The technique reported here allows for the generation of cloned adult *Drosophila*.

The rate at which developmental defects arise and the rate at which viable adult *Drosophila* clones are generated are comparable to that observed in mammals (Wilmut *et al.*. 2002); ~10% of clones survive through embryogenesis, and ~1% develop into viable adults. Failure to properly reprogram mammalian embryonic and somatic nuclei in cloning frequently manifests itself as placental abnormalities, fetal overgrowth, and premature death (Shi *et al.* 2003). Likewise, the abnormalities seen in inviable *Drosophila* clones could be due to incomplete reprogramming of donor nuclei. As the genetic regulation of early development is well characterized in *Drosophila* and there is a wealth of mutations affecting early development and maintenance of differentiated cell states, this method can be used to quickly and easily assess constraints on reprogramming of nuclei when cloning.

3.6 Acknowledgements

We thank L. A. McEachern, N. Gorguy, M. Hart, and V. Walker for discussion and comments on the manuscript. The *H2AvDGFP* stock was generously provided by S. Campbell; we thank the Bloomington *Drosophila* Stock Center for all other stocks. The work was supported by a Natural Sciences and Engineering Research Council discovery grant to V.K.L. and W.A.M. was supported by a fellowship from the Nova Scotia Health Research Foundation.

3.7 Literature Cited

Clarkson,M. and Saint,R. (1999) A His2AvDGFP fusion gene complements a lethal His2AvD mutant allele and provides an in vivo marker for *Drosophila* chromosome behavior. *DNA Cell Biol.*, **18,** 457-462.

Gurdon,J.B. and Uehlinger,V. (1966) "Fertile" intestine nuclei. *Nature,* **210,** 1240-1241.

Illmensee,K. (1972) Developmental potencies of nuclei from cleavage, preblastoderm, and syncytial blastoderm transplanted into unfertilized eggs of *Drosophila melanogaster*. *Wilhelm Roux' Arch,* **170,** 267-298.

Illmensee,K. (1968) Transplantation of embryonic nuclei into unfertilized eggs of *Drosophila melanogaster*. *Nature,* **219,** 1268-1269.

Lee,K.Y., Huang,H., Ju,B., Yang,Z. and Lin,S. (2002) Cloned zebrafish by nuclear transfer from long-term-cultured cells. *Nat.Biotechnol.*, **20,** 795-799.

Lewis,D.L., Farr,C.L., Farquhar,A.L. and Kaguni,L.S. (1994) Sequence, organization, and evolution of the A+T region of *Drosophila melanogaster* mitochondrial DNA. *Mol.Biol.Evol.*, **11,** 523-538.

Shi,W., Zakhartchenko,V. and Wolf,E. (2003) Epigenetic reprogramming in mammalian nuclear transfer. *Differentiation,* **71,** 91-113.

Wilmut,I., Beaujean,N., de Sousa,P., Dinnyes,A., King,T., Paterson,L., Wells,D. and Young,L. (2002) Somatic cell nuclear transfer. *Nature,* **419,** 583-586.

Yasuda,G.K., Schubiger,G. and Wakimoto,B.T. (1995) Genetic characterization of ms(3)K81, a paternal effect gene of *Drosophila melanogaster*. *Genetics,* **140,** 219-229.

4.1 Linking Paragraph

The following chapter examines the genomic imprint in *Drosophila* clones. Imprinted genes exhibit chromatin-based, parent-of-origin specific, monoallelic expression, and loss of imprinting occurs in a number of cloned organisms. We used a well established assay system to examine the status of the imprint in *Drosophila* clones, and found that it is compromised in individuals generated by nuclear transplant. The chapter has been submitted to EMBO Reports and we are awaiting a response.

Loss of Genomic Imprinting in *Drosophila* clones

Andrew J. Haigh and Vett K. Lloyd

4.3 Abstract

Genomic imprinting is a process that genetically distinguishes maternal and paternal genomes, and can result in parent-of-origin-dependent mono-allelic expression of a gene. As such, an otherwise functional maternally inherited allele may be silenced so that the gene is expressed exclusively from the paternal allele, or vice-versa. Once thought to be restricted to mammals, genomic imprinting has been documented in angiosperm plants (Kermicle 1970), zebrafish (Martin and McGowan 1995), insects (Lloyd *et al.* 1999) and *C. elegans* (Bean *et al.* 2004), and in each case it appears to rely on differential chromatin structure. Aberrant imprinting has been implicated in various human cancers (Feinberg *et al.* 2002) and has been detected in a number of cloned mammals (Humpherys *et al.* 2001, Young *et al.* 2003, Zhang *et al.* 2004). Here we show that genomic imprinting associated with a mini-X chromosome is lost in *Drosophila melanogaster* clones.

4.4 Introduction

The birth of the first cloned mammal in 1996 (Wilmut *et al.* 1997) was a profound demonstration of the totipotency of somatic nuclei, and in the years since it has ignited both excitement and controversy in the fields of therapeutic and reproductive cloning. The advent of organismal cloning directly implied the feasibility of stem-cell based therapies for a host of human disorders, through customized tissue transplants engineered to be an identical genetic match to the patient. However, closer inspection of many adult clones has revealed subtle irregularities in the expression of imprinted genes that may present complications when generating either tissues or organisms from cloned embryos.

Insulin-like growth factor 2-H19 (*Igf2-H19*) and *mannose-6-phosphate/Insulin-like growth factor 2 receptor* (*M6P/Igf2r*) gene expression constitute the most intensely studied instances of genomic imprinting in mammals, and in each case, gene expression is thought to be based in part on differentially methylated imprint control regions of DNA. As such, their expression is often examined to assess the status of the genomic imprint in parthenogenetic, anderogenetic, and cloned organisms (Sasaki *et al.* 2005, Young *et al.* 2003). In sheep, both *Igf2-H19* and *M6P/Igf2r* are imprinted, and loss of genomic imprinting at both loci in cloned sheep has been reported; however, the loss of CpG methylation was more pronounced at the *Igf2r* locus (Young *et al.* 2003). This loss of imprinting has been correlated with so-called "large offspring syndrome" (Young *et al.* 2001) – characterized by a large size at birth, breathing difficulties, organ distension, placental abnormalities, and sudden perinatal death (for review see Young *et al.* 1998). Similarly, in cattle, H19 is usually expressed predominantly from the maternal allele, and biallelic expression was reported in cloned cattle suffering from developmental abnormalities (Zhang *et al.* 2004). A high degree of epigenetic instability has been reported at the imprinted *Igf2-H19* region in mice cloned from embryonic stem cell cultures (Humpherys *et al.* 2001), and large offspring syndrome has been reported in cloned mice (Eggan *et al.* 2001). In contrast, Inoue and colleagues (2002) report faithful conservation of *Igf2-H19* imprinting in mice derived from somatic Sertoli cell nuclei, suggesting that the imprint may be manageable in clones.

In humans, loss of imprinting was first associated with cancer when it was discovered that *Igf2 / H19* imprinting was lost in Wilms' tumors of the kidney (Rainier *et al.* 1993, Ogawa *et al.* 1993). Loss of imprinting has since been documented in

embryonal tumors, including hepatoblastoma and rhabdomyosarcoma, and adult colorectal, liver, and lung tumors (for review see Feinberg *et al.* 2002). Moreover, altered imprinting of gene clusters at human chromosome 11p15.5, which include *Igf2*, is thought to be responsible for Beckwith-Wiedemann syndrome, an epigenetic disorder predisposing children to prenatal overgrowth, birth abnormalities, and cancer (Maher and Reik 2000). As loss of imprinting has been well documented in various cancers, and as genomic imprinting appears to be compromised in many organisms and tissue cultures derived from nuclear transplantation, steps towards management of the imprint should be considered before proceeding with stem-cell based therapies for manageable human disorders.

Drosophila melanogaster has long served as a model organism for genetic studies and exhibits genomic imprinting of at least eight regions, which can encompass multiple genes, each involving the production of compacted chromatin domains that silence the associated gene depending on the sex of the parent (Lloyd 2000). The best studied of these involves a mini-X chromosome, *Dp(1;f)LJ9*, that contains multiple imprinted genes, including the *garnet* eye colour gene (Lloyd *et al.* 1999). The chromosome was constructed by an inversion that places the gene near the tip of the X-chromosome, followed by the subsequent removal of most of the euchromatic region (Hardy *et al.* 1984), situating the gene on the border of the imprinted X-chromosome heterochromatin. Maternal transmission of the mini-X chromosome results in full expression of *garnet* in all cells, whereas paternal transmission of the mini-X leads to gene silencing in many cells of the genetically identical offspring (Lloyd *et al.* 1999). Thus, the level of *garnet* transcription differs depending on the sex of the parent, and the result is visually

distinguishable in the eye – paternal transmission of the chromosome leads to variegation in the eye (Figure 4.1a), while maternal transmission of the mini-X results in a wild-type phenotype (Figure 4.1b). Our results indicate that the paternal imprint at the garnet gene appears to be lost upon cloning of *Drosophila*.

We have previously described the generation of the adult *Drosophila* derived by nuclear transplant (Haigh *et al.* 2005). In order to assess the status of the genomic imprint associated with the *Dp(1;f)LJ9* mini-X chromosome, we cloned flies from donor embryos to which the imprint marker, the mini-X chromosome, had been either maternally or paternally transmitted. The eye pigmentation of the resulting male offspring was then evaluated.

4.6 Results and Discussion

Male clones derived from nuclei to which the mini-X chromosome was paternally transmitted (Figure 4.1c) exhibited a significant loss ($p = 1.176*10^{-5}$, n=27) of gene silencing associated with imprinted *garnet* expression, resulting in increased eye pigment variegation when compared with genotypically identical, but sexually derived, offspring (Figure 4.1a). This loss of eye pigment variegation, resulting in near wild-type or wild-type gene expression, suggests that the genomic imprint associated with the paternal transmission of the mini-X chromosome is severely hindered, if not lost entirely, in cloned *Drosophila* adult males. Conversely, male clones derived from nuclei to which the mini-X chromosome was maternally transmitted (Figure 4.1d) exhibited the same wild-type eye phenotype as their sexually derived counterparts (Figure 4.1b).

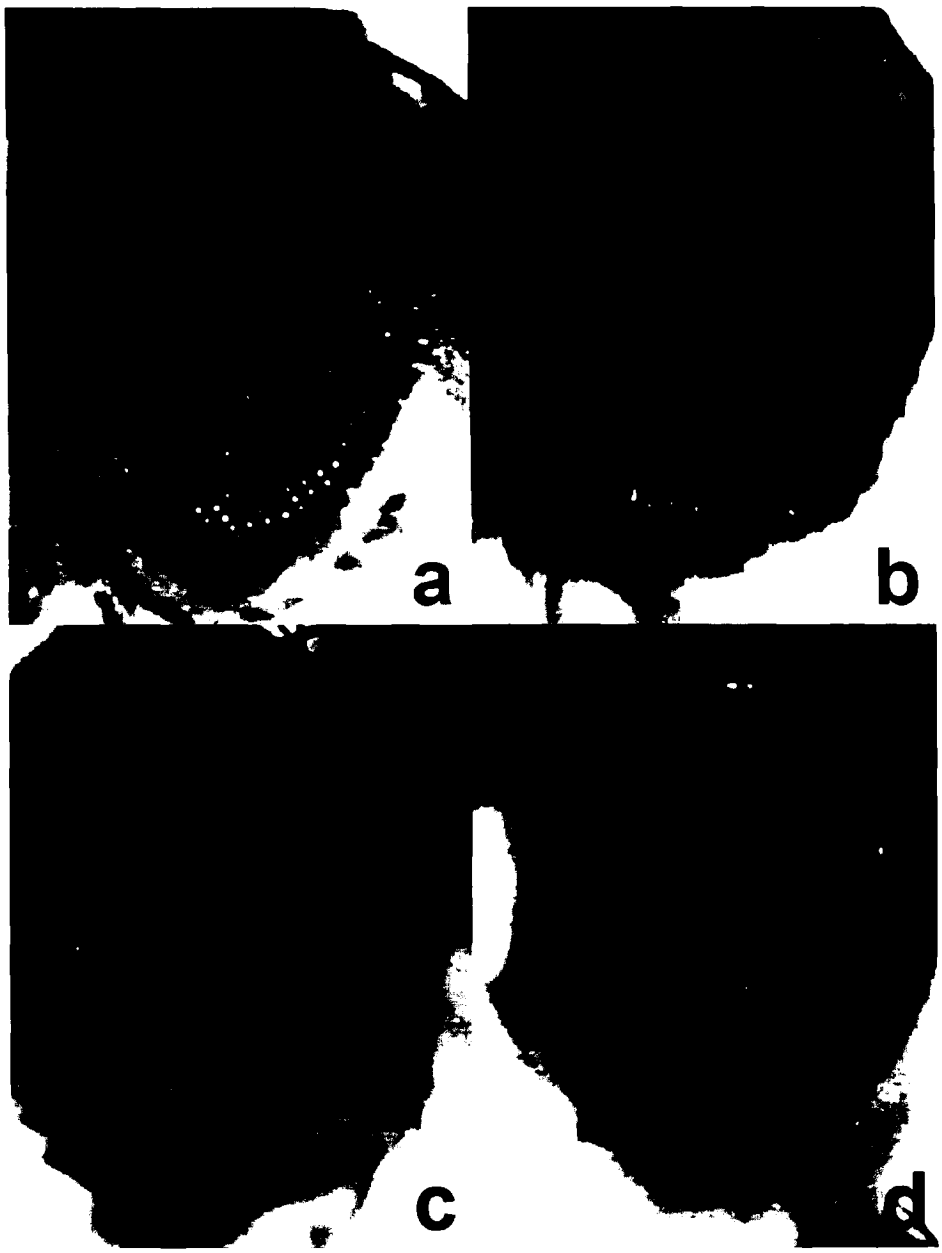

Figure 4.1: Eye colour variegation in sexually derived and cloned *Drosophila*. (a) Paternally transmited mini-X chromosome results in eye colour variegation, while (b) maternally inherited mini-X chromosome leads to wild-type phenotype. (c) *Drosophila* clone to which the mini-X chromosome was paternally transmitted has significant reduction of eye colour variegation, whereas (d) *Drosophila* clone to which the mini-X chromosome was maternally transmitted leads to wild-type phenotype.

The imprinted mini-X chromosome has previously been used to identify genes involved in genomic imprinting in *Drosophila*, and of those tested, members of the *trx* and *Su(var)* groups play a role in the maintenance of the paternal genomic imprint (Joanis and Lloyd 2002). The protiens likely affect the genomic imprint via histone modifications that mediate chromatin-based gene expression. The function of these proteins appears to be conserved across species, although their role in imprinting in species other than *Drosophila* has not yet been examined directly. The results described herein lay the foundation for the potential manipulation of modifiers of imprinting in *Drosophila* to manage the stability of the genomic imprint in cloned flies. Furthermore, as *Drosophila Su(var)* homologues have been linked to chromatin mediated gene expression in mammals (Wreggett *et al.* 1994, Aagaard *et al.* 1999, O'Carroll *et al.* 2000, Norwood *et al.* 2004), this system may provide an efficient, cost-effective means of assessing the impact of genetic and environmental manipulations designed to maintain genomic imprinting in nuclear transplant recipients.

4.6 Methods

Clones were generated as previously described (Haigh *et al.* 2005). Unless otherwise stated, all procedures took place at 22°C. All embryos were dechorionated in 0.3% $NaHCO_3$ for 50 seconds. Recipient embryos were dehydrated with Drierite™ for 6 minutes prior to injection. Both donor and recipient embryos were then covered by a thin layer of Halocarbon™ 200 oil. Cytoplasm containing nuclei was drawn from donor embryos 70-100 minutes after egg laying, and approximately $0.01mm^3$ (between 25-250 nuclei) was transferred to each 10-40 minute old recipient embryo. Embryos were then

incubated at 18°C until larvae hatched, at which point they were raised on standard *Drosophila* media. Control embryos were subjected to identical dechorionation, timing, and temperature regiments.

To assess the paternal imprint in *Drosophila* clones, donor embryos were collected from *Dp(1:f)LJ9* males carrying the mini-X chromosome crossed to $y\ z^{a}\ g^{53d}$ homozygous females. The maternal imprint was assessed by generating clones from donor embryos derived from the reciprocal cross.

Adult eyes were photographed using a Sony DSC-S70 digital camera and a Zeiss Stemi 2000-C. Eye colour variegation was evaluated using Adobe Photoshop 11 in a manner similar to that previously described by (Coveny *et al.* 2002). The lasso tool was used to select the total area of the eye, which was then transferred to a new document. The image was colour balanced, and the level of variegation was determined by selecting pixels of the eye not displaying full *garnet* expression, and therefore orange eyes, using the preset colour range tool. This was compared to the total number of pixels in the eye. Some areas were manually corrected for glare.

Percent eye colour variegation for each individual was calculated as an average of the two eyes. Two tailed, heteroscedastic T-tests compared percent eye colour variegation between cloned and control animal populations.

4.7 Literature Cited

Aagaard,L., Laible,G., Selenko,P., Schmid,M., Dorn,R., Schotta,G., Kuhfittig,S., Wolf,A., Lebersorger,A., Singh,P., Reuter,G. and Jenuwein,T. (1999) Functional mammalian homologues of the *Drosophila* PEV-modifier *Su(var)3-9* encode centromere-associated proteins which complex with the heterochromatin component M31. *EMBO J,* **18,** 1923-1938.

Bean,C.J., Schaner,C.E. and Kelly,W.G. (2004) Meiotic pairing and imprinted X chromatin assembly in *Caenorhabditis elegans*. *Nat.Genet.*, **36,** 100-105.

Coveny,A.M., Dray,T. and Gloor,G.B. (2002) The effect of heterologous insertions on gene conversion in mitotically dividing cells in *Drosophila melanogaster*. *Genetics,* **161,** 249-258.

Eggan,K., Akutsu,H., Loring,J., Jackson-Grusby,L., Klemm,M., Rideout,W.M.,3rd, Yanagimachi,R. and Jaenisch,R. (2001) Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. *PNAS,* **98,** 6209-6214.

Feinberg,A.P., Cui,H. and Ohlsson,R. (2002) DNA methylation and genomic imprinting: insights from cancer into epigenetic mechanisms. *Semin.Cancer Biol.*, **12,** 389-398.

Haigh,A.J., MacDonald,W.A. and Lloyd,V.K. (2005) The generation of cloned *Drosophila melanogaster*. *Genetics,* **169,** 1165-1167.

Hardy,R.W., Lindsley,D.L., Livak,K.J., Lewis,B., Siversten,A.L., Joslyn,G.L., Edwards,J. and Bonaccorsi,S. (1984) Cytogenetic analysis of a segment of the Y chromosome of *Drosophila melanogaster*. *Genetics,* **107,** 591-610.

Humpherys,D., Eggan,K., Akutsu,H., Hochedlinger,K., Rideout,W.M.,3rd, Biniszkiewicz,D., Yanagimachi,R. and Jaenisch,R. (2001) Epigenetic instability in ES cells and cloned mice. *Science,* **293,** 95-97.

Inoue,K., Kohda,T., Lee,J., Ogonuki,N., Mochida,K., Noguchi,Y., Tanemura,K., Kaneko-Ishino,T., Ishino,F. and Ogura,A. (2002) Faithful expression of imprinted genes in cloned mice. *Science,* **295,** 297.

Joanis,V. and Lloyd,V.K. (2002) Genomic imprinting in *Drosophila* is maintained by the products of *Suppressor of variegation* and *trithorax* group, but not *Polycomb* group, genes. *Mol.Genet.Genomics,* **268,** 103-112.

Kermicle,J.L. (1970) Dependence of the R-mottled aleurone phenotype in maize on mode of sexual transmission. *Genetics,* **66,** 69-85.

Lloyd,V. (2000) Parental imprinting in *Drosophila*. *Genetica,* **109,** 35-44.

Lloyd,V.K., Sinclair,D.A. and Grigliatti,T.A. (1999) Genomic imprinting and position-effect variegation in *Drosophila melanogaster*. *Genetics,* **151,** 1503-1516.

Maher,E.R. and Reik,W. (2000) Beckwith-Wiedemann syndrome: imprinting in clusters revisited. *J.Clin.Invest.,* **105,** 247-252.

Martin,C.C. and McGowan,R. (1995) Genotype-specific modifiers of transgene methylation and expression in the zebrafish, *Danio rerio*. *Genet.Res.,* **65,** 21-28.

Norwood,L.E., Grade,S.K., Cryderman,D.E., Hines,K.A., Furiasse,N., Toro,R., Li,Y., Dhasarathy,A., Kladde,M.P., Hendrix,M.J., Kirschmann,D.A. and Wallrath,L.L. (2004) Conserved properties of HP1(Hsalpha). *Gene,* **336,** 37-46.

O'Carroll,D., Scherthan,H., Peters,A.H., Opravil,S., Haynes,A.R., Laible,G., Rea,S., Schmid,M., Lebersorger,A., Jerratsch,M., Sattler,L., Mattei,M.G., Denny,P., Brown,S.D., Schweizer,D. and Jenuwein,T. (2000) Isolation and characterization of *Suv39h2*, a second histone H3 methyltransferase gene that displays testis-specific expression. *Mol.Cell.Biol.,* **20,** 9423-9433.

Ogawa,O., Eccles,M.R., Szeto,J., McNoe,L.A., Yun,K., Maw,M.A., Smith,P.J. and Reeve,A.E. (1993) Relaxation of insulin-like growth factor II gene imprinting implicated in Wilms' tumour. *Nature,* **362,** 749-751.

Rainier,S., Johnson,L.A., Dobry,C.J., Ping,A.J., Grundy,P.E. and Feinberg,A.P. (1993) Relaxation of imprinted genes in human cancer. *Nature,* **362,** 747-749.

Sasaki,H., Ferguson-Smith,A.C., Shum,A.S., Barton,S.C. and Surani,M.A. (1995) Temporal and spatial regulation of H19 imprinting in normal and uniparental mouse embryos. *Development,* **121,** 4195-4202.

Wilmut,I., Schnieke,A., McWhir,J., Kind,A. and Campbell,K. (1997) Viable offspring derived from fetal and adult mammalian cells. *Nature,* **385,** 810-813.

Wreggett,K.A., Hill,F., James,P.S., Hutchings,A., Butcher,G.W. and Singh,P.B. (1994) A mammalian homologue of *Drosophila* heterochromatin protein 1 (HP1) is a component of constitutive heterochromatin. *Cytogenet.Cell Genet.*, **66,** 99-103.

Young,L.E., Fernandes,K., McEvoy,T.G., Butterwith,S.C., Gutierrez,C.G., Carolan,C., Broadbent,P.J., Robinson,J.J., Wilmut,I. and Sinclair,K.D. (2001) Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nat.Genet.*, **27,** 153-154.

Young,L.E., Schnieke,A.E., McCreath,K.J., Wieckowski,S., Konfortova,G., Fernandes,K., Ptak,G., Kind,A.J., Wilmut,I., Loi,P. and Feil,R. (2003) Conservation of IGF2-H19 and IGF2R imprinting in sheep: effects of somatic cell nuclear transfer. *Mech.Dev.*, **120,** 1433-1442.

