An *in vivo* evaluation of aneuploid hematopoietic stem cell fitness

by

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A.B. Molecular Biology
Princeton University, 2008

Submitted to the Department of Biology
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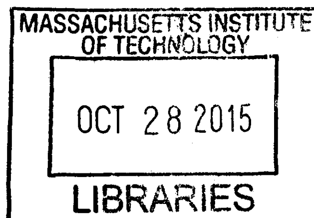
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ABSTRACT

Aneuploidy is an unbalanced cell state associated with developmental conditions such as Down syndrome (DS) as well as cancer, a disease of rapid proliferation. Studies of yeast, mouse and human cells harboring one extra chromosome have demonstrated that aneuploid cells show a number of common phenotypes *in vitro*, notably decreased proliferation. However, the precise role of aneuploidy in cancer has yet to be elucidated, in part due to lack of systematic *in vivo* model systems. Furthermore, evaluation of aneuploidy-associated phenotypes *in vivo* has been difficult because autosomal trisomy is generally embryonic lethal in mice.

Here, I have evaluated hematopoietic stem cells (HSCs) derived from three aneuploid mouse models *in vivo*, two models of autosomal trisomy and one model of chromosome instability. By performing hematopoietic reconstitutions, I found that aneuploid HSCs have a range of fitness *in vivo* that correlates with the amount of extra DNA in each line. My results demonstrate that aneuploidy-associated cellular phenotypes are observed *in vivo* and in the context of a euploid organism. Additionally, I found that aneuploidy is well tolerated in the hematopoietic lineage under normal conditions in two of the three mouse models analyzed. However, even these relatively fit aneuploid cells begin to show more severe phenotypes upon repeated proliferative challenge. In humans, DS is associated with perturbations in the hematopoietic system, often resulting in childhood leukemia. Trisomy is also frequently observed in non-DS leukemias. Establishment of this model system enables future systematic dissection of the source of aneuploidy-associated fitness defects *in vivo* both in hematopoiesis and in the context of cancer.

Thesis Supervisor: Angelika Amon
Title: Kathleen and Curtis Marble Professor of Cancer Research

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Chapter 1: Introduction

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Cellular aneuploidy in cancer and disease

Cancer cells contain a multitude of genetic lesions that endow them with increased proliferative potential and means to evade elimination by apoptosis. Most transformed cells harbor mutations in multiple growth- and proliferation-promoting pathways, and the spectrum of genetic lesions varies significantly between individual cancers (Balmain et al., 2003; Vogelstein and Kinzler, 2004).The genetic heterogeneity of cancer cells —both within tumors and between those derived from different individuals— makes targeting individual components of these pathways challenging (Longo, 2012).

Nevertheless, cancer cells also share features. Notably, the vast majority of solid tumors are aneuploid: they have an inappropriate number of chromosomes (Rajagopalan and Lengauer, 2004). This is surprising, as aneuploidy has severely deleterious consequences when present throughout an organism. In humans, only three autosomal trisomies survive to birth, and of these, only individuals with trisomy 21, or Down syndrome (DS) survive infancy (Torres et al., 2008). The definitive effects of aneuploidy on cell physiology are only beginning to be understood, and therefore its role in tumorigenesis and cancer remains unclear.

In this Introduction, I will first discuss what is known about the effects of aneuploidy on normal cell physiology from *in vitro* cellular systems. Next, I will relate what is known about the role of aneuploidy in cancer from both human case studies and animal models. Then, I will propose a model that could explain these complex effects. Finally, I will discuss the system we chose to investigate the role of aneuploidy *in vivo* for my thesis work and explain how this particular system is a useful tool for testing this model.

Aneuploidy, precisely defined

In the context of a discussion on aneuploidy and its role in tumorigenesis, it is especially important to distinguish "aneuploidy" from "polyploidy." These terms describe two very

different cellular states that have distinct effects on cells and organisms. Aneuploidy —derived from the Greek *an* meaning "not," *eu* meaning "good," and *ploos* meaning "fold"— is a state in which a cell does not contain an exact multiple of the haploid chromosomal complement (literally "not the right fold"; Figure 1). Therefore, aneuploidy refers to an unbalanced genomic state. In contrast, polyploidy refers to a state in which a cell contains a whole number multiple of the entire genome (literally "many fold"). Thus the genomic state is balanced in polyploid cells. Just as "polyploidy" can describe cells with a range of ploidies, from "diploid" to "tetraploid" to "octoploid" and beyond, "aneuploidy" is a general term that can describe a wide range of unbalanced karyotypes (Figure 1). We will describe this spectrum of aneuploid karyotypes with two terms, "high grade" and "low grade". High-grade aneuploidy describes the deviation of many chromosomes from the euploid chromosome number, whereas low-grade aneuploidy refers to the deviation of a few chromosomes from the euploid complement.

In the strictest sense of the word, only changes in chromosome number that are not multiples of the haploid complement should be defined as aneuploidies. However, the term is also commonly used to describe genomic alterations that result in unbalanced copy numbers of sub-chromosomal regions. These copy number variations, non-reciprocal translocations and duplications or deletions of portions of chromosomes are termed "microaneuploidies", "partial aneuploidies" or "segmental aneuploidies", to distinguish them from whole chromosomal aneuploidies.