Young,L.E., Sinclair,K.D. and Wilmut,I. (1998) Large offspring syndrome in cattle and sheep. *Rev.Reprod.*, **3,** 155-163.

Zhang,S., Kubota,C., Yang,L., Zhang,Y., Page,R., O'neill,M., Yang,X. and Tian,X.C. (2004) Genomic imprinting of H19 in naturally reproduced and cloned cattle. *Biol.Reprod.*, **71,** 1540-1544.

5.1 Linking Paragraph

The following chapter evaluates *Drosophila* as a model organism for cloning research, and serves to situate our work in the greater context of epigenetic and cloning research. I examine similarities and differences between cloning *Drosophila* and mammals at both the cellular and genetic level.

5. Drosophila melanogaster *as a Model Organism for Cloning Research*

5.2 Introduction

We have passed a unique crossroads in biological research. For the first time in history we are able to transplant the genetic material of a higher organism, in its entirety, to an embryonic shell and begin life anew. The metaphysical and ethical implications of cloning are staggering – we are no longer limited to the linear progression of life, but as this technology progresses we will have the potential to unilaterally extend our individual genetic pedigree. Although rife with ethical and technical complications, this technology will provide unique opportunities to re-address classical lines of biological inquiry, such as the origin of sexual reproduction, the ongoing debate over the contributions of nature versus nurture to one's individuality, and it will force us to define individuality itself (Brock 2002). Nuclear transplant technology, even in its infancy, represents the forefront of medical research. In recent years, it has been exploited to create stem cells for use in therapeutic cloning; the manipulation of cloned embryos to produce individually tailored tissues or organs, such as skin or liver grafts (Koh and Atala 2004).

Reproductive cloning allows for the generation of live offspring from embryos derived by nuclear transplantation, and we are already capable of reproducing copies of genetically modified mammals engineered to produce biologically important compounds (Wilmut *et al.* 2002). Once the fodder of science fiction, reproductive cloning may allow for the reconstitution of an organism from nuclei cryogenically frozen hundreds or thousands of years previously (Ogura *et al.* 2000). Although reports of the birth of human clones thus far remain unsubstantiated, in the nine short years since the birth of Dolly the sheep in 1996 (Wilmut *et al.* 1997), we have developed the technology to create viable

cloned human embryos through nuclear transplantation (Hwang *et al.* 2004). Although no cloned human embryos have been allowed to develop past the blastocyst stage, a pluripotent embryonic stem cell line has been derived from a cloned human embryo, illustrating that embryonic stem cell lines can be generated from the somatic cells of a living person (Hwang *et al.* 2004). The ethical examination of the issues raised by such technology is a profession in itself – one that has called for a universal moratorium on new biotechnologies until these issues can be satisfactorily addressed. Due to conflicting scientific, industrial, and political ideologies and agendas worldwide, these calls remain largely unheeded.

Organisms generated by nuclear transplantation suffer from a high rate of associated defects (Wilmut *et al.* 2002), thus the question is no longer "should we clone?" but rather "can we clone effectively?" As such, we must ensure that we seek first and foremost to decrease the incidence of nuclear transplant related aberrations in cloned offspring. Through a greater understanding of the underlying developmental reprogramming that occurs during nuclear transplantation we would not only be improving the well-being of cloned organisms brought to term, but increasing the efficiency with which these organisms can be generated. Further, and perhaps more importantly, lessons learned from reproductive cloning may facilitate emerging stem cell therapies.

In the Spring of 2003 we became the first group to successfully clone the fruit fly *Drosophila melanogaster* (Haigh *et al.* 2005). *Drosophila* has traditionally been attractive as a model organism based on its cost-effectiveness, the ease with which its genome can be manipulated, and its rapid generation time. Since Morgan's discovery of a white-eyed

mutant in 1910 (Morgan 1910), the fly's complete genome has been sequenced (Adams *et al.* 2000), and it has been the subject of tens of thousands of primary research articles (The Flybase Consortium 2003) and many major research initiatives, including a comprehensive gene disruption project (Bellen *et al.* 2004). The large repertoire of mutations and the sophisticated genetic tools available in *Drosophila* have made this organism one in which the events of early development are extremely well understood at the cellular and molecular level (Nusslein-Volhard and Weischaus 1980). With our recent work, we can now add the fruit fly to the list of organisms that can be generated by nuclear transplant. This work assesses the potential of *Drosophila melanogaster* as a model organism for nuclear transplant studies.

5.3 Nuclear transplantation

There are four fundamental steps in the generation of cloned organisms: (1) recipient oocyte enucleation, (2) donor nucleus isolation, (3) nuclear transplantation and ooplast (preimplantation embryo) activation, often considered one step because they can happen simultaneously, and (4) embryo culture (Mollard *et al.* 2002, Figure 5.1). As mammalian cloning technology progresses, the range of nuclei available for transplant is increasing (Eggan *et al.* 2004), and each step in the cloning process can be performed a number of ways. Rather than physically transplanting the donor nuclei, often the donor cell is fused to the enucleated oocyte. Nuclei are typically transplanted when arrested in the G_0, G_1, or in the mitotic phase of the cell cycle to prevent nucler damage upon chromosome condensation following nuclear transplant or cell-oocyte fusion (Wilmut *et*

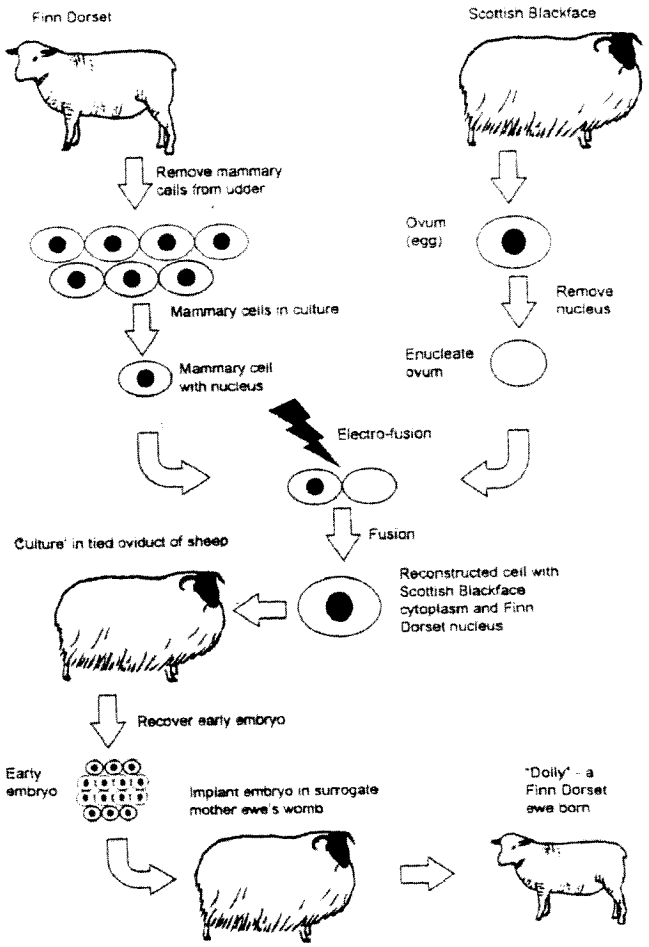

Figure 5.1: Fundamental steps in nuclear transplantation. Includes recipient oocyte enucleation followed by isolation of either embryonic or somatic donor nucleus. Combination of donor nucleus and recipient oocyte can be achieved through electro-fusion or transplantation, followed by embryo culture (Mollard *et al.* 2002, http://www.heaf.freeuk.com/dolly.gif).

al. 1997, Cibelli *et al.* 1998). The resulting recipient oocyte can be activated pre- or post-transplantation, and the embryo is cultured in the oviduct of the host female before being implanted into the surrogate mother's uterus (Mollard *et al.* 2002). In *Drosophila*, as opposed to physically enucleating the recipient oocyte, we employ a male-sterile mutation which generates motile sperm capable of activating the recipient oocyte but with pronuclei unable to participate in syngamy, thus rendering the developing embryo inviable (Yasuda *et al.* 1995, Haigh *et al.* 2005). However, in each case, managing a number of different factors is fundamental to the survival of the clones.

5.4 Is Drosophila *a good model for mammalian nuclear transplantation research?*

Variable contributing to the successful cloning of any organism include experimental technique, the maintenance of euploidy, compatible donor and recipient cell cycle phases, developmental stage of the donor cell, genomic reprogramming, and imprinted gene regulation (Wakayama and Yanagimachi 1999b, Wilmut *et al.* 2002). Furthermore, a detailed understanding of the molecular events that take place upon normal fertilization and nuclear transplantation/fusion is crucial to increasing cloning efficiency.

5.4.1 Experimental technique

Due to fundamental differences in vertebrate and invertebrate embryo physiology and morphology, this system will likely not provide insights into the technical aspects of mammalian cloning, such as ideal needle diameters or injection volumes. Since early *Drosophila* embryos exist as multinucleate syncytial blastoderms, it is necessary to transplant multiple nuclei in order to generate viable clones – a problem unique to

insects. Rearing embryos post-transplantation is relatively simple, yet both pre- and post-transplantation conditions vary greatly between mammalian and fly cloning embryo culture conditions.

5.4.2 Maintenance of ploidy

We have yet to examine the ploidy of nuclear transplant recipients, although given our current methods, haploid embryos do not survive to hatch as first instar larvae (Yasuda *et al.* 1995).

5.4.3 Cell cycle coordination

Furthermore, we do not yet attempt to coordinate cell cycle phases prior to nuclear transplantation. However, there are a number of mutations that could be exploited to study the effect of cell cycle coordination when cloning *Drosophila* from embryonic or somatic nuclei (The Flybase Consortium 2003).

5.4.4 Developmental stage

As *Drosophila* have yet to be cloned from cultured somatic cells, this model is not suitable for identifying donor cells at ideal developmental stages to promote chromatin rearrangement.

5.4.5 Nuclear reprogramming

At present, we have cloned fruit flies from early embryonic nuclei, and little, if any, nuclear reprogramming is necessary to generate cloned offspring. While the embryonic axes have been established and broad domains of presumptive gene expression have been set, donor nuclei divide under maternal control within the syncytial blastoderm, and only following the mid-blastula transition at the thirteenth nuclear division does zygotic gene expression commence (Foe *et al.* 1993). When cloning, donor

nuclei are aspirated 80 to 110 minutes after egg laying (Haigh *et al.* 2005), prior to the thirteenth nuclear division, and thus are primed to support embryonic development and large scale nuclear reprogramming is not necessarily needed to facilitate development.

5.4.6 Genomic imprinting

Drosophila may be attractive as a model organism for examining aspects of imprinted gene regulation. Our results suggest that like their mammalian counterparts, genomic imprinting in cloned *Drosophila* is not always faithfully maintained.

Drosophila exhibit genomic imprinting of the *Dp(1:f)LJ9* mini-X chromosome resulting in an imprinted variegated eye colour phenotype due to mosaic *garnet* expression when the chromosome is paternally inherited, so that the eye appears light orange with patches of wild-type gene expression (Figure 5.2a). Males derived from nuclear transplantation carrying the *Dp(1:f)LJ9* chromosome show little or no *garnet* silencing in the eye, suggesting that even when cloned at such early stages the genomic imprint is not faithfully maintained (Figure 5.2b). Therefore, it may be possible to identify environmental or genetic conditions conducive to imprint maintenance when cloning *Drosophila*. Because the imprinting mechanism appears to be highly conserved, any findings in *Drosophila* could be applicable when generating mammalian clones. Thus, although at present *Drosophila* does not provide a comprehensive model addressing all aspects of nuclear transplantation, it may be used to study specific epigenetic gene interactions following cloning – one of the most complex and least understood areas of development.

In order for *Drosophila* to serve as a model organism for cloning research, the developmental irregularities associated with mammalian and *Drosophila* clones must be

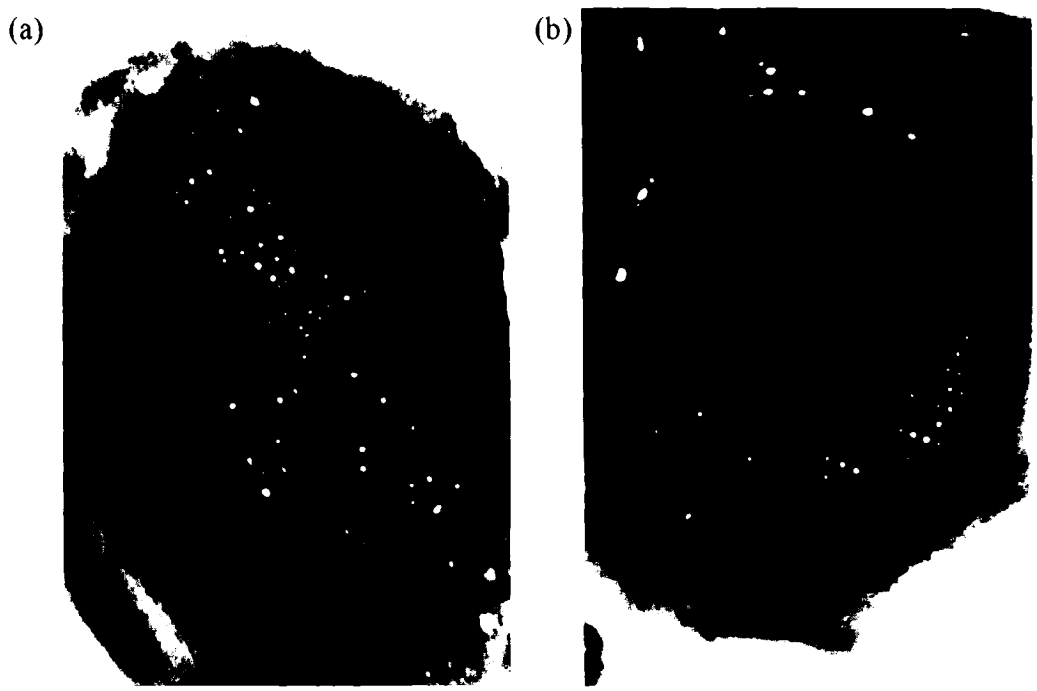

Figure 5.2: Imprinting of the *garnet* gene in *Drosophila melanogaster*. (a) When the Dp(1:f)LJ9 chromosome is paternally inherited, the *garnet* gene is silenced in all or most cells of male offspring resulting in variegation. (b) Males derived from nuclear transplantation carrying the Dp(1:f)LJ9 chromosome show little or no variegation in eye colour, suggesting that the genomic imprint is not faithfully maintained.

due to comparable genetic or epigenetic anomalies, and the underlying processes regulating gene expression in mammals and *Drosophila* must be sufficiently conserved. Here we examine the potential underlying causes of irregularities observed in some cloned mammals within the context of the loss of genomic imprinting seen in *Drosophila* clones.

5.5 Irregularities associated with cloning

The list of cloned animals reared to adulthood currently includes the frog (Gurdon 1962), sheep (Wilmut *et al.* 1997), rhesus monkey (Meng *et al.* 1997), mouse (Wakayama *et al.* 1998) , cow (Cibelli *et al.* 1998), goat (Baguisi *et al.* 1999), pig (Polejaeva *et al.* 2000), rabbit (Chesne *et al.* 2002), cat (Shin *et al.* 2002), zebrafish (Lee *et al.* 2002), mule (Woods *et al.* 2003), horse (Galli *et al.* 2003), rat (Zhou *et al.* 2003), fruit fly (Haigh *et al.* 2005), and dog (Lee *et al.* 2005). The process of nuclear transplantation, whether applied to an insect or a mammal, suffers from a low efficiency rate and incurs a high rate of associated irregularities in those organisms that are brought to term. Typically success rates for generating adult mammalian clones vary between 0.5 and 5% (Wilmut and Peterson 2002). Differences across species likely do not reflect intrinsic interspecific differences, rather, differences in the age and source of donor nuclei, varying experimental conditions, and the available technical resources. Many nuclear transplant recipients are lost at early embryonic stages, and in mammals the cloning process is associated with high rates of fetal, perinatal, and neonatal loss (Wilmut *et al.* 2002). Characteristics of abnormal mammalian clones brought to term include overgrown placentas, distended internal organs, and general fetal overgrowth, which are

collectively referred to as "large offspring syndrome" (Young *et al.* 1998, Figure 5.3). Such aberrations are thought to be due, in part, to misregulation of imprinted growth factors and receptors, such as *Insulin-like growth factor 2* (*Igf2*) and *Insulin-like growth factor 2 receptor* (*Igf2r*), which can lose their imprinted status during the cloning process (Young *et al.* 2003). Misregulation of at least some of the other approximately 40 imprinted mammalian genes (Beechey *et al.* 2003) likely hinders clone development.

About 97% of *Drosophila* transplant recipients die during embryogenesis. Of those that do hatch, only approximately one third eclose as adults, while the remainder die as 1st, 2nd, or 3rd instar larvae. Gross morphological embryonic defects can be readily observed, such as tracheal disorganization, and the absence of specific anterior structures, such as mouth hooks, is apparent (Haigh *et al.* 2005). The underlying cause of these aberrations is not yet known. Both mammalian and insect clone survival is thought to be adversely affected by a number of factors, including mechanical damage to the embryo, discordant donor and recipient cell cycle, and disrupted epigenetic gene regulation. If the underlying epigenetic mechanisms were sufficiently conserved between fruit flies and mammals, cloning in *Drosophila* could serve as a useful tool for studying this aspect of nuclear transplantation.

5.6 Epigenetics

Epigenetics describes events that cause mitotically and meiotically heritable changes in gene function without a change in the DNA sequence. With few exceptions, each cell in a higher eukaryote contains a complete genomic DNA complement, and what distinguishes one cell type from another is the relative level of individual gene expression

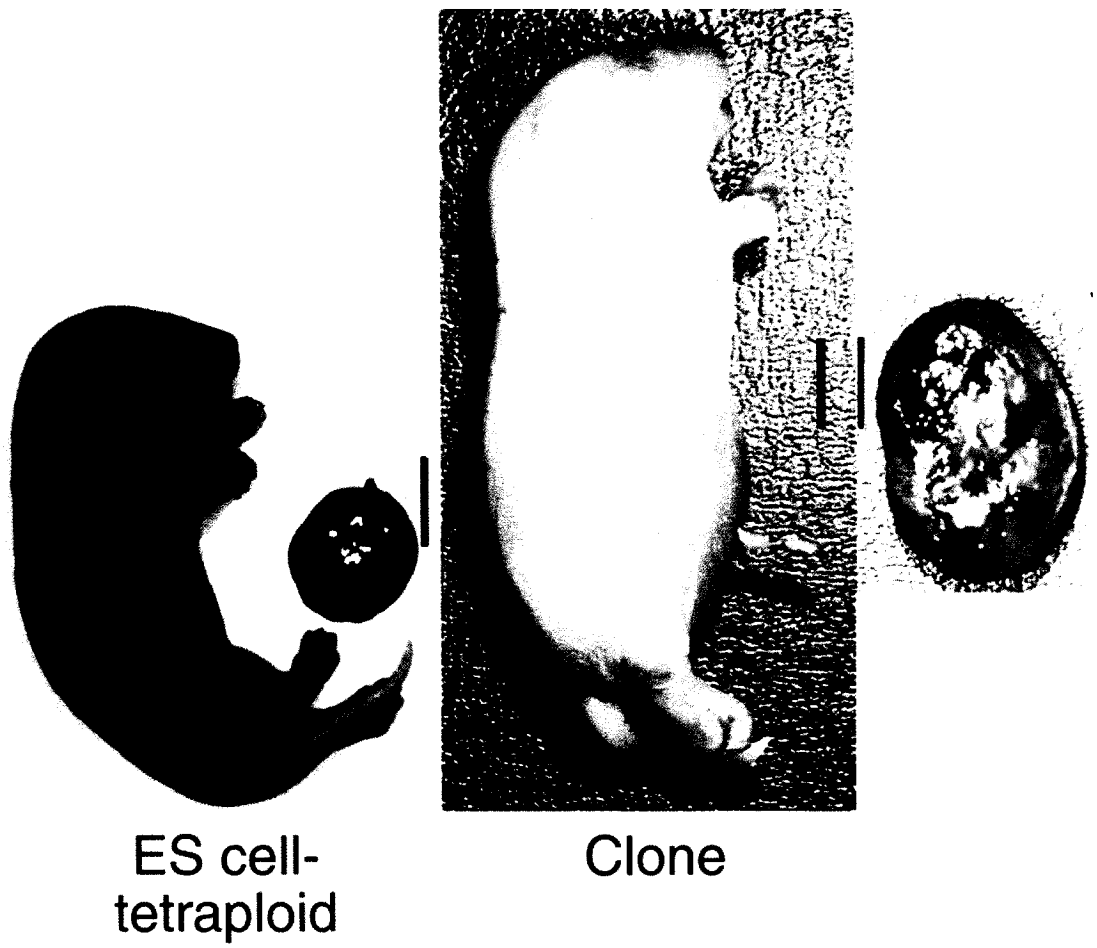

Figure 5.3: Large Offspring Syndrome. (a) Control mouse embryo and placenta, (b) cloned mouse embryo exhibiting "large offspring syndrome." Overgrowth of individual and placenta is apparent. Clone and control are to scale (Adapted from Eggan *et al.* 2001).

As an organism develops from an embryo to adulthood, transcription factors, chromatin proteins, chromatin modification proteins, and non-coding RNAs interact with DNA to spatially and temporally control gene expression, thus defining the developmental stage and identity of each cell (Lippman and Martienssen 2004). If these interactions are not carefully orchestrated, aberrations in gene expression often give rise to serious abnormalities.

The determined, and later, the differentiated state of a cell, reflects epigenetic regulation of gene expression and is maintained primarily through packaging of genes into differentially accessible chromatin conformations (for review see Czermin and Imhof 2003). Nucleosomes are the primary structural subunits of chromatin, consisting of DNA wound twice around an octamer of histone proteins to generate a fibre approximately 10 nm in diameter. DNA then further compacts into a spiral or solenoid configuration with six nucleosomes per rotation, measuring 10-40 (30) nm in diameter. This solenoid is then compacted through interactions with the nuclear matrix and nuclear membrane, in which the chromatin fibre is anchored to the non-histone nuclear scaffold at various points, creating "looped" domains (Eissenberg *et al.* 1985, Figure 5.4). The more highly compacted forms of chromatin are traditionally termed heterochromatin, whereas the more loosely compacted forms are considered euchromatin (Elgin and Workman 2002). Depending on the degree to which the DNA is compacted, the degree of histone modification, subsequent covalent DNA modification, and the location of the DNA within the nucleus, transcriptional machinery may be less able to access specific genes and so a given gene may be silenced (Eissenberg and Elgin 2000). Thus, differential chromatin packaging can epigenetically regulate genes, and areas of tightly packaged

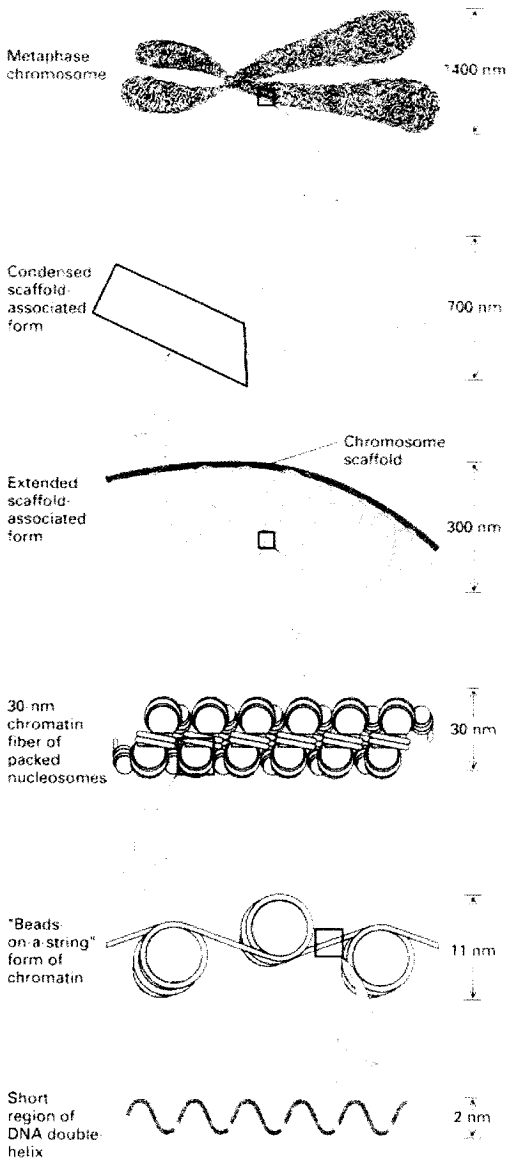

Figure 5.4: Chromatin structure. Chromatin is genomic DNA associated with histone and non-histone proteins. Nucleosomes are the primary structural subunits of chromatin, consisting of DNA wound around an octamer of histone sub-units. DNA further compacts into a spiral or solenoid configuration with six nucleosomes per rotation and is then tethered to the nuclear scaffold (adapted from Lodish *et al.* 1999).

heterochromatin contain genes that are predominantly transcriptionally silenced, whereas euchromatic regions are associated with transcriptionally active genes (Wallrath *et al.* 1994).

Two classes of highly conserved proteins interact with nucleosomes to modulate gene expression: those that utilize energy derived from ATP to disrupt the nucleosome or alter nucleosome phasing exposing DNA for transcription, and those that covalently modify histones at specific amino acid residues to alter their association with DNA (Peterson 2002, Peterson 2004). The ATP-dependent SWI/SNF may disrupt chromatin structure by applying superhelical torsion on DNA at nucleosomes, resulting in small but accessible DNA loops upon relaxation, thus facilitating gene transcription (Peterson 2002). Covalent post-translational histone modification also affects gene expression. Histones are modified in a number of ways, including ribosylation, ubiquination, and sumoylation, but best understood is either acetylation or methylation at lysine residues. Hypo-acetylated and consequently hyper-methylated histones are associated with a more condensed chromatin structure than hyper-acetylated and hypo-methylated histones. Thus, deacetylation results in DNA less accessible to transcription machinery, resulting in gene silencing (Peterson and Laniel 2004).

Covalent DNA modification, through methylation at cytosine residues (CpG in mammals, CpA and CpT in *Drosophila*)(Kunert *et al.* 2003), mediates chromatin recruitment by blocking transcription factor binding and facilitating binding of chromatin modifying complexes (Pikkard and Lawrence 2002), including histone-deacetylases (HDACs). Histone deacetylation at Lys9 of histone 3 (H3-Lys-9) allows for histone methylation by histone methyltransferases (HMTase) at the same site, promoting a closed

chromatin conformation (Pikaard and Lawrence 2002). In turn, H3-Lys9 methylation reinforces DNA methylation at cytosine residues (Tamaru and Selker 2001). Therefore, the protein interactions governing epigenetic gene silencing are inter-related and self-reinforcing (Pikaard and Lawrence 2002). In *Drosophila* (Joanis and Lloyd 2002) and mice (Szabo *et al.* 2004), some of the proteins that modify chromatin structure maintain and establish, respectively, genomic imprinting,

5.7 Genomic imprinting

Classical Mendelian genetics supposes that gene expression is independent of the parent from whom the gene is inherited. Imprinting can result in parent-of-origin-dependent expression of a gene, and thus is in violation of Mendel's first law. Genomic imprinting can cause an otherwise functional maternally inherited allele to be silenced, so the gene would have to be expressed exclusively from the paternal allele, or vice-versa. Once thought to be restricted to mammals, genomic imprinting occurs in angiosperm plants (Kermicle 1970), zebrafish (Martin and McGowan 1995), insects (Lloyd *et al.* 2000), and *C. elegans* (Bean *et al.* 2004), and in each case appears to rely on differential chromatin structure. Furthermore, aberrant genomic imprinting has been observed in clones exhibiting a variety of defects (Alberio and Campbell 2003).

Genomic imprinting involves three stages: the establishment of the imprint, its maintenance, and its subsequent resetting in the germ cell line of the individual. Molecular analysis of imprinted gene regulation is yielding some insight into how these processes function. The epigenetic "tag" in the maternal or paternal germline that determines which genes are imprinted appears to be established by germline specific

proteins that set up differential chromatin domains (Surani 1998, Szabo *et al.* 2004) that persist through meiosis. DNA methylation and histone modification, via acetylation or methylation, promoting either an "open" or "closed" chromatin conformation, respectively, appears to play a crucial role in the maintenance of imprinted genes in the somatic cells of the progeny.

The groundwork for imprint establishment is laid in the primordial germ cell line, and germ cell transmission is thought to be necessary for establishing monoallelic expression of imprinted genes (Tucker *et al.* 1996). In mammals, during meiosis, the highly methylated nucleus undergoes a wave of global de-methylation that includes differentially methylated domains, followed some time later by selective re-methylation during gametogenesis (Reik *et al.* 2001). With the exception of methylated imprinted genes, upon fertilization but prior to syngamy, the paternal genome is actively demethylated while the maternal genome is passively demethylated (Reik *et al.* 2001). Passive methylation occurs when DNA methylation patterns are not maintained through replication and cell division, whereas active demethylation occurs before DNA replication and is most likely enzymatic in nature (Oswald *et al.* 2000). This demethylation is concordant with the remodelling of the sperm nucleus to form the male pronucleus. The male and female pronuclei undergo chromatin decondensation and protein exchange in the oocyte and then fuse. Remethylation of the genome, with the exception of unmethylated imprinted genes, is coincident with implantation (Reik *et al.* 2001). In cloned organisms, the amount of disruption of imprinted gene methylation between the embryo proper and extra-embryonic tissues can differ significantly (Humpherys *et al.* 2001) – possibly explaining why some cloned offspring suffer gross

placental irregularities yet remain viable. This is likely due to the fact that transplanted nuclei are not re-transmitted through the germ cell line. Nevertheless, that derived offspring were viable is testament to the developmental potential of both embryonic and somatic nuclei.

Some estimates suggest that imprinting evolved 1200 million years ago (mya), coinciding with the evolution of sexual reproduction (Pardo-Manuel de Villena *et al.* 2000). Based on phylogenetic analysis of *IGF2*, others have suggested that imprinting evolved in therian mammals during the Jurassic period 150 mya (Killian *et al.* 2001a). However, this assertion is challenged by the finding that *IGF2* is imprinted in some chick embryos (Koski *et al.* 2000), and birds are thought to have diverged from the mammalian lineage approximately 350 mya (Killian *et al.* 2001a). These conflicting results can be reconciled by the finding that imprinting at specific loci can be either gained or lost in relatively short evolutionary time (Pearsall *et al.* 1999). Analysis of imprinting at the molecular level appears to be consistent with an ancient and conserved origin of genomic imprinting, as mammalian imprint control regions can facilitate transgene silencing in *Drosophila* (Lyko *et al.* 1997, Lyko *et al.* 1998). The specific imprinting mechanism likely varies slightly from species to species, but analysis of the process in *Drosophila* and mammals highlights many similarities.

5.8 Imprinting in mammals

Thus far, in humans, over thirty genes have been found to be imprinted, but it is postulated that up to two hundred exist (Jirtle *et al.* 1999, Barlow 1995, Lucifero *et al.* 2004), and aberrant genomic imprinting is the causative agent in many genetic diseases,

including Prader-Willi/Angelman syndrome, Beckwith-Wiedemann syndrome, and several types of pediatric and other cancers (Hall 1999, Falls *et al.* 1999). However, the most intensely studied case of genomic imprinting in mammals involves the *H19*, *Insulin-2 (Ins-2)*, and *Insulin-like growth factor 2 (Igf2)* genes in mice. *Igf2* encodes a mammalian growth factor, *Ins-2* a growth hormone, and the *H19* gene encodes a non-translatable RNA that serves to lower the concentration of IGF2 and INS-2 by suppressing their transcription on the maternal chromosome (Leighton *et al.* 1996). The *Ins-2* and *Igf2* genes lie adjacent to each other in a 300kb region on the distal portion of chromosome 7, *Igf2* lies downstream from *Ins-2*, and *H19* lies 100kb further downstream (Figure 5.5). The *Ins-2* and *Igf2* genes are separated from *H19* by a differentially methylated imprint control region which lies upstream from an enhancer that differentially regulates *H19* and *Ins-2/Igf2*. As a result, while each individual has two copies of each of the genes, one on each chromosome, maternal expression of *H19* contributes to the silencing of the adjacent maternal copies of *Ins-2* and *Igf2*, rendering the organism effectively haploid.

Central to *Igf2/H19* gene regulation is CTCF, an insulator protein that recruits a complex of associated chromatin proteins that bind to the maternal unmethylated imprint control region at four distinct sites (Lewis and Murrell 2004, Szabo *et al.* 2004). CTCF copurifies with nucleophosmin/B23, and it is this protein interaction that is thought to physically block downstream enhancers from interacting with the *Igf2* and *Ins-2* promoters, facilitating the insulator function of the protein complex (Yusufzai *et al.* 2004).

Nucleophosmin/B23 interacts preferentially with histone 3, promoting nucleosome formation (Okuwaki *et al.* 2001). When bound to the imprint control region,

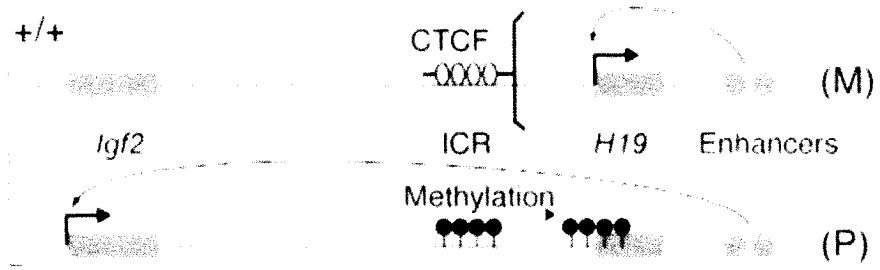

Figure 5.5: Imprinting at the *Igf2/H19* locus. Lack of methylation at the maternal (top) imprint control region (ICR) allows CTCF to bind, recruiting insulator proteins that block downstream enhancers from interacting with the *Igf2* promoter allowing *H19* expression. Methylation at the paternal (bottom) ICR blocks CTCF binding, allowing the downstream enhancer to interact with the *Igf2* promoter facilitating gene expression (Adapted from Szabo *et al.* 2004).

the CTCF chromatin protein complex acts as an insulator, possibly by tethering the imprint control region to the nuclear matrix. As a result *cis*-acting enhancers are kept from interacting with the *Igf/Ins-2* promoter, thus maternal *H19* is transcribed and *Igf2/Ins-2* are not (Lewis and Murrell 2004). CTCF is only present in the maternal germline, so when the imprinted gene cluster is inherited from the paternal germline, CTCF does not bind to the imprint control region. Therefore, the imprint control region becomes methylated and enhancer sequences are not blocked, giving the downstream enhancer unimpeded interaction with the *Igf2/Ins-2* promoters. In this way, CTCF facilitates monoallelic expression of *Igf2*, *Ins-2*, and *H19*.

These genes are similarly imprinted in humans. Beckwith-Wiedemann syndrome is associated with a loss of DNA methylation, correlated with a loss of H3-Lys9 methylation, at the differentially methylated region of the maternally inherited copy of chromosome 11 at the *Igf2/Ins-2/H19* locus (Higashimoto *et al.* 2003). This appears to cause a loss of imprinting, and therefore biallelic expression, at the *Igf2* locus (Jirtle *et al.* 1999), which likely contributes to the diagnostic pathology of the disease, including increased neonatal size, organ distention, and abdominal abnormalities.

Parent-of-origin specific expression of the mouse *Igf-2 receptor* (*Igf2r*) and its anti-sense transcript *Air* (Figure 5.6) appears to be regulated by similar processes. Genomic imprinting of these genes is associated with two differentially methylated intronic imprint control regions. Cytosine methylation at the first imprint control region, in concert with *Air* transcription, blocks *Igf2* transcription on the paternal allele while allowing transcription from the maternal allele. Lack of cytosine methylation at the second intronic *Igf2r* paternal imprint control region allows for *Air* transcription, which

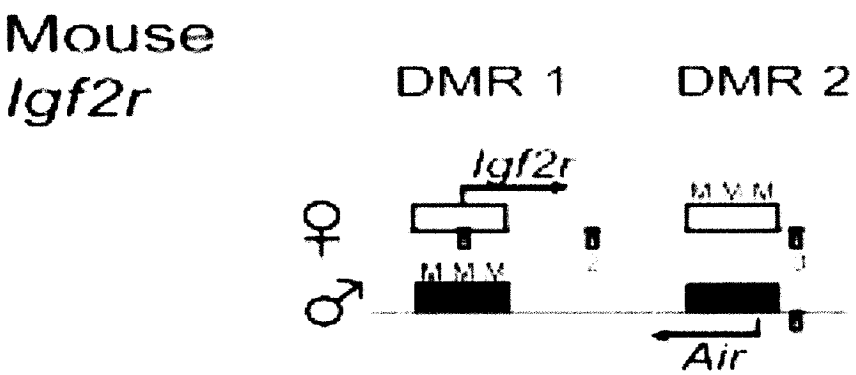

Figure 5.6: Imprinting at the *Igf2r* locus. Gene expression is regulated by two differentially methylated intronic imprint control regions. Cytosine methylation (M) at the first ICR, in concert with the *Air* transcript, blocks *Igf2* transcription on the paternal allele, while allowing transcription from the maternal allele. No cytosine methylation at the second intronic *Igf2r* paternal imprint control region facilitates *Air* transcription which further represses transcription of paternal *Igf2r* (Adapted from Vu *et al.* 2004).

serves to further repress transcription of *Igf2r* and two other genes on the same paternal chromosome (Delaval and Feil 2004, Vu *et al.* 2004). In mice and humans, methylation of Lys9 of histone H3 is indicative of an inactive *Igf2r* allele, and histone acetylation is associated with the active *Igf2r* promoter (Vu *et al.* 2004). Thus, mammalian genomic imprinting appears to be dependent, in part, on interplay between DNA methylation, non-coding RNA, and differential histone modification, which establishes parent-of-origin-specific open or closed chromatin conformations.

5.9 Imprinting in Drosophila

Drosophila melanogaster exhibits genomic imprinting of at least 8 regions, each involving the production of compacted chromatin domains that silence the associated gene, depending on the sex of the parent (Lloyd 2000). The best studied of these involves the artificial mini-X chromosome containing a number of genes, including the *garnet* eye colour gene. The chromosome was constructed by an inversion that places the *garnet* gene near the tip of the X-chromosome, followed by the subsequent removal of most of the euchromatic region (Hardy *et al.* 1984), situating the gene on the border of the presumably endogenously imprinted X-chromosome heterochromatin (Lloyd *et al.* 1999). Maternal transmission of the mini-X chromosome results in full expression of *garnet* in all cells, whereas paternal transmission of the mini-X leads to gene silencing in all or most cells of the genetically identical offspring (Lloyd *et al.* 1999, Figure 5.2a). As the level of *garnet* expression is visually distinguishable, the imprinted mini-X chromosome has been used to identify genes involved in the maintenance of the genomic imprint. Of

those genes tested, members of the *trx* and *Su(var)* groups have been found to play a role in the maintenance of the paternal genomic imprint (Joanis and Lloyd 2002).

The *trx* group of genes encode early acting proteins that maintain the expression patterns of homeotic (*hox*) and many other genes (Simon 1995), and contain both PHD (plant homeodomain) and SET (*Su(var)3-9*, *Enhancer of zeste*, and *trx*) domains characteristic of chromatin associated factors (Jones and Gelbert 1993, Aasland *et al.* 1995). As such, mutations in *trx-g* genes are pleiotropic, affecting a variety of early processes in the developing embryo. *Mixed lineage leukemia* (*Mll*) is the murine homologue of *Drosophila trx* and *Mll* deficient mice exhibit abnormal segment identity and altered *hox* gene expression, mimicking the segment transformations associated with *trx* mutations in *Drosophila* (Yu *et al.* 1995, The FlyBase Consortium 2003). The mammalian protein contains PHD and SET domains characteristic of chromatin-associated proteins (Yu *et al.* 1995). *hbrm*, the human homologue of the *Drosophila brm* gene, likewise functions as a strong transcriptional activator; however, its role in imprinting is not yet known (Muchardt and Yaniv 1993).

TRX-G proteins may interact with the SU(VAR) group of proteins, many of which also contain SET domains (Schotta *et al.* 2003a). Mutations in *Su(var)* genes abolish maintenance of the paternal imprint (Joanis and Lloyd 2002). Of the 11 *Su(var)* genes found to regulate imprinting in *Drosophila*, *Su(var)3-9* and *Su(var)2-5* are best characterized (Joanis and Lloyd 2002). Mutations in *Su(var)* genes result in relaxed chromatin condensation allowing transcription of otherwise silenced genes (Grigliatti 1991). As genes that serve to epigenetically lock the status of the genome are highly

conserved (Singh *et al.* 1991), it is reasonable to presume they serve similar functions in arthropods, plants, fish, yeasts, and mammals.

In *Drosophila*, *Su(var)3-9* encodes a histone methyltransferase that catalyzes H3-Lys9 methylation and recruits heterochromatin protein 1 (HP1) to DNA (Schotta *et al.* 2002b, Figure 6). The mouse orthologues of the *Drosophila Su(var)3-9* gene, *Suv39h1* or *Suv39h2*, likewise encode histone methyltransferases that catalyze H3-Lys9 methylation (Bultman and Magnuson 2000, O'Carroll *et al.* 2000). The functional conservation of this gene is illustrated by the enhancement of chromatin associated gene silencing in transgenic flies expressing a mammalian copy of the gene (Aagaard *et al.* 1999).

HP1 is a protein enriched in heterochromatic regions, involved in gene expression, and in *Drosophila* is encoded by the *Su(var)2-5* gene (Eissenberg and Elgin 2000, The Flybase Consortium 2003, Figure 5.7). Thus far, three mammalian homologues of *Su(var)2-5* have been found: *M31*, *M32* and $HP1^{Hs\alpha}$ (Singh *et al.* 1991, Norwood *et al.* 2004). The human and $HP1^{Hs\alpha}$ rescues lethality associated with homozygous *Su(var)2-5* null mutation in *Drosophila* (Norwood *et al.* 2004), again illustrating strong functional conservation. Furthermore, the mammalian homologues of SU(VAR)3-9 associate with M31 (Aagaard *et al.* 1999) and mammalian SU(VAR)2-5 homologues and SUV39h1/SUV39h2 colocalize to areas of heterochromatin in mammalian cells (Wreggett *et al.* 1994, Aagaard *et al.* 1999, O'Carroll *et al.* 2000, Norwood *et al.* 2004). The biochemical interactions of these proteins are being intensely studied, but they are believed to form multi-unit complexes that differentially methylate histones resulting in adoption of heterochromatin structure which silences genes (Ebert *et al.* 2004, Figure 5.7).

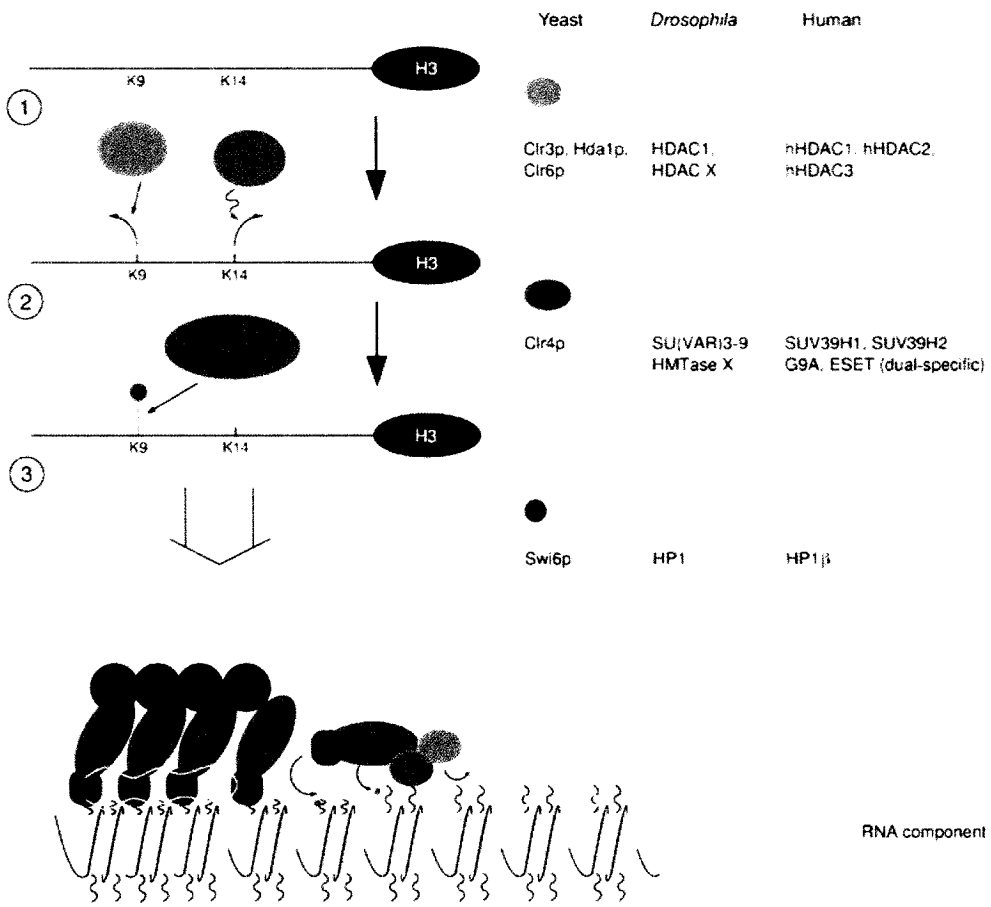

Figure 5.7: Chromatin associated protein interactions. Histone 3 can be acetylated (blue flag)/methylated (red circle) at lysine (K) 9 or 14. (1) Acetylated H3 is (2) deacetylated by HDAC, allowing for (3) H3 methylation by SU(VAR)3-9. H3 methylation recruits HP1 (encoded by *Su(var)2-5* in *Drosophila*). H3/HP1 complexes with other proteins and RNA to compact chromatin, resulting in gene silencing (Schotta *et al* 2003a).

Thus, in *Drosophila*, genes involved in genomic imprinting appear to do so via histone modifications that can mediate chromatin-based gene expression. In mammals, DNA methylation appears to be instrumental for genomic imprinting as it can serve to recruit chromatin and chromatin associated proteins, many of which interact with *Drosophila* homologues involved with imprint maintenance. The function of these proteins appears to be conserved across species, although their role in imprinting in species other than *Drosophila* has not been directly examined.

5.10 Imprinted genes in clones

Imprinted gene regulation is often disrupted during nuclear transplantation, and can have dire consequences for the organism. In sheep, as in mice and humans, *H19/Igf2* and *Igf2r* exhibit differentially methylated imprint control regions and allelic expression is parent of origin dependent (Young *et al.* 2003). Likewise, *H19* is expressed predominately or exclusively from the maternal allele in cows (Zhang *et al.* 2004), and the *Ins* and *Igf2* genes are only expressed from the paternal chromosome in pigs (Nezer *et al.* 2003). Killian and colleagues (2001b) report that imprinting at the *Igf2r* locus is evolutionarily conserved in all rodentia and marsupialia.

To successfully clone an organism, it is necessary to modify chromatin in order to epigenetically reset the donor nuclei, yet faithfully maintain the expression pattern of imprinted genes. Erasing the genomic imprint without subsequent reestablishment can lead to biallelic expression of maternally or paternally imprinted genes resulting in various developmental abnormalities. Treatment of nuclei prior to transplantation can

disrupt the genomic imprint (Young *et al.* 2001), and as nuclei do not pass through the germ line following transplantation, the imprint is not re-established.

Some defects reported in cloned mammals resemble symptoms associated with naturally occurring mammalian diseases attributed to a loss of genomic imprinting. Symptoms like placental overgrowth, increased birth weight, and other fetal abnormalities characteristic of "large offspring syndrome" (Young *et al.* 1998) – such as macroglossia (large tounge); visceromegaly (large organs), including liver distension; and macrosomia (large body size)(Walter and Paulsen 2003) – are reminiscent of Beckwith-Widemann syndrome. However, unlike Beckwith-Widemann syndrome, large offspring syndrome appears to be associated with reduced expression of *Igf2r* rather than over-expression of *Igf2*. Under-expression of *Igf2r* has been reported in sheep embryos cultured for extended periods before implantation, potentially contributing to the disorder (Young *et al.* 2001). Loss of methylation at the second intronic imprint control region may facilitate biallelic expression of *Air*, suppressing *Igf2r* expression (Figure 5.6). This suggests that loss of imprinting affecting other genes in the *Igf* signal pathway, or other imprinted genes, is responsible for the observed developmental aberrations in clones.

In mice, imprinted genes are not limited to *H19/Igf2* and *Igf2r*, and over 70 other murine examples have been documented (Beechey *et al.* 2003). In some cases, examination of cloned mice and the embryonic stem cells from which they are derived indicated a high degree of epigenetic instability. Aberrant expression levels of *H19/Igf2* and other imprinted genes were reported in the placentas and organs of cloned offspring, even though they were derived from a single mouse embryonic stem cell line (Humpherys *et al.* 2001). Furthermore, aberrant methylation patterns at one imprint

control region did not necessarily reflect aberrant methylation patterns at another (Humpherys *et al.* 2001). In contrast, Inoue and colleagues (2002) report faithful conservation of genomic imprinting in mice derived from somatic Sertoli cells, the testis cells that nourish spermatids. This suggests that some imprinting defects result from varying donor cell types or culture conditions prior to transplantation, thus not inherent to all clones, and therefore may be manageable prior to nuclear transplantation or implantation. However, Humpherys and colleagues (2002) report that mice derived from embryonic stem cells and cumulus cells, the ovum support cells, show anomalous expression is about 4% of genes expressed in clone placentas when assessed on microarrays, and that epigenetic errors inherited from the donor nucleus are passed on faithfully. Aberrant genomic imprinting has been reported in cloned cattle that died shortly after birth or exhibited severe developmental defects (Zhang *et al.* 2004). Young and colleagues (2003) report loss of genomic imprinting at the *H19/Igf2* and *Igf2r* loci in cloned sheep; however, the loss of CpG methylation was more pronounced at the *Igf2r* locus.

In *Drosophila*, males derived from nuclear transplantation carrying a theoretically paternally imprinted Dp(1:f)LJ9 mini-X chromosome show little or no *garnet* silencing in the eye, suggesting that even when cloned at early developmental stages the genomic imprint is not faithfully maintained. This concurs with the finding that cloned mice derived from embryonic nuclei suffer from aberrant genomic imprinting (Humpherys *et al.* 2001).

Thus, it should be possible to use this assay system to identify environmental or genetic conditions conducive to imprint maintenance when cloning *Drosophila*. Because

the imprinting mechanism appears to be highly conserved, any findings in the fly could be applicable when generating mammalian clones. While at present *Drosophila* does not provide a comprehensive model addressing all aspects of nuclear transplantation, it may be used to study specific epigenetic gene interactions following cloning, one of the most complex and least understood areas of development.

5.11 Conclusion

The future of nuclear transplant technology depends on managing the complex interplay between genetic and epigenetic variables to increase its overall efficiency. A better understanding of the epigenetic remodelling of the genome during normal development and induced development in cloning could potentially allow us to monitor or even selectively manipulate the epigenetic state of cloned embryos prior to nuclear transplantation. If either therapeutic or reproductive cloning is to become a widely employed practice, increasing nuclear transplant efficiency is crucial to ensuring the process is economically feasible. In doing so, it may be possible to increase the effectiveness of stem cell treatments and the efficiency with which cloned offspring can be generated, and improve the health and well-being of individuals brought to term.

In the last century, *Drosophila melanogaster* has been instrumental in increasing our understanding of many aspects of modern developmental and genetic processes. In the past decade, a greater understanding of the events of early development has led to the advent of reproductive and therapeutic cloning – technology that has the potential to revolutionize modern medicine. *Drosophila* has long been used as model organism in biology and medicine, and the advent of fly cloning presents a unique opportunity to

address some of the more complex challenges associated with therapeutic and reproductive cloning.

6.1 Linking paragraph

This work documents ongoing research that began in September of 2001 at Dalhousie University, Halifax, Nova Scotia. Initially I set out to determine the intracellular location of the *Drosophila* White protein by generating transgenic flies that express white fused to a florescent marker protein. By April of 2003 I had achieved this goal. A number of circumstances led me to attempt the cloning of *Drosophila melanogaster*, as suggested by my supervisor Dr. Vett Lloyd. While the intracellular localization of white and the cloning experiments are not related in theory, they are based on the same microinjection procedure. Following my initial cloning success, during the summer or 2003 I transferred into a Ph.D. program based on a number of projects utilizing our new technique. The *white* paper is included as the following chapter as it was part of my graduate studies at Dalhousie, but will not be considered in the Introduction and Discussion of this thesis. The following chapter will be published as part of a larger research project with Dr. Ian Meinertzhagen.

6. *Sub-cellular localization of the* Drosophila melanogaster *WHITE protein*

6.2 Introduction

The first gene discovered in *Drosophila* was the eye colour gene *white* (Morgan 1910). Since its initial description, *white* has become one of the most intensively studied genes at the genetic, cytological, and molecular levels because of its dramatic phenotype; null mutants have startling white eyes and hypomorphs have eye colours ranging from white through every shade of red (reviewed by Judd 1987, Hazelrigg 1987, Green 1996). As early as 1952, it was suggested that the *white* protein interacts with the *brown* and *scarlet* gene products to impart the characteristic brick-red colour to the wild-type *Drosophila* eye (Nolte 1952). Despite the intense investigation of the gene in its own right, and its extensive use as a marker in crosses and genetic transformation of *Drosophila*, it was not until 1995 that a neural role for the gene was suggested. Zhang and Odenwald (1995) reported that male flies experiencing heat-shock driven expression of the *white* gene began to vigorously court each other, forming long courtship chains (Figure 6.1). Acquisition of homosexual courtship behavior was not a phenotype expected in flies over-expressing a gene previously thought to be involved only in the pigmentation of eyes and internal organs.

The predicted structure of the *white* gene product yields insight into the biological basis of the behavioral phenotype – *white* encodes a protein that belongs to the ATP binding cassette (ABC) family of transmembrane pumps (Mount 1987, Pepling and Mount 1990). These proteins use energy generated by ATP hydrolysis to transport specific substrate molecules through lipid bilayers (Hyde *et al.* 1990). Each pump is

Figure 6.1: Male-male courtship behavior in *Drosophila* induced by *white* gene over-expression. Courtship displays include touching partners with forelegs, wing extension, genital licking, and curling of the abdomen to achieve genital-genital contact. Arrow indicates female fly excluded from male courtship behavior (taken from Zhang and Odenwald 1994).

characterized by two hydrophilic ATP-binding folds and two membrane-spanning domains, each consisting of five to six membrane-spanning alpha helices (Hyde *et al.* 1990). These elements can be present in one protein, such as the multiple drug resistance proteins in humans, or split between two polypeptides that then dimerize, as is the case in *Drosophila* (Dreesen *et al.* 1988). Each nucleotide binding fold contains a Walker A and Walker B motif – highly conserved N-terminus amino acid residues presumably involved in the hydrolysis of ATP (Walker *et al.* 1982). Each *Drosophila* channel subunit consists of six trans-membrane α-helices and the nucleotide binding folds are found at the N-terminus of the protein (Figure 6.2, Ewart *et al.* 1994).

Substrate specificity of the White protein pump is imposed by the protein's binding partner. Mackenzie and colleagues (2000) have proposed that the White-Scarlet heterodimer acts to pump tryptophan-derived 3-hydroxykynurenine into the pigment granules, where it is then converted to the brown ommochrome pigment xanthommatin (Figure 6.3). White is also believed to dimerize with the Brown protein to form a guanosine triphosphate (GTP) transporter (Dreesen *et al.* 1988, Ewart *et al.* 1994). In pigmented cells GTP is converted into the red pteridine pigment drosopterin (Summers *et al.* 1982). By analyzing the sequence of *white* mutant alleles, specific amino acid residues in the trans-membrane α–helices necessary for proper protein dimerization have been identified (Ewart *et al.* 1994, Mackenzie *et al.* 1999). Further, nucleotide changes in the C-terminus hydrophobic region of the protein affect White-Brown interaction, but not White-Scarlet interaction (Ewart *et al.* 1994). No functional significance is prescribed to the C-terminus hydrophilic region. While the *white* gene is most heavily expressed in the eyes and other pigmented organs (Fosje *et al.* 1984), lower levels of *white* mRNA are

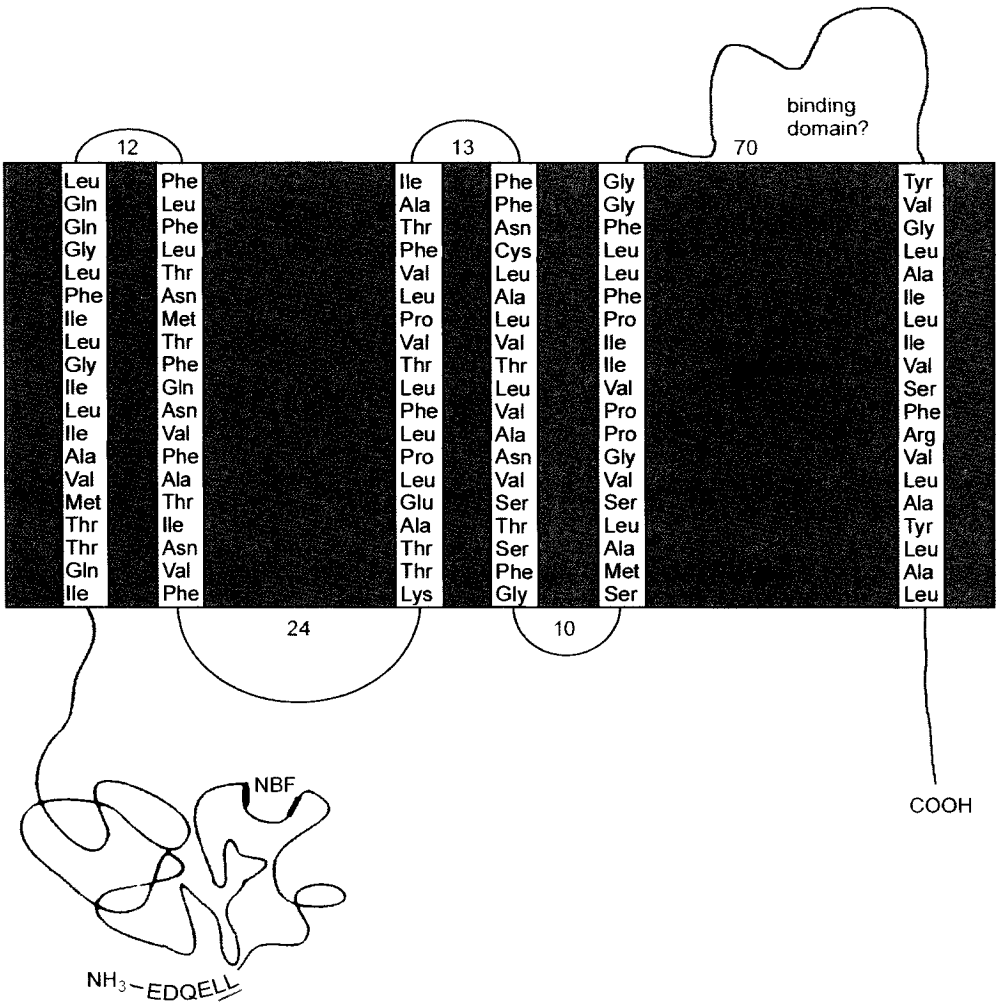

Figure 6.2: Predicted structure of the White protein. Includes six transmembrane alpha-helicies, dileucine residue (as indicated by LL), and the nucleotide binding fold (NBF)(adapted from Ewart *et al.* 1994).

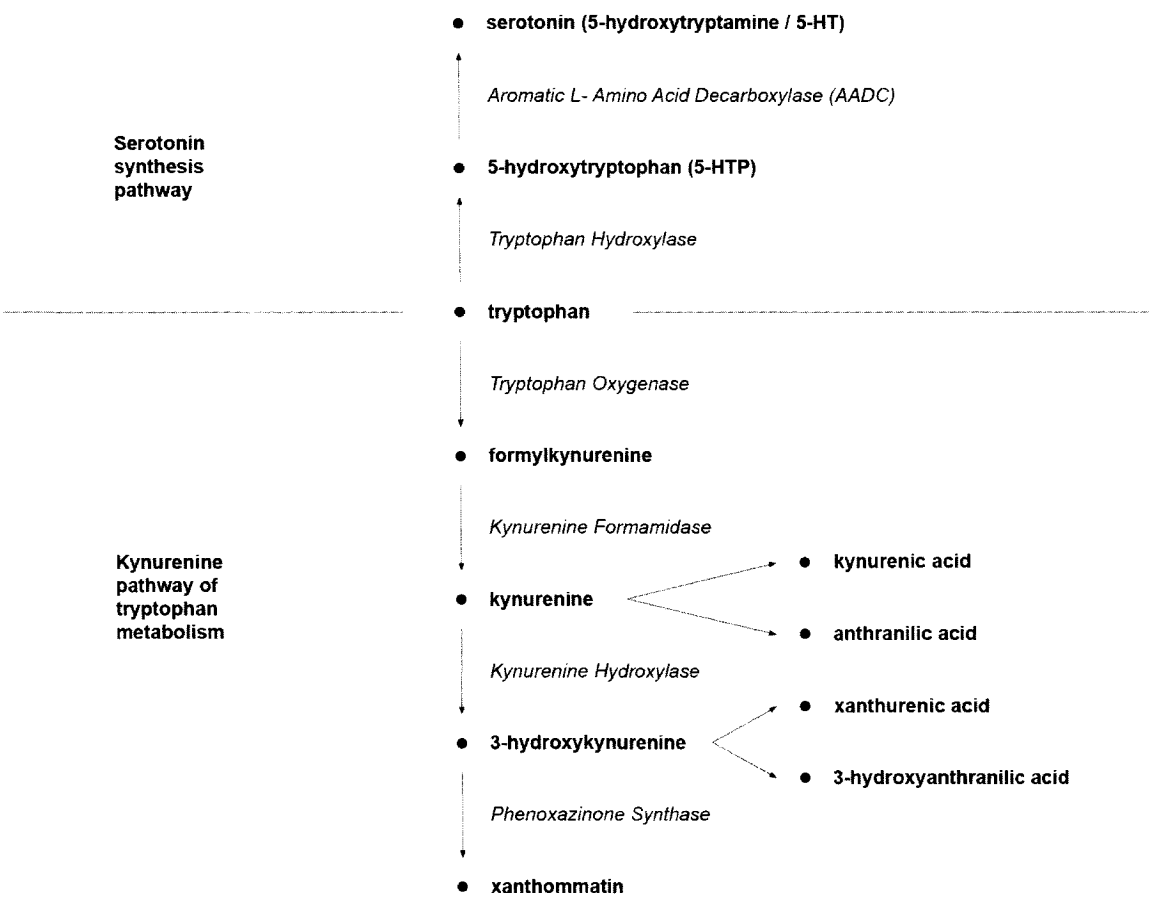

Figure 6.3: Pathways of tryptophan metabolism. In *Drosophila* tryptophan can be metabolized via the serotonin or kynurenine pathways. Enzymes are indicated by italics (Savvateeva 1991, Joh 1997).

detected in the heads of *sine oculis* mutants, which completely lack pigmented cells in the head, by RT-PCR (Campbell and Nash 2001), thus further suggesting a neurobiological role for the *white* gene. Accumulation of the protein pump's proposed substrate, 3-hydroxykynurenine, in *cardinal* mutants, adversely affects learning and memory in *Drosophila* (Savateeva *et al.* 2000), and we have observed learning deficits in *white* mutants (Lloyd *et al.* unpublished observation). Thus, it may be that *white* is necessary for the transport and/or catabolism of 3-hydroxykynurenine in the kynurenine pathway of tryptophan metabolism in the *Drosophila* brain.

Alternately, tryptophan and GTP are involved in amine neurotransmitter production in the brain. Tryptophan is the precursor of 5-hydroxytryptophan, the direct precursor of serotonin (5-hydroxytryptamine, 5-HT, Figure 6.3). GTP serves as the precursor of tetrahydrobiopterin (BH_4), an essential cofactor for the production of tryptophan and dopamine (Joh 1997). In serotoninergic neurons, tryptophan is converted into 5-HT via the serotonin metabolic pathway and then loaded into synaptic vesicles by the *Drosophila* Vesicular Monoamine Transporter (dVMAT, Greer *et al.* 2005, Figure 6.4a). Numerous studies link serotonin to sexual behavior, and *Drosophila fruitless* mutants exhibit reduced neural 5-HT levels and homosexual courtship behavior (Lee and Hall 2001). This suggests that the male-male courtship behavior observed in flies over-expressing the *white* gene is due to disruption of 5-HT synthesis.

The observation that mutants for the classical eye pigment gene *garnet* exhibit male-male courtship indicates how over-expression of *white* could give rise to altered courtship behavior (Lloyd *et al.* 2002). The *garnet* gene encodes the δ-subunit of the AP-3 adapter complex (Ooi *et al.* 1997, Simpson *et al.* 1997, Lloyd *et al* 1999), which is

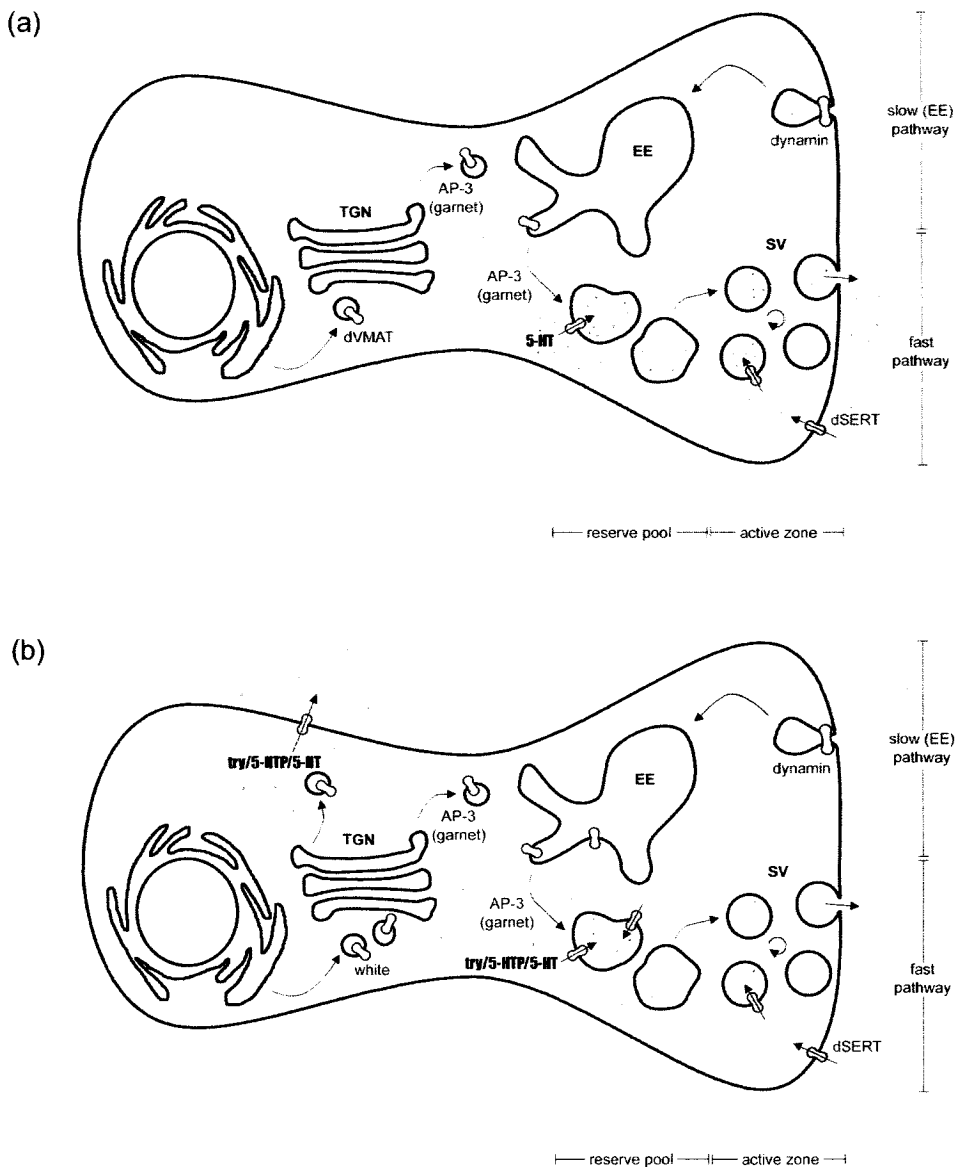

Figure 6.4: Serotonin synthesis and deposition in the nerve cell. (a) Garnet targets *Drosophila* Vesicular Monoamine Transporter (dVMAT) to the endosomal membrane. Serotonin (5-HT, red) is synthesized in the cytoplasm and translocated into synaptic vesicles by dVMAT. Following release of 5-HT into the synaptic cleft, it is recycled through the action of the *Drosophila* Serotonin Transporter (dSERT) or via dynamin-dependent endocytosis. (b) When *white* is overexpressed in serotoninergic neurons it displaces 5-HT or 5-HT precursor molecules, such as tryptophan (try) or 5-hydroxytrptophan (5-HTP), at the endosomal or plasma membrane, resulting in a decrease in cytoplasmic 5-HT levels (EE early endosome; SV synaptic vesicles).

involved in transporting proteins from the trans-Golgi network to endosomes, and therefore to endosomally derived vesicles (Odorizzi *et al.* 1998).

In pigmented cells, the pigment granules are derived from endosomes, and in neurons synaptic vesicles receive their cargo from the endosomes. The presence of a diagnostic di-leucine residue in the N-terminal region of White suggests that the protein is targeted for transport to the endosomal compartment in pigmented cells by the AP-3 complex (Simpson *et al.* 1997), and Mackenzie and colleagues (2000) have localized White to the pigment granule membrane. Likewise, *dVMAT* contains a di-leucine residue, presumably to target the protein to synaptic vesicles. Therefore, faulty delivery of both White and dVMAT in *garnet* mutants may be responsible for the observed eye pigment deficit and behavioral anomalies. Here I incorporate recent findings regarding *white* over-expression into existing models of White function.

Zhang and Odenwald (1995) ubiquitously over-expressed the *white* gene in *Drosophila* via a heat-shock promoter. While *white* is expressed in the brain, it may not usually be expressed in serotoninergic neurons. Thus, heat-shock induced ubiquitous expression would cause *white* expression in tissues where it is normally silenced. As a protein pump involved in transporting tryptophan derived metabolites, it is conceivable that *white* over-expression causes mis-localization of serotonin or serotonin precursor molecules (Figure 6.4b). Further, when *white* is ubiquitously over-expressed from the heat shock promoter, the protein may overwhelm its usual sorting pathway to the endosomes, and the excess protein would be transported from the Golgi body to the plasma membrane via the constitutive default intracellular protein transport pathway. Due to the polarity of the protein at the plasma membrane, it would then pump serotonin or

serotonin precursor molecules out of the cytoplasm, resulting in a global decrease in serotonin availability. This model predicts that (1) genetic anomalies resulting in either *white* over-expression or reduced serotonin production will show male-male courtship behavior, (2) White will be localized to endosomally derived compartments, and that (3) synaptic vesicles in flies over-expressing *white* will be depleted of serotonin.

The goal of this work was to investigate at the molecular and cellular level how over-expression of the *white* eye-colour gene induces homosexual courtship behavior in *Drosophila*. I confirmed that over-expression of the *white* gene induces male-male courtship behavior. I show that a DsRed-tagged version of the White protein and GFP-tagged Garnet appear to co-localize in *Drosophila* S2 and mammalian COS cells, and they are presumably localized to endosomes. In motor neurons, DsRed-tagged White protein co-localizes with synaptic vesicle marker GFP-tagged Syntapotagmin (Zhang *et al* 2002). These data support a model which suggests the *white* gene encodes a transporter that functions at the endosomal membrane.

6.3 Materials and methods

6.3.1 Mutant strains and chromosomes

With the following exceptions, all the mutations used in this study are described in Flybase (1999) and were obtained from the Indiana *Drosophila* stock center. The construction of *DsRed*- and *EGFP*-tagged *white*$^{+}$ and *DsRed*-tagged *garnet* transgenic strains, hereafter referred to as w^{+}*-DsRed*, w^{+}*-EGFP*, and g^{8}*-DsRed*, respectively, is described below.

Molecular analyses

Restriction digests, ligations, and polymerase chain reactions were performed with enzymes and buffers supplied by MBI Fermentas by following standard procedures (Sambrook *et al.* 1989). DNA was precipitated by the addition of NaCl following standard procedures (Sambrook *et al.* 1989) or with the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech Inc.) following the manufacturer's instructions. DNA fragments isolated from (usually) 1% agarose gels were isolated using a GFX™ PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech Inc.) following manufacturer's instructions. DH5α subcloning efficiency competent *E. coli* cells (Invitrogen) were used for standard transformations and plasmid DNA was isolated using an alkaline lysis mini-preparation (Sambrook *et al.* 1989).

6.3.2 Plasmid Construction

Construction of the w^+-DsRed and w^+-EGFP vectors for S2 and COS-1 cell transformation

The pDsRed1-N1-w^+ and pEGFP-N1-w^+ plasmids were created by isolating the mini-*white* gene as a 3396 base pair (bp) *Hind*III-*Apa*I fragment from the 7776 bp pP(CaSper) vector (Genbank accession number X81644). This fragment was then ligated into the pDsRed1-N1 and pEGFP-N1 vector (Clontech) multiple cloning site between the *Hind*III and *Apa*I restriction sites. This resulted in the substitution of the last 3 amino acids of the White protein with either the DsRed or EGFP proteins.

Construction of the g^S-DsRed vector for COS-1 cell transformation

The pEGFP-N1-g^8 plasmid was created by isolating the *garnet* cDNA as a 2265 bp *Eco*RI-*Sal*I fragment from the 6005 bp pUC19-*garnet* vector (Genbank accession number, U31351, Lloyd *et al.*. 1999). This fragment was then ligated into the pDsRed1-N1 vector (Clontech) multiple cloning site between the *Eco*RI and *Sal*I restriction sites.

Construction of the pRmHa-3- w^+-DsRed vector for S2 cell transformation

The *white* gene was isolated as a 3396 base pair (bp) *Hind*III-*Apa*I fragment from the 7776 bp pP(CaSper) vector described below. This fragment was then ligated into the pBluescript II KS +/- (pBs) vector (Stratagene) multiple cloning site between the *Hind*III and *Apa*I restriction sites. DNA sequencing using the T7 and T3 primers confirmed the presence and orientation of the *white* gene. The *DsRed1* gene was amplified via polymerase chain reaction (PCR) from the pDsRed1-N1 vector using primers 5'CCGCGGGCCCGGGATCCACCGGTCGCC3' and 5'GGCGGGCCCTGATCTAGAGTCGCGGCC3' which introduced an *Apa*1 site at the 3' end of the gene. The 738 bp PCR product was isolated, digested with *Apa*I and ligated into the *Apa*I site in the pBs–w^+ plasmid polylinker 3' to the *white* gene. This resulted in the substitution of the last 3 amino acids of the White protein with either DsRed or EGFP amino acids. Presence of in-frame *DsRed* was initially detected by restriction digestion with *Apa*I, and then by DNA sequencing using the T7 and T3 primers. A 4150 bp *Eco*R1–*Kpn*I fragment from the resulting pBs-w^+-DsRed plasmid construct was isolated and ligated into the pRmHa-3 multiple cloning site between the *Eco*RI and *Kpn*I restriction enzyme sites.

Construction of the pP(UAST)-w^+-DsRed vector for microinjection

The w^+-*DsRed* gene construct was isolated as a 4150 bp *Eco*R1–*Kpn*I fragment from pBs-w^+-*DsRed.* The construct was ligated into the multiple cloning site of pP(UAST). Presence of the gene construct was confirmed by restriction digest.

6.3.3 Crosses

All crosses were performed at 22^{O} C unless otherwise stated. Culture medium was standard cornmeal/molasses medium supplemented with 0.04% tegosept as a mold inhibitor (http://fly.bio.indiana.edu/molasses-food.htm).

6.3.4 Transformation

Transformation of COS-1 cells

COS-1 African green monkey kidney cells were maintained in a water-jacketed incubator with 5% carbon dioxide in 25 mL vented culture flasks (Falcon) to allow gas exchange. Cells were raised on Dulbecco's Modified Eagles Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (Gibco) and 2% penicillin/streptomycin 4'-6-Diamidino-2-phenylindole antibiotic mixture (Gibco). Cells were subcultured approximately every 4 to 5 days. For transfection, 700 ng plasmid DNA was added to 4 μL Superfect^{TM} transfection reagent (Qiagen) and vigorously mixed before being added to a COS-1 subculture in a sterile six-well culture plate (Falcon), with each well containing a sterile cover slip. Cells were then cultured for 48 to 72 hours before fixation, 4'-6-Diamidino-2-phenylindole (DAPI) staining to label nuclei, and inspection using either a Zeiss LSM 410 confocal or a Leitz Aristoplan microscope.

Transformation of S2 cells

Drosophila S2 (Schneider's 2) cells were maintained following standard procedures (Cherbas *et al.* 1997) at 27°C in 25 mL vented culture flasks (Falcon), and grown in Shield and Sang's (M3) media (Sigma) supplemented with 10% fetal bovine serum (Gibco) and 2% penicillin/streptomycin antibiotic mixture (Gibco). S2 cells were subcultured approximately every three days when the cells reached 70% confluence. Transfection was carried out by vortexing for 30 sec., 1 mL M3 media containing no sera or antibiotics, 150 ng plasmid DNA and Cellfectin™ transfection reagent (Invitrogen) in a 10 mL sterile tube (Falcon). After 30 minutes, concentrated S2 cells, harvested at 50-60% confluence, were added and the tube incubated horizontally at 27°C for 4 hours. Following incubation, the cells were pelleted and gently resuspended in 3 mL M3 containing 10% fetal bovine serum and 2% penicillin/streptomycin, and aliquoted into 3 wells of a sterile six-well culture plate (Falcon), with each well containing a sterile cover slip. The culture plate was wrapped in Parafilm and placed at 27°C overnight. The following day sterilized $CuSO_4$-$5H_20$ was added to each well to a final concentration of 0.5 - 0.7 mM. Plates were wrapped in Parafilm and returned to the 27°C incubator for 72 hours before fixation, DAPI staining and inspection using a Zeiss LSM 150 confocal microscope. Fixation, staining with DAPI, and detection of DsRed fluorescence was done according to standard procedure (Clontech).

Generation of transgenic Drosophila lines

w[1118]/Dp(1;Y)y[+]; ry[506] Dr[1] P{ry[+t7.2]=Delta2-3}99B/TM6C, Sb[1] *Drosophila* embryos (Sved *et al.*. 1990) were injected with 0.5μg/uL pP(UAST)-w^+-

DsRed plasmid purified by two successive phenol-chloroform extractions, precipitation, and concentration, using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech Inc.). Generation of transgenic *Drosophila* was performed essentially as described by Spradling (1986). Embryos surviving injection to hatch were selected as first-instar larvae and were cultured on standard *Drosophila* medium and crossed to w^{1118} individuals of the appropriate sex to detect successful transformants. The chromosome into which the pP(UAST)-w^{+}-*DsRed* had inserted was determined through standard crosses with a *w, dp, e, ci* strain marked on all 4 chromosome sets.

6.3.5 Intracellular localization of DsRed-*tagged* White

An X chromosome insert of w^{+}-*DsRed* was combined with an X chromosome *synaptotagmin-EGFP* transgene (Broadie *et al.* 2002) and a third chromosome ubiquitously expressed *tubulin-GAL4* driver transgene. Fillets of w^{+}-*DsRed* third-instar larvae were prepared as described by Bellen and Budnik (2000) and fluorescence microscopy was used to detect the DsRed-tagged White and EGFP-tagged Synaptotagmin protein at the neuromuscular junctions.

6.4 Results

6.4.1 Intracellular localization of the white protein

In order to investigate the intracellular localization of the White protein, *DsRed* tagged versions of the *white* gene were generated for expression in *Drosophila* S2 cells

and mammalian COS cells, and COS cells were co-transfected with an *EGFP*-tagged *white* gene and a *DsRed*-tagged *garnet* gene.

When expressed in *Drosophila* S2 embryonic cells, DsRed fluorescence is seen in a punctate perinuclear distribution consistent with localization to the endosomal compartment (Figure 6.5a). When expressed in the larger mammalian COS-1 cells the fluorescence of the DsRed-tagged White protein is again punctuate and perinuclear (Figure 6.5b). The distribution of EGFP-tagged White largely overlaps that of DsRed-tagged Garnet protein co-transfected into the COS-1 cells (Figure 6.6).

To investigate White protein expression in specialized cells such as pigment cells and neurons, flies transgenic for *DsRed* tagged *white* gene were generated. In these flies, the coding region of the fluorescent-tagged *white* gene was placed downstream from a GAL-4 inducible promoter, so that transgene expression was induced by crossing flies containing *DsRed-w*$^{+}$ to flies transgenic for the yeast GAL4 transcriptional activator expressed in a suitable tissue (Brand and Perrimon 1993). Ubiquitous expression of DsRed-tagged White protein and EFGP-tagged Synaptotagmin using the same *tubulin* GAL4 driver strain allowed us to examine co-localization of White and Synaptotagmin at the neuromuscular junction. Synaptotagmin is found in synaptic vesicles and, at a lesser level, in a diffuse distribution on the plasma membrane, presumably because it is recycled following synaptic transmission (Zhang *et al.* 2002). We found White and Synaptotagmin appear to co-localization in the boutons of the third-instar neuromuscular junction (Figure 6.7).

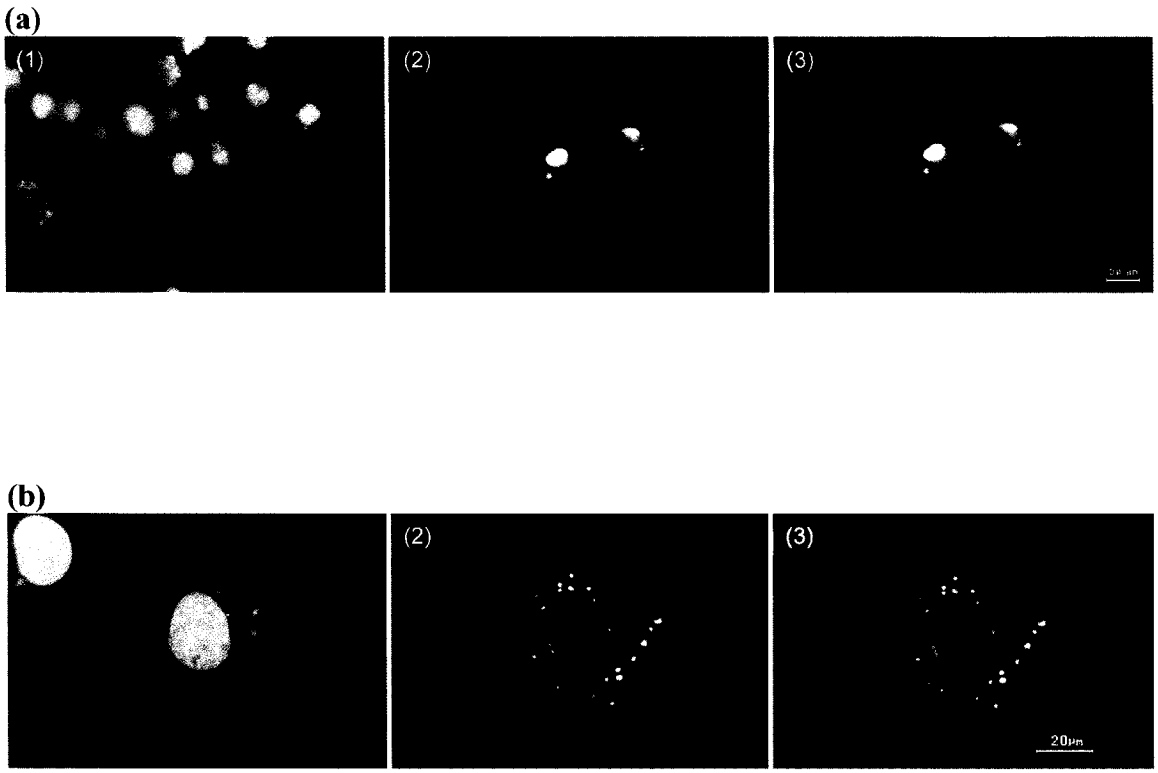

Figure 6.5: Intracellular localization of White in S2 and COS cells. (a) The pRmHa-3-white-DsRed vector was expressed in S2 cells. (1) Cell nuclei are stained blue (DAPI) and (2) red florescence indicates White expression. (3) Merged images. (b) The pDsRed1-N1-w^{+} was expressed in COS cells. (1) Cell nuclei are stained blue (DAPI) and (2) red florescence indicates White expression. (3) Merged images. White protein expression can be observed surrounding the cell nucleus and in the cytoplasm. Florescent microscopy, Leitz Aristoplan, Leica A and I3 filter blocks, respectively.

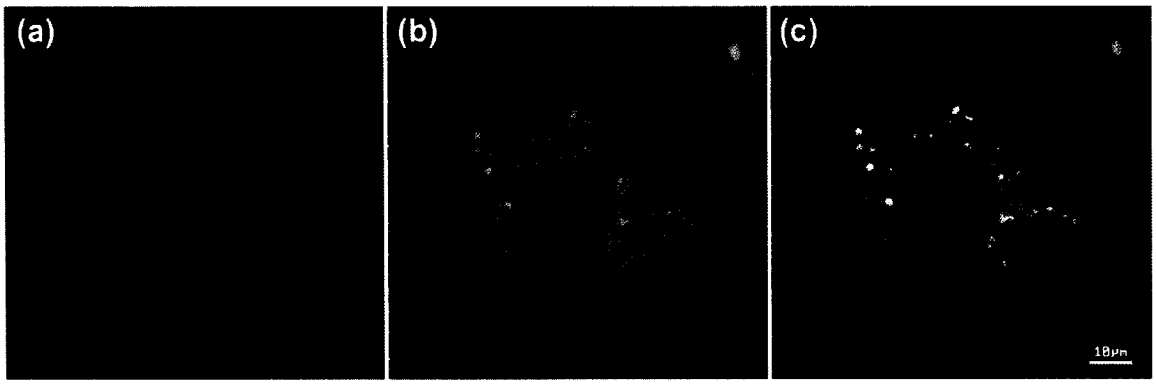

Figure 6.6: COS cells transfected with pDsRed1-N1-g^8 and pEGFP-N1-w^+. (a) COS cell expressing the pDsRed1-N1-g^8 (red) (b) COS cell expressing the pEGFP-N1-w^+ gene construct (green) (c) Merged images. Combined pDsRed1-N1-w^+ and pEGFP-N1-g^8 gene construct expression. Overlapping florescence appears yellow indicating colocalization. Note punctate distribution throughout the cytoplasm. Confocal microscopy; LSM 410, FITC and rhodamine filters, respectively.

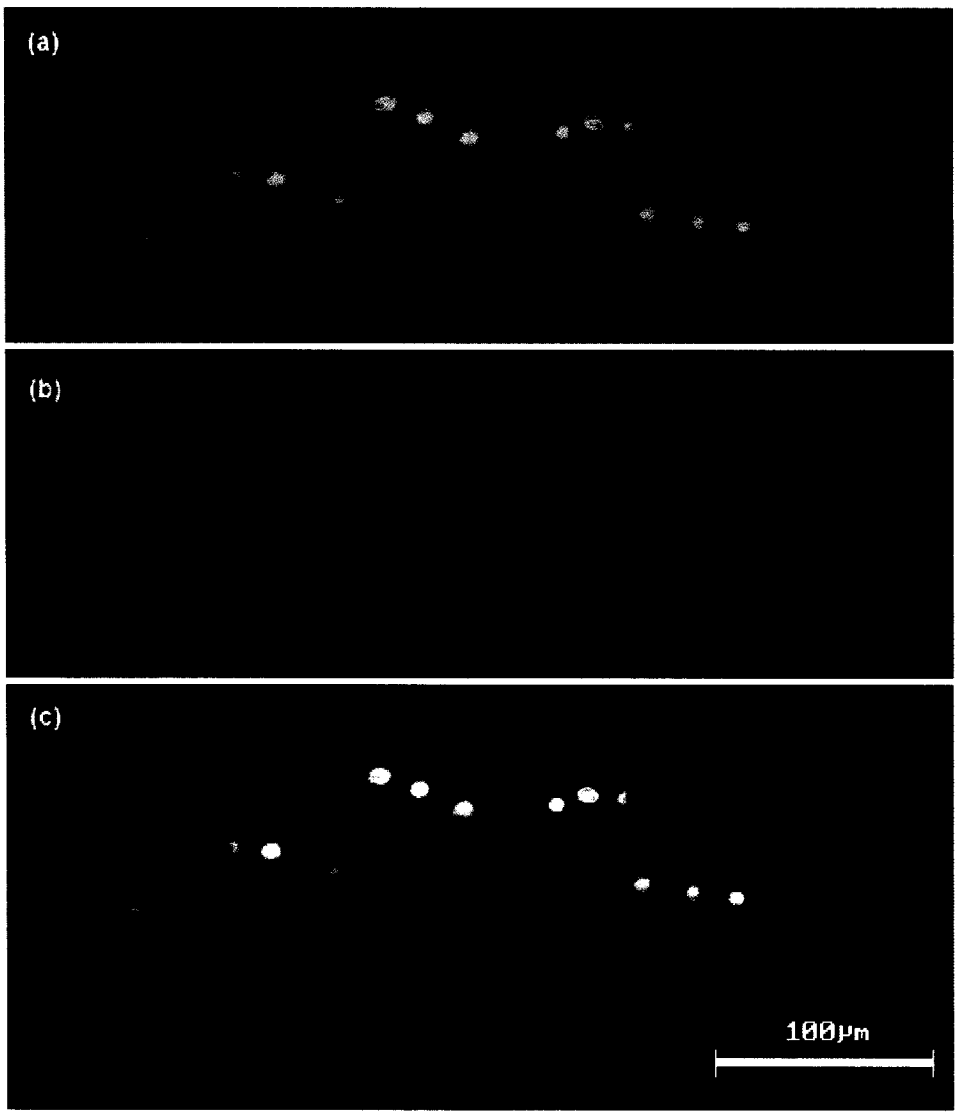

Figure 6.7: Colocalization of White and Synaptotagmin at the *Drosophila* 3rd instar larval neuromuscular junction. Larval fillets—midline runs down center of image. (a) EGFP tagged Synaptotagmin indicates synaptic vesicles at the neuromuscular junction (b) pP(UAST)-*white-DsRed* expression at the neuromuscular junction driven by *tubulin*-GAL4. (c) Areas of White and Synaptotagmin colocalization appear yellow. Confocal microscopy; LSM 410, FITC and rhodamine filters, respectively.

6.5 *Discussion*

The initial observation of energetic male-male courtship behavior in mini-*w* *Drosophila* (Zhang and Odenwald 1995) was surprising and provided the first evidence that the *white* gene may have a role other than in pigmentation. The observed male-male courtship behavior is not due to misinterpretation of sensory cues in the external environment (Zhang and Odenwald 1995, Hing and Carlson 1996) and the reversible nature of the effect precluded anatomical modifications. Thus, the alteration in behavior is likely due to internal physiological changes caused by over-expression of the *white* gene. Examination of the behavior of individuals mutant for the *white* gene, individuals mutant for the genes involved in serotonin production and transport, and the intracellular localization of White and dVMAT, offers insight into the gene's effect on neural function and the cellular and biochemical basis for the observed male-male courtship behavior.

While *white* appears to play a neurobiological role in the fly (Campbell and Nash 2001), heat-shock induced over-expression of the *white* gene causing male-male courtship may be due to ectopic expression in serotoninergic neurons. When expressed, White may transport tryptophan-derived substrates, as it appears to do in the eye, such as serotonin or serotonin precursor molecules. The protein pump may transport 5-hydroxytrptophan into endosomally derived vesicles, thus reducing cytoplasmic levels of the precursor necessary for serotonin production. Alternately, *white* over-expression may result in the relocation of surplus White transmembrane channel protein to the plasma membrane. There, it could drain the cell of neurotransmitter or neurotransmitter precursors normally needed for loading into synaptic vesicles through the correctly localized dVMAT, thus lowering the overall cytoplasmic concentration of the amine

metabolite. White appeared to colocalize with the synaptic vesicle protein Synaptotagmin at the neuromuscular junction, suggesting that when expressed in neurons, the *white* protein may be transported to the synaptic vesicle membrane.

Reduction in intracellular serotonin levels is linked to male-male courtship behavior in *Drosophila* and mammals. Most notably, feeding male rats *p*-chlorophenylalanine, an inhibitor of serotonin biosynthesis, and feeding rabbits a diet lacking tryptophan, the amino acid precursor of serotonin, induced male homosexual mounting behavior (Tagliamonte *et al.* 1969, Fratta *et al.* 1977) superficially similar to that observed in *Drosophila*. Additionally, by selectively disrupting synaptic vesicle transmission in the central and peripheral neurons by blocking the synaptic vesicle recycling action of dynamin in *Drosophila* Kitamoto (2002) was able to induce male-male courtship behavior similar to that observed in flies over-expressing the *white* gene. *Drosophila fruitless* mutants, which fail to innervate the abdominal ganglion with serotoninergic neurons, also exhibit male-male courtship behavior (Lee and Hall 2001).

HPLC studies have indicated that *Drosophila white*, *scarlet*, and *brown* mutants exhibit reduced intracellular and/or intravesicular serotonin levels (Meinertzhagen *et al.* personal communication). While there may not yet be an established functional link between the kynurenine and serotonin pathways of tryptophan metabolism, the possibility exists that the *white*, *scarlet*, and *brown* genes and the *dVMAT* gene are coordinately regulated. Thus, mutation or mis-expression in protein pumps involved in the kynurenine metabolism pathway may affect expression of *dVMAT*, causing reduced vesicular amine neurotransmitter levels and male-male courtship behavior in *Drosophila*. It is conceivable that genetic regulation of the serotonin pathway of tryptophan metabolism, established

later in development, is dependent on the kynurenine pathway of tryptophan metabolism, as the latter is the primary pathway in the developing embryo (Savvateeva *et al.* 2000). The finding that *brown* and *scarlet* mutants show male-male courtship behavior (Barkova 2002) also suggests that discordant regulation of genes involved in tryptophan metabolism is be responsible for this behavior. Certainly, evidence exists for coordinate gene regulation involving the *white* locus (Rabinow *et al.* 1991, Bhadra *et al.* 1997). Alternately, White heterodimers may transport amine neurotransmitters in some neurons while dVMAT functions in others, and genetic disruption of *white* or its binding partners may upset 5-HT production or transport.

That the *white* gene was the focus of hundreds of studies over the course of 85 years before its role in behavior was discovered is remarkable. One reason for this oversight is that it is only over-expression of the *white* gene that produces a change in the sexual behavior of male flies; null and hypomorphic *white* mutations do not induce any obvious change in male or female sexual behavior. However, it appears that *white* null mutants exhibit learning defects, perhaps due to build-up of 3-hyroxykenurenine, as is the case in *cardinal* mutants (Savvateeva *et al.* 2000).

This work provides the first evidence that the Garnet and White proteins colocalize, and suggests that White appears to be found primarily in endosomally derived structures such as synaptic vesicles. Moreover, that the Garnet and White proteins localize to endosome-like structures in mammalian COS-1 cells shows a remarkable conservation of protein structure and function in the AP-3 intracellular sorting mechanism. The AP-3 complex is heterotetrameric, and in order for the tagged *Drosophila garnet*/delta AP-3 subunit to function when expressed in COS-1 cells it must

form a working complex with the other three mammalian subunits. The co-localization of the Garnet and White fluorochromes implies that the *Drosophila* DsRed-tagged Garnet protein is recognized by the other members of the mammalian AP-3 complex and that this hybrid AP-3 complex functions correctly and recognizes the tagged White protein.

Interestingly, two human homologues of the *Drosophila white* gene have been identified recently and, as in *Drosophila*, the homologous human gene is expressed throughout the body and in the brain (Croop *et al.* 1997, ABCG4 paper). Polymorphisms in one of them, *ABCG1* (*ABC8*), are associated with mood and panic disorders in males (Nakamura *et al.* 1999). *ABCG1* is implicated in other conditions such as cholesterol and phospholipid metabolism problems, a form of deafness, and as a contributor to the etiology of Down's syndrome (Nakamura *et al.* 1999, Klucken *et al.* 2000, Haiming *et al.* 1996). Investigation of the action of this highly conserved gene will offer insight into the pathways of tryptophan metabolism in the cell and contribute to our understanding of the genetic basis of behavior.

7.1 Linking Paragraph

The following is a discussion of the cloning work presented herein. I look at the differences between our approach and that of previous attempts to clone *Drosophila*, and what ultimately led to our success. I present some data from other cloning experiments performed over the past two years.

7.2 Discussion

This work describes the first successful generation of a cloned adult insect. *Drosophila melanogaster* has long been the subject of genetic studies, and given the generation time, cost effectiveness, and ease with which the genome can be manipulated, it was the logical candidate for the extension of nuclear transfer research to insects. Genomic imprinting is well characterized in *Drosophila*, and there exists a wealth of genetic mutations that may affect nuclear reprogramming when cloning flies. Furthermore, stem cell populations and *Drosophila* cell cultures are well characterized, and could potentially serve as nuclear donors. This work includes *Drosophila* in the group of organisms that can be cloned, and represents the first step in potentially adopting the fruit fly as a model organism for cloning research.

Attempts to clone the fruit fly were initiated in the late sixties (Illmensee 1968), but were hindered by the embryology of the organism. Early *Drosophila* embryos exist as syncytial blastoderms, and undergo extensive nuclear replication prior to cellularization. Thus, physically enucleating the multinucleate developing embryo prior to nuclear transplantation is difficult, if not impossible, to accomplish, and early attempts involved injecting blastula stage nuclei into unfertilized embryos or embryos fertilized by irradiated sperm. Extensive efforts produced very few larvae and no pupae or adults (Illmensee 1970), and further attempts to clone *Drosophila* by Illmensee and other researchers were abandoned.

The advent of mammalian cloning from somatic cells in the late nineties renewed interest in generating *Drosophila* clones, but the theoretical problem of how to enucleate the fruit fly embryo prior to nuclear transplantation still remained. However, since the

first unsuccessful attempts to clone *Drosophila*, a wealth of information about genetic mutations affecting gametogenesis and early development was introduced. The *ms(3)K81*^{1} mutation is unusual among male-sterile mutations as it is one of the few that produces developmental arrest after the sperm enters the egg, rather than during sperm production or mating (Yasuda *et al.* 1995). We theorized that by using a genetic mutation that blocks syngamy, the fusion of the sperm and the egg nucleus, we could create normally fertilized and activated, yet non-viable, recipient embryos. Thus, the resulting haploid embryo is physiologically activated upon sperm entry, but invariably suffers from developmental arrest, and is therefore unable to hatch as a first instar larva. By introducing diploid blastula-stage nuclei into newly laid, activated non-viable embryos by microinjection, I was able to generate adult *Drosophila* clones (Haigh *et al.* 2005).

Once produced, demonstrating that flies were clones and not either chimeras, a mixture of donor and recipient nuclei, or due to contamination resulting from a mix-up of donor and recipient embryos, proved somewhat challenging since the cloned embryos are theoretically identical to donor embryos with respect to genomic DNA content. Abnormalities in telomere length have been reported in some clones, but were not investigated in this study. In order to definitively demonstrate that I had generated cloned *Drosophila* I relied on three characteristics intrinsic to our initial method. First, all clones were derived from recipient embryos generated by *ms(3)K81*^{1} males mated to w^{1118} females, so that none of the non-viable recipient embryos, during experimental trials or otherwise, should hatch as viable larvae without outside intervention. Therefore, any embryos that hatched following nuclear transplantation would have developed under the control of the donor nuclei. The recipient stocks were constantly monitored for the

presence of larvae, potentially indicative of either the presence of non-virgin females or males that were not of the *ms(3)K81*^{1} genotype. Such contamination occurred once and was detected by the observation of a chimeric *H2A-GFP*/wild-type larva. Examination of the recipient stock revealed that it was contaminated by a wild-type male and viable larvae were found. All recipient stocks and larvae derived from experimental trials potentially affected by the contamination were destroyed. Second, in each case the cloned offspring were phenotypically identical to the donor stock rather than the recipient. In initial trials donor nuclei contained a marker transgene expressing both green florescent protein (GFP) and the *Drosophila white* gene, and as such, donor flies exhibited both GFP florescence and pigmented eyes. These nuclei were injected into recipient embryos derived from white eyed (w^{1118}) females not exhibiting GFP. All cloned offspring ubiquitously exhibited GFP florescence and eye pigmentation characteristic of the nuclear donor. Third, cloned offspring contained the nuclear DNA of the donor strain and primarily or exclusively the mitochondrial DNA of the recipient. Tandem mitochondrial DNA repeat sequences vary between populations of *Drosophila*, and this difference is detectable using PCR. By using acrylamide gel electrophoresis, I resolved an approximately 14 base pair divergence in a variable stretch of mitochondrial DNA that fortuitously differed between the donor and recipient strains. Thus, I demonstrated that clonally derived offspring contained the nuclear DNA of their donor while retaining the mitochondrial DNA of the recipient embryo (Haigh *et al.* 2005).

Our results concur with Illmensee's conclusion that blastula stage nuclei can support development of an array of somatic structures and are therefore not determined at this stage (Illmensee 1972). However, we cannot yet speculate on the totipotency of

somatic nuclei in *Drosophila*. All cloned offspring generated in this study were derived from nuclei 70-100 minutes old at 22°C, and thus were transplanted before or around the time of the mid-blastula transition – the point at which zygotic gene expression commences and the embryo is no longer under complete maternal control (Foe *et al.* 1993). As such, extensive nuclear dedifferentiation or dedetermination is likely unnecessary to support embryonic development through to adulthood. Further studies to determine the developmental capacity of late-blastula, gastrula, and later somatic and germ-cell nuclei, should follow this work.

After refining the initial cloning technique I attempted to increase cloning efficiency through genetic manipulation. Chromatin formation is essential for the determination and differentiation of cells and tissue types through terminal silencing of various developmental genes. In order to successfully clone an organism from a late stage nucleus it is necessary to epigenetically "reset" the nucleus prior to transplantation, which involves chromatin rearrangement. We theorized that because *Suppressor of variegation* (*Su(var)*), *trithorax* (*trx*), and *Polycomb* (*Pc*) group proteins interact with chromatin to "lock in" cell fate by interacting with histone proteins (Schotta *et al.* 2003a), mutations in these genes may relax chromatin-associated nuclear determination. Thus, nuclei mutant for these genes may be more amenable for nuclear transplant. In each case we used donor nuclei that had the potential to produce viable offspring, including *Su(var)205*^{1}, *Su(var)205*^{4}, *Su(var)3-9*, *l(2)gl*, and *trx/brm*; however, we did not observe an increase in cloning efficiency, as determined by χ^2 and G-tests (Table 7.1). If we eventually generate cloned adults from somatic nuclei, manipulation of genes such as these may have an

Table 7.1: Development *Drosophila* clones with genetic mutations: Donors carried the genetic mutation and the GFP marker transgene. Total number injected of each genotype is shown, as is total number exhibiting GFP (driven by intrinsic GAL-4), number that hatched as larvae, and number that eclosed as adults.

	Kr-Gal-4 **(control)**	*Su(var)205*^{1}	*Su(var)205*^{4}	*Su(var)3-9*	*l(2)gl*	*trx/brm*	**Total**
injected	193	174	180	190	316	188	1241
GFP	21	12	7	7	10	5	62
hatch	7	1	3	0	1	0	12
Adult (G, χ^2)	2	0(2.063, 1.813)	2(0.004, 0.005)	0(2.201, 1.979)	1(0.864, 1.060)	0(2.184, 1.958)	5

effect on cloning efficiency, but using embryonic nuclei I did not produce a significant increase in cloning efficiency.

Genomic imprinting can be lost in mammals derived from embryos manipulated prior to implantation, including those cloned from either embryonic or somatic nuclei. This loss of imprint has been reported in a number of mammalian clones (Humphereys *et al.* 2001, Zhang *et al.* 2004) and often results in "large offspring syndrome", a term used to describe a collection of symptoms including foetal overgrowth and organ distension (Young *et al.* 1998), presumably due to disruption of imprinted genes in the *insulin-like growth factor 2* signalling pathway. Genomic imprinting in *Drosophila* can be detected as differences in eye colour variegation due to imprinted expression, and therefore partial silencing of the *garnet* gene on a synthetic mini-X chromosome when the gene is placed next to a presumably endogenous imprinted region. When the chromosome is maternally inherited, the *garnet* gene is expressed in every cell of the eye, but paternal transmission of the gene results in mosaic *garnet* expression, and thus variegated eye colouration. I determined that the paternal genomic imprint at the *garnet* locus was lost during nuclear transplant.

My results indicate that the genomic imprint associated with the mini-X chromosome is lost in *Drosophila* derived from embryonic nuclear transplant. Of the seven male clones derived from donor nuclei that should exhibit a variegated eye phenotype, all showed little or no eye colour variegation, indicating that the genomic imprint had been lost. Similar loss of genomic imprinting in clones derived from embryonic nuclei was reported for mice (Humpherys *et al.* 2001, Humpherys *et al.* 2002).

This result suggests that *Drosophila* will be a useful tool when examining the epigenetic alterations associated with cloning.

Drosophila has served as a model organism for genetics since the advent of the field itself. Following the initial report of a white-eyed mutant almost 100 years ago, we have fully deciphered the organism's genome, conducted numerous large scale screens for genetic mutations, and painstakingly documented every stage of development. We are now able to generate *Drosophila* by nuclear transplantation, providing us a new technique with which to investigate the epigenetics surrounding cell determination and differentiation. Moreover, as genomic imprinting is compromised in cloned *Drosophila*, this provides a unique opportunity to assess potential genetic or environmental manipulations aimed at imprint re-establishment or maintenance. This technology is already being adopted by other laboratories around the world, and I am confident that its employment will result in useful contributions to the fields of genetics, cloning, and developmental biology.

8. *References*

Aagaard,L., Laible,G., Selenko,P., Schmid,M., Dorn,R., Schotta,G., Kuhfittig,S., Wolf,A., Lebersorger,A., Singh,P., Reuter,G. and Jenuwein,T. (1999) Functional mammalian homologues of the *Drosophila* PEV-modifier *Su(var)3-9* encode centromere-associated proteins which complex with the heterochromatin component M31. *EMBO J,* **18,** 1923-1938.

Aaron,C.S. (1977) Homosexual behavior induced in *Drosophila* by sodium tungstate. *Drosophila Information Service,* **52,** 57.

Aasland,R., Gibson,T.J. and Stewart,A.F. (1995) The PHD finger: implications for chromatin-mediated transcriptional regulation. *Trends Biochem.Sci.,* **20,** 56-59.

Adams,M.D., Celniker,S.E., Holt,R.A., Evans,C.A., Gocayne,J.D., Amanatides,P.G., Scherer,S.E., Li,P.W., Hoskins,R.A., Galle,R.F., George,R.A., Lewis,S.E., Richards,S., Ashburner,M., Henderson,S.N., Sutton,G.G., Wortman,J.R., Yandell,M.D., Zhang,Q., Chen,L.X., Brandon,R.C., Rogers,Y.H., Blazej,R.G., Champe,M., Pfeiffer,B.D., Wan,K.H., Doyle,C., Baxter,E.G., Helt,G., Nelson,C.R., Gabor,G.L., Abril,J.F., Agbayani,A., An,H.J., Andrews-Pfannkoch,C., Baldwin,D., Ballew,R.M., Basu,A., Baxendale,J., Bayraktaroglu,L., Beasley,E.M., Beeson,K.Y., Benos,P.V., Berman,B.P., Bhandari,D., Bolshakov,S., Borkova,D., Botchan,M.R., Bouck,J., Brokstein,P., Brottier,P., Burtis,K.C., Busam,D.A., Butler,H., Cadieu,E., Center,A., Chandra,I., Cherry,J.M., Cawley,S., Dahlke,C., Davenport,L.B., Davies,P., de Pablos,B., Delcher,A., Deng,Z., Mays,A.D., Dew,I., Dietz,S.M., Dodson,K., Doup,L.E., Downes,M., Dugan-Rocha,S., Dunkov,B.C., Dunn,P., Durbin,K.J., Evangelista,C.C., Ferraz,C., Ferriera,S., Fleischmann,W., Fosler,C., Gabrielian,A.E., Garg,N.S., Gelbart,W.M., Glasser,K., Glodek,A., Gong,F., Gorrell,J.H., Gu,Z., Guan,P., Harris,M., Harris,N.L., Harvey,D., Heiman,T.J., Hernandez,J.R., Houck,J., Hostin,D., Houston,K.A., Howland,T.J., Wei,M.H., Ibegwam,C., Jalali,M., Kalush,F., Karpen,G.H., Ke,Z., Kennison,J.A., Ketchum,K.A., Kimmel,B.E., Kodira,C.D., Kraft,C., Kravitz,S., Kulp,D., Lai,Z., Lasko,P., Lei,Y., Levitsky,A.A., Li,J., Li,Z., Liang,Y., Lin,X., Liu,X., Mattei,B., McIntosh,T.C., McLeod,M.P., McPherson,D., Merkulov,G., Milshina,N.V., Mobarry,C., Morris,J., Moshrefi,A., Mount,S.M., Moy,M., Murphy,B., Murphy,L., Muzny,D.M., Nelson,D.L., Nelson,D.R., Nelson,K.A., Nixon,K., Nusskern,D.R., Pacleb,J.M., Palazzolo,M., Pittman,G.S., Pan,S., Pollard,J., Puri,V., Reese,M.G., Reinert,K., Remington,K., Saunders,R.D., Scheeler,F., Shen,H., Shue,B.C., Siden-Kiamos,I., Simpson,M., Skupski,M.P., Smith,T., Spier,E., Spradling,A.C., Stapleton,M., Strong,R., Sun,E., Svirskas,R., Tector,C., Turner,R., Venter,E., Wang,A.H., Wang,X., Wang,Z.Y., Wassarman,D.A., Weinstock,G.M., Weissenbach,J., Williams,S.M., WoodageT,

Worley,K.C., Wu,D., Yang,S., Yao,Q.A., Ye,J., Yeh,R.F. and Zav(TRUNCATED). (2000) The genome sequence of *Drosophila melanogaster*. *Science,* **287,** 2185-2195.

Alberio,R. and Campbell,K.H. (2003) Epigenetics and nuclear transfer. *Lancet,* **361,** 1239-1240.

Alcaniz,S. and Silva,F.J. (1997) Phenylalanine hydroxylase participation in the synthesis of serotonin and pteridines in *Drosophila melanogaster*. *Comp. Bioc. and Phys. C.,* **116,** 205-212.

Baguisi,A., Behboodi,E., Melican,D.T., Pollock,J.S., Destrempes,M.M., Cammuso,C., Williams,J.L., Nims,S.D., Porter,C.A., Midura,P., Palacios,M.J., Ayres,S.L., Denniston,R.S., Hayes,M.L., Ziomek,C.A., Meade,H.M., Godke,R.A., Gavin,W.G., Overstrom,E.W. and Echelard,Y. (1999) Production of goats by somatic cell nuclear transfer. *Nat.Biotechnol.,* **17,** 456-461.

Barkova,E. (2002) Characterization of hypersexual behavior associated with granule group mutations and serotinin levels in *Drosophila melanogaster*. BScHon thesis. Dalhousie University.

Barlow,D.P. (1995) Gametic imprinting in mammals. *Science,* **270,** 1610-1613.

Bean,C.J., Schaner,C.E. and Kelly,W.G. (2004) Meiotic pairing and imprinted X chromatin assembly in *Caenorhabditis elegans*. *Nat.Genet.,* **36,** 100-105.

Beaujean,N., Hartshorne,G., Cavilla,J., Taylor,J., Gardner,J., Wilmut,I., Meehan,R. and Young,L. (2004) Non-conservation of mammalian preimplantation methylation dynamics. *Curr.Biol.,* **14,** R266-7.

Beaujean,N., Taylor,J., Gardner,J., Wilmut,I., Meehan,R. and Young,L. (2004) Effect of limited DNA methylation reprogramming in the normal sheep embryo on somatic cell nuclear transfer. *Biol.Reprod.,* **71,** 185-193.

Beechey,C.V., Cattanach,B.M., Blake,A. and Peters,J. (2003) Mouse Imprinting Data and References *MRC Mammalian Genetics Unit, Harwell, Oxfordshire.,* http://www.mgu.har.mrc.ac.uk/research/imprinting.

Beetschen,J.C. and Fischer,J.L. (2004) Yves Delage (1854-1920) as a forerunner of modern nuclear transfer experiments. *Int.J.Dev.Biol.,* **48,** 607-612.

Bellen,H.J. and Budnik,V. (2000) The neuromuscular junction. In Sullivan,W., Ashburner,M. and Hawley,R.S. (eds.), *Drosophila Protocols.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 175-199.

Bellen,H.J., Levis,R.W., Liao,G., He,Y., Carlson,J.W., Tsang,G., Evans-Holm,M., Hiesinger,P.R., Schulze,K.L., Rubin,G.M., Hoskins,R.A. and Spradling,A.C. (2004) The BDGP gene disruption project: single transposon insertions associated with 40% of *Drosophila* genes. *Genetics,* **167,** 761-781.

Bhadra,U., Pal-Bhadra,M. and Birchler,J.A. (1997) A trans-acting modifier causing extensive overexpression of genes in *Drosophila melanogaster. Mol.Gen.Genet.,* **254,** 621-634.

Bodzioch,M., Orso,E., Klucken,J., Langmann,T., Bottcher,A., Diederich,W., Drobnik,W., Barlage,S., Buchler,C., Porsch-Ozcurumez,M., Kaminski,W.E., Hahmann,H.W., Oette,K., Rothe,G., Aslanidis,C., Lackner,K.J. and Schmitz,G. (1999) The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. *Nat.Genet.,* **22,** 347-351.

Bondioli,K.R., Westhusin,M.E. and Looney,C.R. (1990) Production of identical bovine offspring by nuclear transfer. *Theriogenology,* **33,** 165-174.

Brand,A.H. and Perrimon,N. (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development,* **118,** 401-415.

Briggs,R. and King,T.J. (1952) Transplantation of living nuclei from blastula cells into enucleated frogs' eggs. *Proc. Natl. Acad. Sci. USA,* **38,** 455-463.

Brock,D. (2002) Human cloning and our sense of self. *Science,* **296,** 314-316.

Budiansky,S. (1984) Karl Illmensee: NIH withdraws research grant. *Nature,* **309,** 738.

Bultman,S. and Magnuson,T. (2000) Molecular and genetic analysis of the mouse homolog of the *Drosophila* suppressor of position-effect variegation 3-9 gene. *Mamm.Genome,* **11,** 251-254.

Campbell,J.L. and Nash,H.A. (2001) Volatile general anesthetics reveal a neurobiological role for the *white* and *brown* genes of *Drosophila melanogaster. J.Neurobiol.,* **49,** 339-349.

Campbell,K.H., McWhir,J., Ritchie,W.A. and Wilmut,I. (1996) Sheep cloned by nuclear transfer from a cultured cell line. *Nature,* **380,** 64-66.

Campbell,K.H.S. (1999) Nuclear transfer in farm animal species. *Semin.Cell Dev.Biol.,* **10,** 245-252.

Chen,H., Rossier,C., Lalioti,M.D., Lynn,A., Chakravarti,A., Perrin,G. and Antonarakis,S.E. (1996) Cloning of the cDNA for a human homologue of the *Drosophila white* gene and mapping to chromosome 21q22.3. *Am.J.Hum.Genet.,* **59,** 66-75.

Cherbas,L., Moss,R. and Cherbas,P. (1994) Transformation techniques for *Drosophila* cell lines. *Methods Cell Biol.,* **44,** 161-179.

Chesne,P., Adenot,P.G., Viglietta,C., Baratte,M., Boulanger,L. and Renard,J.P. (2002) Cloned rabbits produced by nuclear transfer from adult somatic cells. *Nat.Biotechnol.,* **20,** 366-369.

Cho,K.S., Elizondo,L.I. and Boerkoel,C.F. (2004) Advances in chromatin remodeling and human disease. *Curr.Opin.Genet.Dev.,* **14,** 308-315.

Cibelli,J.B., Stice,S.L., Golueke,P.J., Kane,J.J., Jerry,J., Blackwell,C., Ponce de Leon,F.A. and Robl,J.M. (1998) Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science,* **280,** 1256-1258.

Clarkson,M. and Saint,R. (1999) A His2AvDGFP fusion gene complements a lethal His2AvD mutant allele and provides an in vivo marker for *Drosophila* chromosome behavior. *DNA Cell Biol.,* **18,** 457-462.

Collas,P. and Robl,J.M. (1990) Factors affecting the efficiency of nuclear transplantation in the rabbit embryo. *Biol.Reprod.,* **43,** 877-884.

Coveny,A.M., Dray,T. and Gloor,G.B. (2002) The effect of heterologous insertions on gene conversion in mitotically dividing cells in *Drosophila melanogaster*. *Genetics,* **161,** 249-258.

Croop,J., Tiller,G., Fletcher,J., Lux,M., Raab,E., Goldenson,D., Son,D., Arciniegas,S. and and Wu,R. (1997) Isolation and characterization of a mammalian homolog of the *Drosophila white* gene. *Gene,* **185,** 77-85.

Czermin,B. and Imhof,A. (2003) The sounds of silence--histone deacetylation meets histone methylation. *Genetica,* **117,** 159-164.

Dean,W., Santos,F., Stojkovic,M., Zakhartchenko,V., Walter,J., Wolf,E. and Reik,W. (2001) Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned embryos. *Proc.Natl.Acad.Sci.U.S.A.*, **98,** 13734-13738.

Delaval,K. and Feil,R. (2004) Epigenetic regulation of mammalian genomic imprinting. *Curr.Opin.Genet.Dev.*, **14,** 188-195.

Dreesen,T.D., Johnson,D.H. and Henikoff,S. (1988) The brown protein of *Drosophila melanogaster* is similar to the white protein and to components of active transport complexes. *Mol.Cell.Biol.*, **8,** 5206-5215.

DuPraw,E.J. (1967) The Honey Bee Embryo. In Wilt,F.H. and Wessells,N.K. (eds.), *Methods in Developmental Biology.* Thomas Y. Crowell Company, New York, pp. 183-217.

Ebert,A., Schotta,G., Lein,S., Kubicek,S., Krauss,V., Jenuwein,T. and Reuter,G. (2004) *Su(var)* genes regulate the balance between euchromatin and heterochromatin in *Drosophila*. *Genes Dev.*, **18,** 2973-2983.

Eggan,K., Baldwin,K., Tackett,M., Osborne,J., Gogos,J., Chess,A., Axel,R. and Jaenisch,R. (2004) Mice cloned from olfactory sensory neurons. *Nature,* **428,** 44-49.

Eggan,K., Akutsu,H., Loring,J., Jackson-Grusby,L., Klemm,M., Rideout,W.M.,3rd, Yanagimachi,R. and Jaenisch,R. (2001) Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. *PNAS,* **98,** 6209-6214.

Eissenberg,J. and Elgin,S. (2000) The HP1 protein family: getting a grip on chromatin. *Curr Opin Genet Dev,* **10,** 204-210.

Eissenberg,J.C., Cartwright,I.L., Thomas,G.H. and Elgin,S.C.R. (1985) Selected topics in chromatin structure. *Annu.Rev.Genet.*, **19,** 485-536.

Elgin,S. and Workman,J. (2002) *Chromatin Structure and Gene Expression* Oxford University Press, U.K.

Ewart,G.D., Cannell,D., Cox,G.B. and Howells,A.J. (1994) Mutational analysis of the traffic ATPase (ABC) transporters involved in uptake of eye pigment precursors in *Drosophila melanogaster*. Implications for structure-function relationships. *J.Biol.Chem.*, **269,** 10370-10377.

Falls,J.G., Pulford,D.J., Wylie,A.A. and Jirtle,R.L. (1999) Genomic imprinting: implications for human disease. *Am.J.Pathol.*, **154,** 635-647.

Feinberg,A.P., Cui,H. and Ohlsson,R. (2002) DNA methylation and genomic imprinting: insights from cancer into epigenetic mechanisms. *Semin.Cancer Biol.*, **12,** 389-398.

Fjose,A., Polito,L.C., Weber,U. and and Gehring,W.J. (1984) Developmental expression of the *white* locus of *Drosophila melanogaster*. *EMBO J.*, **3,** 2087-2094.

Foe,V., Odell,G. and Edgar,B. (1993) Mitosis and morphogenesis in the *Drosophila* embryo: point and counterpoint. In Bate,M. and Martinez Arias,A. (eds.), *The Development of Drosophila melanogaster*. Cold Spring Harbour Laboratory Press, Cold Spring Harbour NY, pp. 149-300.

Fratta,W., Biggio,G. and Gessa,G. (1977) Homosexual mounting behavior induced in male rats and rabbits by a tryptophan free diet. *Life Sci.*, **21,** 379-384.

Furgeson-Smith,A., Sasaki,H., Cattanach,B. and Surani,M. (1993) Parental-origin-specific epigenetic modification of the mouse *H19* gene. *Nature,* **362,** 751-755.

Galli,C., Lagutina,I., Crotti,G., Colleoni,S., Turini,P., Ponderato,N., Duchi,R. and Lazzari,G. (2003) Pregnancy: a cloned horse born to its dam twin. *Nature,* **424,** 635.

Gessa,G.L., Krishna,G., Forn,J., Tagliamonte,A. and Brodie,B.B. (1970) Behavioral and vegetative effects produced by dibutyryl cyclic AMP injected into different areas of the brain. *Adv.Biochem.Psychopharmacol.*, **3,** 371-381.

Gessa,G.L. and Tagliamonte,A. (1974) Possible role of brain serotonin and dopamine in controlling male sexual behavior. *Adv.Biochem.Psychopharmacol.*, **11,** 217-228.

Gessa,G.L., Tagliamonte,A. and Brodie,B.B. (1970) Essential role of testosterone in the sexual stimulation induced by p-chlorophenylalanine in male animals. *Nature,* **227,** 616-617.

Green,M.M. (1996) The "Genesis of the White-Eyed Mutant" in *Drosophila melanogaster*: A Reappraisal. *Genetics,* **142,** 329-331.

Greer,C.L., Grygoruk,A., Patton,D.E., Ley,B., Romero-Calderon,R., Chang,H.Y., Houshyar,R., Bainton,R.J., Diantonio,A. and Krantz,D.E. (2005) A splice variant of the *Drosophila* vesicular monoamine transporter contains a conserved trafficking domain and

functions in the storage of dopamine, serotonin, and octopamine. *J.Neurobiol.*, **64,** 239-258.

Grewal,S.I., Bonaduce,M.J. and Klar,A.J. (1998) Histone deacetylase homologs regulate epigenetic inheritance of transcriptional silencing and chromosome segregation in fission yeast. *Genetics,* **150,** 563-576.

Grigliatti,T. (1991) Position-effect variegation--an assay for nonhistone chromosomal proteins and chromatin assembly and modifying factors. *Methods Cell Biol.*, **35,** 587-627.

Gurdon,J.B. (1962) The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. *J.Embryol.Exp.Morphol.*, **10,** 622-640.

Gurdon,J.B. and Uehlinger,V. (1966) "Fertile" intestine nuclei. *Nature,* **210,** 1240-1241.

Haigh,A.J., MacDonald,W.A. and Lloyd,V.K. (2005) The generation of cloned *Drosophila melanogaster*. *Genetics,* **169,** 1165-1167.

Haldane,J.B.S. (1963) Biological possibilities for the human species for the next ten thousand years. In Wolstenholme,G. (ed.), *Man and His Future.* Little, Brown & Company (Canada) Limited, Toronto, pp. 352.

Hall,J.G. (1999) Human diseases and genomic imprinting. *Results Probl.Cell Differ.*, **25,** 119-132.

Hardy,R.W., Lindsley,D.L., Livak,K.J., Lewis,B., Siversten,A.L., Joslyn,G.L., Edwards,J. and Bonaccorsi,S. (1984) Cytogenetic analysis of a segment of the Y chromosome of *Drosophila melanogaster*. *Genetics,* **107,** 591-610.

Hazelrigg,T., Levis,R. and Rubin,G.M. (1984) Transformation of *white* locus DNA in *Drosophila*: dosage compensation, zeste interaction, and position effects. *Cell,* **36,** 469-481.

Heyman,Y., Chavatte-Palmer,P., LeBourhis,D., Camous,S., Vignon,X. and Renard,J.P. (2002) Frequency and occurrence of late-gestation losses from cattle cloned embryos. *Biol.Reprod.*, **66,** 6-13.

Higashimoto,K., Urano,T., Sugiura,K., Yatsuki,H., Joh,K., Zhao,W., Iwakawa,M., Ohashi,H., Oshimura,M., Niikawa,N., Mukai,T. and Soejima,H. (2003) Loss of CpG methylation is strongly correlated with loss of histone H3 lysine 9 methylation at DMR-LIT1 in patients with Beckwith-Wiedemann syndrome. *Am.J.Hum.Genet.*, **73,** 948-956.

Hochedlinger,K. and Jaenisch,R. (2002) Monoclonal mice generated by nuclear transfer from mature B and T donor cells. *Nature,* **415,** 1035-1038.

Hochedlinger,K. and Jaenisch,R. (2002) Nuclear transplantation: lessons from frogs and mice. *Curr.Opin.Cell Biol.,* **14,** 741-748.

Hoppe,P.C. and Illmensee,K. (1977) Microsurgically produced homozygous-diploid uniparental mice. *Proc.Natl.Acad.Sci.U.S.A.,* **74,** 5657-5661.

Humpherys,D., Eggan,K., Akutsu,H., Friedman,A., Hochedlinger,K., Yanagimachi,R., Lander,E.S., Golub,T.R. and Jaenisch,R. (2002) Abnormal gene expression in cloned mice derived from embryonic stem cell and cumulus cell nuclei. *Proc.Natl.Acad.Sci.U.S.A.,* **99,** 12889-12894.

Humpherys,D., Eggan,K., Akutsu,H., Hochedlinger,K., Rideout,W.M.,3rd, Biniszkiewicz,D., Yanagimachi,R. and Jaenisch,R. (2001) Epigenetic instability in ES cells and cloned mice. *Science,* **293,** 95-97.

Hwang,W., Ryu,Y., Park,J., Park,E., Lee,E., Koo,J., Jeon,H., Lee,B., Kang,S., Kim,S., Ahn,C., Hwang,J., Park,K., Cibelli,J. and Moon,S. (2004) Evidence of a pluripotent human embryonic stem cell line derived from a cloned blastocyst *Science,* **303,** 1669-1674.

Hyde,S.C., Emsley,P., Hartshorn,M.J., Mimmack,M.M., Gileadi,U., Pearce,S.R., Gallagher,M.P., Gill,D.R., Hubbard,R.E. and Higgins,C.F. (1990) Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. *Nature,* **346,** 362-365.

Illmensee,K. (1976) Nuclear and cytoplasmic transplantation in *Drosophila.* In Lawrence,P.A. (ed.), *Insect Development.* Blackwell Scientific Publications, Oxford, pp. 76-96.

Illmensee,K. (1972) Developmental potencies of nuclei from cleavage, preblastoderm, and syncytial blastoderm transplanted into unfertilized eggs of *Drosophila melanogaster. Wilhelm Roux' Arch,* **170,** 267-298.

Illmensee,K. (1999) Controversy over the cloning of mice. *Nature,* **398,** 19-20.

Illmensee,K. (1968) Transplantation of embryonic nuclei into unfertilized eggs of *Drosophila melanogaster. Nature,* **219,** 1268-1269.

Illmensee,K. and Hoppe,P.C. (1981) Nuclear transplantation in Mus musculus: developmental potential of nuclei from preimplantation embryos. *Cell,* **23,** 9-18.

Illmensee,K., Hoppe,P.C. and Croce,C.M. (1978) Chimeric mice derived from human-mouse hybrid cells. *Proc.Natl.Acad.Sci.U.S.A.,* **75,** 1914-1918.

Inoue,K., Kohda,T., Lee,J., Ogonuki,N., Mochida,K., Noguchi,Y., Tanemura,K., Kaneko-Ishino,T., Ishino,F. and Ogura,A. (2002) Faithful expression of imprinted genes in cloned mice. *Science,* **295,** 297.

Joanis,V. and Lloyd,V.K. (2002) Genomic imprinting in *Drosophila* is maintained by the products of Suppressor of variegation and trithorax group, but not Polycomb group, genes. *Mol.Genet.Genomics,* **268,** 103-112.

Joh,T.H. (1997) Tryptophan hydroxylase: molecular biology and regulation. In Baumgarten,H.G. and Gothert,M. (eds.), *Serotoninergic Neurons and 5-HT Receptors in the CNS (Handbook of experimental pharmacology; v.129).* Springer-Verlag, Berlin, pp. 117-129.

Judd,B.H. (1987) The white locus of *Drosophila melanogaster. Results Probl.Cell Differ.,* **14,** 81-94.

Kermicle,J.L. (1970) Dependence of the R-mottled aleurone phenotype in maize on mode of sexual transmission. *Genetics,* **66,** 69-85.

Killian,J.K., Nolan,C.M., Stewart,N., Munday,B.L., Andersen,N.A., Nicol,S. and Jirtle,R.L. (2001) Monotreme IGF2 expression and ancestral origin of genomic imprinting. *J.Exp.Zool.,* **291,** 205-212.

Killian,J.K., Nolan,C.M., Wylie,A.A., Li,T., Vu,T.H., Hoffman,A.R. and Jirtle,R.L. (2001) Divergent evolution in M6P/IGF2R imprinting from the Jurassic to the Quaternary. *Hum.Mol.Genet.,* **10,** 1721-1728.

Kitamoto,T. (2002) Conditional disruption of synaptic transmission induces male-male courtship behavior in *Drosophila. Proc.Natl.Acad.Sci.U.S.A.,* **99,** 13232-13237.

Klucken,J., Buchler,C., Orso,E., Kaminski,W.E., Porsch-Ozcurumez,M., Liebisch,G., Kapinsky,M., Diederich,W., Drobnik,W., Dean,M., Allikmets,R. and Schmitz,G. (2000) *ABCG1 (ABC8),* the human homolog of the *Drosophila white* gene, is a regulator of

macrophage cholesterol and phospholipid transport. *Proc.Natl.Acad.Sci.U.S.A.*, **97,** 817-822.

Kodama,R. and Eguchi,G. (1994) The loss of gap junctional cell-to-cell communication is coupled with dedifferentiation of retinal pigmented epithelial cells in the course of transdifferentiation into the lens. *Int.J.Dev.Biol.*, **38,** 357-364.

Koh,C.J. and Atala,A. (2004) Therapeutic cloning applications for organ transplantation. *Transpl.Immunol.*, **12,** 193-201.

Kolata,G. (1998) *Clone : the road to Dolly, and the path ahead.* W. Morrow & Co, New York.

Koski,L.B., Sasaki,E., Roberts,R.D., Gibson,J. and Etches,R.J. (2000) Monoalleleic transcription of the insulin-like growth factor-II gene (Igf2) in chick embryos. *Mol.Reprod.Dev.*, **56,** 345-352.

Kunert,N., Marhold,J., Stanke,J., Stach,D. and Lyko,F. (2003) A Dnmt2-like protein mediates DNA methylation in *Drosophila*. *Development,* **130,** 5083-5090.

Kuo,M.H. and Allis,C.D. (1999) *In vitro* cross-linking and immunoprecipitation for studying dynamic protein:DNA associations in a chromatin environment. *Methods,* **19,** 425-433.

Lee,B.C., Kim,M.K., Jang,G., Oh,H.J., Yuda,F., Kim,H.J., Shamim,M.H., Kim,J.J., Kang,S.K., Schatten,G. and Hwang,W.S. (2005) Dogs cloned from adult somatic cells. *Nature,* **436,** 641.

Lee,G. and Hall,J.C. (2001) Abnormalities of male-specific FRU protein and serotonin expression in the CNS of fruitless mutants in *Drosophila*. *J.Neurosci.*, **21,** 513-526.

Lee,K.Y., Huang,H., Ju,B., Yang,Z. and Lin,S. (2002) Cloned zebrafish by nuclear transfer from long-term-cultured cells. *Nat.Biotechnol.*, **20,** 795-799.

Leighton,P.A., Saam,J.R., Ingram,R.S. and Tilghman,S.M. (1996) Genomic imprinting in mice: its function and mechanism. *Biol.Reprod.*, **54,** 273-278.

Lewis,A. and Murrell,A. (2004) Genomic imprinting: CTCF protects the boundaries. *Curr.Biol.*, **14,** R284-6.

Lewis,D.L., Farr,C.L., Farquhar,A.L. and Kaguni,L.S. (1994) Sequence, organization, and evolution of the A+T region of *Drosophila melanogaster* mitochondrial DNA. *Mol.Biol.Evol.*, **11,** 523-538.

Lippman,Z. and Martienssen,R. (2004) The role of RNA interference in heterochromatic silencing. *Nature,* **431,** 364-370.

Lloyd,V.K. (2000) Parental imprinting in *Drosophila*. *Genetica,* **109,** 35-44.

Lloyd,V.K., Sinclair,D.A., Alperyn,M. and Grigliatti,T.A. (2002) Enhancer of garnet/deltaAP-3 is a cryptic allele of the white gene and identifies the intracellular transport system for the white protein. *Genome,* **45,** 296-312.

Lloyd,V.K., Sinclair,D.A. and Grigliatti,T.A. (1999) Genomic imprinting and position-effect variegation in *Drosophila melanogaster*. *Genetics,* **151,** 1503-1516.

Lodish,H., Berk,A., Zipursky,S.L., Matsudaria,P., Baltimore,D. and Darnell,J.E. (1999) *Molecular Cell Biology.* W. H. Freeman and Company, New York.

Lucifero,D., Chaillet,J.R. and Trasler,J.M. (2004) Potential significance of genomic imprinting defects for reproduction and assisted reproductive technology. *Hum.Reprod.Update,* **10,** 3-18.

Lyko,F., Brenton,J.D., Surani,M.A. and Paro,R. (1997) An imprinting element from the mouse H19 locus functions as a silencer in *Drosophila*. *Nat.Genet.*, **16,** 171-173.

Lyko,F., Buiting,K., Horsthemke,B. and Paro,R. (1998) Identification of a silencing element in the human 15q11-q13 imprinting center by using transgenic *Drosophila*. *Proc.Natl.Acad.Sci.U.S.A.*, **95,** 1698-1702.

Mackenzie,S.M., Brooker,M.R., Gill,T.R., Cox,G.B., Howells,A.J. and Ewart,G.D. (1999) Mutations in the *white* gene of *Drosophila melanogaster* affecting ABC transporters that determine eye colouration. *Biochim.Biophys.Acta,* **1419,** 173-185.

Mackenzie,S.M., Howells,A.J., Cox,G.B. and Ewart,G.D. (2000) Sub-cellular localisation of the white/scarlet ABC transporter to pigment granule membranes within the compound eye of *Drosophila melanogaster*. *Genetica,* **108,** 239-252.

Maher,E.R. and Reik,W. (2000) Beckwith-Wiedemann syndrome: imprinting in clusters revisited. *J.Clin.Invest.*, **105,** 247-252.

Martin,C.C. and McGowan,R. (1995) Genotype-specific modifiers of transgene methylation and expression in the zebrafish, Danio rerio. *Genet.Res.*, **65,** 21-28.

McGann,C.J., Odelberg,S.J. and Keating,M.T. (2001) Mammalian myotube dedifferentiation induced by newt regeneration extract. *Proc.Natl.Acad.Sci.U.S.A.*, **98,** 13699-13704.

McGrath,J. and Solter,D. (1984) Inability of mouse blastomere nuclei transferred to enucleated zygotes to support development in vitro. *Science*, **226,** 1317-1319.

McGrath,J. and Solter,D. (1984) Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell*, **37,** 179-183.

Mollard,R., Denham,M. and Trounson,A. (2002) Technical advances and pitfalls on the way to human cloning. *Differentiation*, **70,** 1-9.

Meng,L., Ely,J.J., Stouffer,R.L. and Wolf,D.P. (1997) Rhesus monkeys produced by nuclear transfer. *Biol.Reprod.*, **57,** 454-459.

Morgan,T.H. (1910) Sex limited inheritance in *Drosophila Science*, **32,** 120-122.

Mount,S.M. (1987) Sequence similarity. *Nature*, **325,** 487.

Muchardt,C. and Yaniv,M. (1993) A human homologue of *Saccharomyces cerevisiae SNF2/SWI2* and *Drosophila brm* genes potentiates transcriptional activation by the glucocorticoid receptor. *EMBO J.*, **12,** 4279-4290.

Nagy,A., Rossant,J., Nagy,R., Abramow-Newerly,W. and Roder,J.C. (1993) Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc.Natl.Acad.Sci.U.S.A.*, **90,** 8424-8428.

Nakamura,M., Ueno,S., Sano,A. and Tanabe,H. (1999) Polymorphisms of the human homologue of the *Drosophila* white gene are associated with mood and panic disorders. *Mol.Psychiatry*, **4,** 155-162.

Nezer,C., Collette,C., Moreau,L., Brouwers,B., Kim,J.J., Giuffra,E., Buys,N., Andersson,L. and Georges,M. (2003) Haplotype sharing refines the location of an imprinted quantitative trait locus with major effect on muscle mass to a 250-kb chromosome segment containing the porcine IGF2 gene. *Genetics*, **165,** 277-285.

Nicholas,J.S. and Hall,B.V. (1942) Experiments on developing rats. II. The development of isolated blastomeres and fused eggs. *J. exp. Zool.*, **90,** 441-459.

No authors listed. (1999) Science told to slow down and let ethics debates catch up. *Nature*, **399,** 12-12.

Nolte,D.J. (1952) The eye pigmentary system of *Drosophila*: III. The action of eye-colour genes. *J. Genetics*, **51,** 142-186.

Norwood,L.E., Grade,S.K., Cryderman,D.E., Hines,K.A., Furiasse,N., Toro,R., Li,Y., Dhasarathy,A., Kladde,M.P., Hendrix,M.J., Kirschmann,D.A. and Wallrath,L.L. (2004) Conserved properties of HP1(Hsalpha). *Gene*, **336,** 37-46.

Nusslein-Volhard,C. and Wieschaus,E. (1980) Genetic analysis of embryogenesis in *Drosophila melanogaster*. *Res. Rep., EMBL*, 18-21.

O'Carroll,D., Scherthan,H., Peters,A.H., Opravil,S., Haynes,A.R., Laible,G., Rea,S., Schmid,M., Lebersorger,A., Jerratsch,M., Sattler,L., Mattei,M.G., Denny,P., Brown,S.D., Schweizer,D. and Jenuwein,T. (2000) Isolation and characterization of *Suv39h2*, a second histone H3 methyltransferase gene that displays testis-specific expression. *Mol.Cell.Biol.*, **20,** 9423-9433.

Odelberg,S.J. (2002) Inducing cellular dedifferentiation: a potential method for enhancing endogenous regeneration in mammals. *Semin.Cell Dev.Biol.*, **13,** 335-343.

Odorizzi,G., Cowles,C.R. and Emr,S.D. (1998) The AP-3 complex: a coat of many colours. *Trends Cell Biol.*, **8,** 282-288.

Ogawa,O., Eccles,M.R., Szeto,J., McNoe,L.A., Yun,K., Maw,M.A., Smith,P.J. and Reeve,A.E. (1993) Relaxation of insulin-like growth factor II gene imprinting implicated in Wilms' tumour. *Nature*, **362,** 749-751.

Ogura,A., Inoue,K., Ogonuki,N., Noguchi,A., Takano,K., Nagano,R., Suzuki,O., Lee,J., Ishino,F. and Matsuda,J. (2000) Production of male cloned mice from fresh, cultured, and cryopreserved immature sertoli cells. *Biol Reprod*, **62,** 1579-1584.

Okuwaki,M., Matsumoto,K., Tsujimoto,M. and Nagata,K. (2001) Function of nucleophosmin/B23, a nucleolar acidic protein, as a histone chaperone. *FEBS Lett.*, **506,** 272-276.

Ooi,C.E., Moreira,J.E., Dell'Angelica,E.C., Poy,G., Wassarman,D.A. and Bonifacino,J.S. (1997) Altered expression of a novel adaptin leads to defective pigment granule biogenesis in the *Drosophila* eye colour mutant garnet. *EMBO J.*, **16,** 4508-4518.

Oswald,J., Engemann,S., Lane,N., Mayer,W., Olek,A., Fundele,R., Dean,W., Reik,W. and Walter,J. (2000) Active demethylation of the paternal genome in the mouse zygote. *Curr.Biol.*, **10,** 475-478.

Pardo-Manuel de Villena,F., de la Casa-Esperon,E. and Sapienza,C. (2000) Natural selection and the function of genome imprinting: beyond the silenced minority. *Trends Genet.*, **16,** 573-579.

Pearsall,R.S., Plass,C., Romano,M.A., Garrick,M.D., Shibata,H., Hayashizaki,Y. and Held,W.A. (1999) A direct repeat sequence at the *Rasgrf1* locus and imprinted expression. *Genomics,* **55,** 194-201.

Pepling,M. and Mount,S.M. (1990) Sequence of a cDNA from the *Drosophila melanogaster* white gene. *Nucleic Acids Res.*, **18,** 1633.

Peterson,L. and Wilmut,I. (2002) Somatic cell nuclear transfer (cloning) efficiency. 5. http://www.roslin.ac.uk/public/webtablesGR.pdf

Peterson,C.L. (2002) Chromatin Remodeling: Nucleosomes Bulging at the Seams. *Current Biology,* **12,** R245-R247.

Peterson,C.L. and Laniel,M. (2004) Histones and histone modifications. *Current Biology,* **14,** R546-R551.

Pikaard,C.S. and Lawrence,R.J. (2002) Uniting the paths to gene silencing. *Nat.Genet.*, **32,** 340-341.

Pirrotta,V. (1988) Vectors for P-mediated transformation in *Drosophila. Biotechnology,* **10,** 437-456.

Polejaeva,I.A., Chen,S., Vaught,T.D., Page,R.L., Mullins,J., Ball,S., Dai,Y., Boone,J., Walker,S., Ayares,D.L., Colman,A. and Campbell,K.H.S. (2000) Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature,* **407,** 86-90.

Prather,R.S., Barnes,F.L., Sims,M.M., Robl,J.M., Eyestone,W.H. and First,N.L. (1987) Nuclear transplantation in the bovine embryo: assessment of donor nuclei and recipient oocyte. *Biol.Reprod.*, **37,** 859-866.

Rabinow,L., Nguyen-Huynh,A.T. and Birchler,J.A. (1991) A trans-acting regulatory gene that inversely affects the expression of the white, brown and scarlet loci in *Drosophila*. *Genetics,* **129,** 463-480.

Rainier,S., Johnson,L.A., Dobry,C.J., Ping,A.J., Grundy,P.E. and Feinberg,A.P. (1993) Relaxation of imprinted genes in human cancer. *Nature,* **362,** 747-749.

Reik,W., Dean,W. and Walter,J. (2001) Epigenetic reprogramming in mammalian development. *Science,* **293,** 1089-1093.

Robl,J.M., Prather,R., Barnes,F., Eyestone,W., Northey,D., Gilligan,B. and First,N.L. (1987) Nuclear transplantation in bovine embryos. *J.Anim.Sci.,* **64,** 642-647.

Sambrooke,J., Fritsh,E. and Maniatas,T. (1989) *Molecular Cloning: A Laboratory manual. Cold Spring Harbour Laboratory, Cold Spring Harbor, NY.* Cold Spring Harbour Laboratory Press, Cold Spring Harbor, NY.

Sasaki,H., Ferguson-Smith,A.C., Shum,A.S., Barton,S.C. and Surani,M.A. (1995) Temporal and spatial regulation of *H19* imprinting in normal and uniparental mouse embryos. *Development,* **121,** 4195-4202.

Savvateeva,E. (1991) Kynurenines in the regulation of behavior in insects. *Adv.Exp.Med.Biol.,* **294,** 319-328.

Savvateeva,E., Popov,A., Kamyshev,N., Bragina,J., Heisenberg,M., Senitz,D., Kornhuber,J. and Riederer,P. (2000) Age-dependent memory loss, synaptic pathology and altered brain plasticity in the *Drosophila* mutant cardinal accumulating 3-hydroxykynurenine. *J.Neural Transm.,* **107,** 581-601.

Schmitz,G., Kaminski,W.E., Porsch-Ozcurumez,M., Klucken,J., Orso,E., Bodzioch,M., Buchler,C. and Drobnik,W. (1999) ATP-binding cassette transporter A1 (ABCA1) in macrophages: a dual function in inflammation and lipid metabolism? *Pathobiology,* **67,** 236-240.

Schotta,G., Ebert,A., Dorn,R. and Reuter,G. (2003a) Position-effect variegation and the genetic dissection of chromatin regulation in *Drosophila*. *Semin.Cell Dev.Biol.,* **14,** 67-75.

Schotta,G., Ebert,A. and Reuter E.R. (2003b) SU(VAR)3-9 is a conserved key function in heterochromatic gene silencing. *Genetica,* **117,** 149-158.

Schotta,G., Ebert,A., Krauss,V., Fischer,A., Hoffmann,J., Rea,S., Jenuwein,T., Dorn,R. and Reuter,G. (2002) Central role of *Drosophila* SU(VAR)3-9 in histone H3-K9 methylation and heterochromatic gene silencing. *EMBO J.*, **21,** 1121-1131.

Seidel,F. (1952) Die Entwicklugspotzen einen isolierten Blastomere des Zweizellenstadiums im Saugetierei. *Naturwissenschaft,* **39,** 355-356.

Shi,W., Zakhartchenko,V. and Wolf,E. (2003) Epigenetic reprogramming in mammalian nuclear transfer. *Differentiation,* **71,** 91-113.

Shin,T., Kraemer,D., Pryor,J., Liu,L., Rugila,J., Howe,L., Buck,S., Murphy,K., Lyons,L. and Westhusin,M. (2002) Cell biology: A cat cloned by nuclear transplantation. *Nature,* **415,** 859-859.

Simon,J. (1995) Locking in stable states of gene expression: transcriptional control during *Drosophila* development. *Curr.Opin.Cell Biol.*, **7,** 376-385.

Simpson,F., Peden,A.A., Christopoulou,L. and Robinson,M.S. (1997) Characterization of the adaptor-related protein complex, AP-3. *J.Cell Biol.*, **137,** 835-845.

Sims,M. and First,N.L. (1994) Production of calves by transfer of nuclei from cultured inner cell mass cells. *Proc.Natl.Acad.Sci.U.S.A.*, **91,** 6143-6147.

Singh,P.B., Miller,J.R., Pearce,J., Kothary,R., Burton,R.D., Paro,R., James,T.C. and Gaunt,S.J. (1991) A sequence motif found in a *Drosophila* heterochromatin protein is conserved in animals and plants. *Nucleic Acids Res.*, **19,** 789-794.

Smith,G. (1969) Letters of Aldous Huxley. 348.

Smith,L.C. and Wilmut,I. (1989) Influence of nuclear and cytoplasmic activity on the development in vivo of sheep embryos after nuclear transplantation. *Biol.Reprod.*, **40,** 1027-1035.

Smith,L.C., Wilmut,I. and Hunter,R.H. (1988) Influence of cell cycle stage at nuclear transplantation on the development in vitro of mouse embryos. *J.Reprod.Fertil.*, **84,** 619-624.

Solter,D. (1999) Cloning claims challenged. *Nature,* **399,** 13.

Solter,D. (1996) Lambing by nuclear transfer. *Nature,* **380,** 24-25.

Spemann,H. (1967) *Embryonic Development and Induction.* Hafner Publishing Company, New York.

Spradling,A.C. (1986) P element-mediated transformation. In Roderts,D.B. (ed.), *Drosphila a practical approach.* IRL Press, Oxford, UK, pp. 175-197.

Sullivan,E., Kasinathan,S., Kasinathan,P., Robl,J. and Collas,P. (2004) Cloned Calves from Chromatin Remodeled In Vitro *Biol Reprod,* **70,** 146-153.

Summers,K.M., Howells,A.J. and Pyliotis,N.A. (1982) Biology of eye pigmentation in insects. *Adv. Insect Physiol.,* **16,** 119-166.

Surani,M.A., Barton,S.C. and Norris,M.L. (1984) Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature,* **308,** 548-550.

Surani,M.A. (1998) Imprinting and the initiation of gene silencing in the germ line. *Cell,* **93,** 309-312.

Sved,J.A., Eggleston,W.B. and Engels,W.R. (1990) Germ-line and somatic recombination induced by in vitro modified P elements in *Drosophila melanogaster. Genetics,* **124,** 331-337.

Szabo,P.E., Tang,S.H., Silva,F.J., Tsark,W.M. and Mann,J.R. (2004) Role of CTCF binding sites in the Igf2/H19 imprinting control region. *Mol.Cell.Biol.,* **24,** 4791-4800.

Tagliamonte,A., Tagliamonte,P., Gessa,G.L. and Brodie,B.B. (1969) Compulsive sexual activity induced by p-chlorophenylalanine in normal and pinealectomized male rats. *Science,* **166,** 1433-1435.

Tamaru,H. and Selker,E.U. (2001) A histone H3 methyltransferase controls DNA methylation in *Neurospora crassa. Nature,* **414,** 277-283.

Tamashiro,K.L., Wakayama,T., Akutsu,H., Yamazaki,Y., Lachey,J.L., Wortman,M.D., Seeley,R.J., D'Alessio,D.A., Woods,S.C., Yanagimachi,R. and Sakai,R.R. (2002) Cloned mice have an obese phenotype not transmitted to their offspring. *Nat.Med.,* **8,** 262-267.

Tarkowski,A.K. and Wroblewska,J. (1967) Development of blastomeres of mouse eggs isolated at the 4- and 8-cell stage. *J.Embryol.Exp.Morphol.,* **18,** 155-180.

Tearle,R.G., Belote,J.M., McKeown,M., Baker,B.S. and Howells,A.J. (1989) Cloning and characterization of the scarlet gene of *Drosophila melanogaster*. *Genetics,* **122,** 595-606.

The Bloomington *Drosophila* Stock Center at Indiana University. Cornmeal-Molasses-Yeast Medium. http://fly.bio.indiana.edu/molasses-food.htm

The FlyBase Consortium. (2003) The FlyBase database of the *Drosophila* genome projects and community literature. *Nucleic Acids Res,* **31,** 172-175.

Tsunoda,Y. and Kato,Y. (1998) Not only inner cell mass cell nuclei but also trophectoderm nuclei of mouse blastocysts have a developmental totipotency. *J.Reprod.Fertil.,* **113,** 181-184.

Tsunoda,Y., Yasui,T., Shioda,Y., Nakamura,K., Uchida,T. and Sugie,T. (1987) Full-term development of mouse blastomere nuclei transplanted into enucleated two-cell embryos. *J.Exp.Zool.,* **242,** 147-151.

Tucker,K.L., Beard,C., Dausmann,J., Jackson-Grusby,L., Laird,P.W., Lei,H., Li,E. and Jaenisch,R. (1996) Germ-line passage is required for establishment of methylation and expression patterns of imprinted but not of nonimprinted genes. *Genes Dev.,* **10,** 1008-1020.

van der Kooy, D. and Weiss,S. (2000) Why Stem Cells? *Science,* **287,** 1439-1441.

Vignon,X., Chesne,P., Le Bourhis,D., Flechon,J.E., Heyman,Y. and Renard,J.P. (1998) Developmental potential of bovine embryos reconstructed from enucleated matured oocytes fused with cultured somatic cells. *C.R.Acad.Sci.III.,* **321,** 735-745.

Wakayama,T., Perry,A.C., Zuccotti,M., Johnson,K.R. and Yanagimachi,R. (1998) Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature,* **394,** 369-374.

Wakayama,T., Rodriguez,I., Perry,A.C., Yanagimachi,R. and Mombaerts,P. (1999) Mice cloned from embryonic stem cells. *Proc.Natl.Acad.Sci.U.S.A.,* **96,** 14984-14989.

Wakayama,T., Shinkai,Y., Tamashiro,K.L., Niida,H., Blanchard,D.C., Blanchard,R.J., Ogura,A., Tanemura,K., Tachibana,M., Perry,A.C., Colgan,D.F., Mombaerts,P. and Yanagimachi,R. (2000a) Cloning of mice to six generations. *Nature,* **407,** 318-319.

Wakayama,T., Tateno,H., Mombaerts,P. and Yanagimachi,R. (2000b) Nuclear transfer into mouse zygotes. *Nat.Genet.*, **24,** 108-109.

Wakayama,T. and Yanagimachi,R. (1999a) Cloning of male mice from adult tail-tip cells. *Nat.Genet.*, **22,** 127-128.

Wakayama,T. and Yanagimachi,R. (1999b) Cloning the laboratory mouse. *Semin.Cell Dev.Biol.*, **10,** 253-258.

Walker,J., Saraste,M., Runswick,M. and Gay,N. (1982) Distantly related sequences in the α- and β- subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.*, **1,** 945-951.

Wallrath,L.L., Lu,Q., Granok,H. and Elgin,S.C. (1994) Architectural variations of inducible eukaryotic promoters: preset and remodeling chromatin structures. *Bioessays,* **16,** 165-170.

Walter,J. and Paulsen,M. (2003) Imprinting and disease. *Semin.Cell Dev.Biol.*, **14,** 101-110.

Weismann,A. (1889) On Heredity, 1883. In Poulton,E., Sconland,S. and Shipley,A. (eds.), *Essays upon heredity and kindred biological problems.* Claredon Press, Oxford, pp. 67-105.

Willadsen,S.M. (1981) The developmental capacity of blastomeres from 4- and 8- cell sheep embryos. *J. Embryol. exp. Morph.*, **65,** 165-172.

Willadsen,S.M. (1980) The Viability of early cleavage stages containing half the normal number of blastomeres in the sheep. *J. Reprod. Fert.*, **59,** 357-362.

Willadsen,S.M. (1986) Nuclear transplantation in sheep embryos. *Nature,* **320,** 63-65.

Willadsen,S.M. (1979) A method for culture of micromanipulated sheep embryos and its use to produce monozygotic twins. *Nature,* **277,** 298-300.

Wilmut,I., Beaujean,N., de Sousa,P., Dinnyes,A., King,T., Paterson,L., Wells,D. and Young,L. (2002) Somatic cell nuclear transfer. *Nature,* **419,** 583-586.

Wilmut,I., Schnieke,A., McWhir,J., Kind,A. and Campbell,K. (1997) Viable offspring derived from fetal and adult mammalian cells. *Nature,* **385,** 810-813.

Wilson,I.B. and Stern,M.S. (1975) Organisation in the Pre-Implantation Embryo. In Balls,M. and Wild,A.E. (eds.), *The Early Development of Mammals.* Cambridge University Press, Cambridge, pp. 81-95.

Woods,G.L., White,K.L., Vanderwall,D.K., Li,G.P., Aston,K.I., Bunch,T.D., Meerdo,L.N. and Pate,B.J. (2003) A mule cloned from fetal cells by nuclear transfer. *Science,* **301,** 1063.

Wreggett,K.A., Hill,F., James,P.S., Hutchings,A., Butcher,G.W. and Singh,P.B. (1994) A mammalian homologue of *Drosophila* heterochromatin protein 1 (HP1) is a component of constitutive heterochromatin. *Cytogenet.Cell Genet.,* **66,** 99-103.

Yang,Y., Li,T., Vu,T., Ulaner,G., Hu,J.F. and Hoffman,A. (2003) The Histone Code Regulating Expression of the Imprinted Mouse *Igf2r* Gene. *Endocrinology,* **144,** 5658-5670.

Yasuda,G.K., Schubiger,G. and Wakimoto,B.T. (1995) Genetic characterization of ms (3) K81, a paternal effect gene of *Drosophila melanogaster*. *Genetics,* **140,** 219-229.

Young,L.E., Fernandes,K., McEvoy,T.G., Butterwith,S.C., Gutierrez,C.G., Carolan,C., Broadbent,P.J., Robinson,J.J., Wilmut,I. and Sinclair,K.D. (2001) Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nat.Genet.,* **27,** 153-154.

Young,L.E., Schnieke,A.E., McCreath,K.J., Wieckowski,S., Konfortova,G., Fernandes,K., Ptak,G., Kind,A.J., Wilmut,I., Loi,P. and Feil,R. (2003) Conservation of IGF2-H19 and IGF2R imprinting in sheep: effects of somatic cell nuclear transfer. *Mech.Dev.,* **120,** 1433-1442.

Young,L.E., Sinclair,K.D. and Wilmut,I. (1998) Large offspring syndrome in cattle and sheep. *Rev.Reprod.,* **3,** 155-163.

Yu,B.D., Hess,J.L., Horning,S.E., Brown,G.A. and Korsmeyer,S.J. (1995) Altered Hox expression and segmental identity in Mll-mutant mice. *Nature,* **378,** 505-508.

Yusufzai,T.M., Tagami,H., Nakatani,Y. and Felsenfeld,G. (2004) CTCF tethers an insulator to subnuclear sites, suggesting shared insulator mechanisms across species. *Mol.Cell,* **13,** 291-298.

Zhang,S., Kubota,C., Yang,L., Zhang,Y., Page,R., O'neill,M., Yang,X. and Tian,X.C. (2004) Genomic imprinting of H19 in naturally reproduced and cloned cattle. *Biol.Reprod.*, **71,** 1540-1544.

Zhang,S.D. and Odenwald,W.F. (1995) Misexpression of the *white* (*w*) gene triggers male-male courtship in *Drosophila*. *Proc.Natl.Acad.Sci.U.S.A.*, **92,** 5525-5529.

Zhang,Y.Q., Rodesch,C.K. and Broadie,K. (2002) Living synaptic vesicle marker: synaptotagmin-GFP. *Genesis*, **34,** 142-145.

Zhou,Q., Renard,J.P., Le Friec,G., Brochard,V., Beaujean,N., Cherifi,Y., Fraichard,A. and Cozzi,J. (2003) Generation of fertile cloned rats by regulating oocyte activation. *Science*, **302,** 1179.

Appendix A - Fly Culture Medium

Fly Culture Medium (4800ml)

5576ml H_2O

400ml molasses

59g agar

165g yeast

400g cornmeal

9g tegosept

- The first four ingredients were mixed and brought to a boil. Cornmeal was added and mixture was simmered 15 minutes.
- Mixture was removed from heat and tegosept was added prior to pouring.
- Medium was allowed to solidify before use.

Appendix B - Grape Juice Agar Plates

Grape Juice Agar Plates

500ml grape juice
29g sucrose
40g agar
10M NaOh to final pH > 5.0
ddH_2O to 1L

- The above ingredients were combined and autoclaved.
- Mixture was allowed to cool and poured into 60mm Petri plates (Fisher Scientific).
- Plates were allowed to set over night and refrigerated prior to use.

Appendix C – Yeast Paste

Yeast Paste

- Dry active yeast was mixed with one part ddH_20 to one part 5% acetic acid to achieve a soft, peanut-butter-like consistency.