An accurate use of terms is particularly important when describing cancer cells. We suggest using the terms as they are defined and to refrain from calling cancer cells "tetraploid" or "polyploid." Although tetraploidization can precede the acquisition of aneuploidy in the development of some types of cancer (Ganem et al., 2007) and many cancer cell lines have

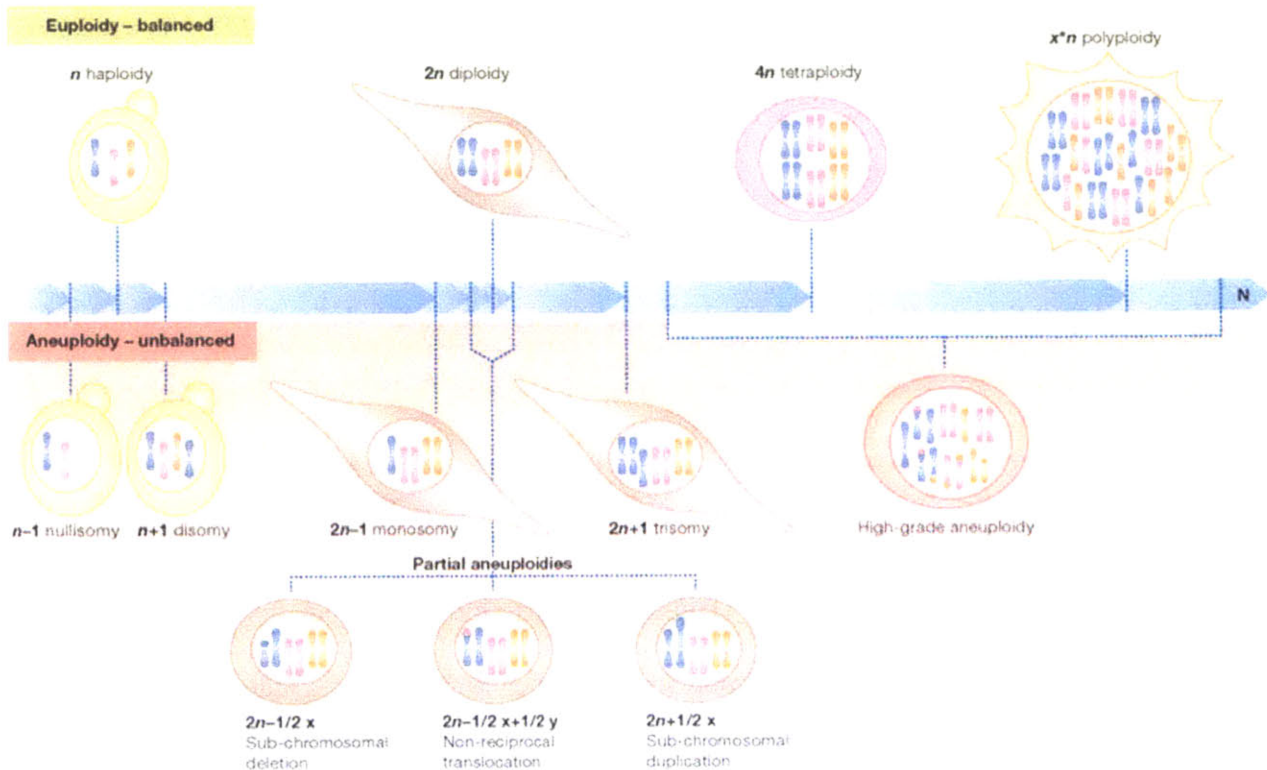

Figure 1. *Aneuploidy defined*

Euploidy defines a species-specific karyotype. Depending on the species, euploidy can describe a haploid, diploid or polyploid karyotype. Euploidy refers to a balanced genomic state. By contrast, aneuploidy is an unbalanced genomic state that describes a range of karyotypes. Whole chromosomes can be lost (nullisomy or monosomy) or gained (disomy or trisomy). Additionally, sub-chromosomal regions can be amplified, deleted or translocated (partial aneuploidies). 'High-grade aneuploidy' occurs when complex aneuploidies are present, often a combination of chromosome losses and gains, as well as sub-chromosomal rearrangements.

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increased base-ploidy (contain a number of chromosomes that approaches a multiple of the haploid complement), cancer cells very rarely —if ever— contain exact multiples of the haploid genome. Rather, cancer cells seem to invariably harbor complex aneuploid karyotypes —such as four copies of one chromosome, two of another, one of a third chromosome, *et cetera*— as shown in (Davidson et al., 2000; Ferti et al., 2004; Griffin et al., 2007). Because the terms

"tetraploid" or "polyploid" imply a sense of balance, we suggest describing cancer cells as "complex aneuploid cells" or as "harboring high grade aneuploidy" to both capture the complexity of their genomes and emphasize their unbalanced genomic complements, without stating their precise chromosomal composition (Figure 1).

Effects of aneuploidy on cell physiology

Before one can understand how aneuploidy contributes to tumorigenesis, it is important to determine the effects of aneuploidy *per se* on cellular processes. Two types of models are currently being used to analyze the effects of aneuploidy on cell physiology. Some studies analyze cells that contain defined chromosomal aneuploidies created through single chromosome transfers or spontaneous meiotic non-disjunction. We will refer to these systems as *chronic defined aneuploidies* because the identity of the aneuploid chromosome is known and it is present from the genesis of the cell or organism. Other studies use cells that have CIN, that is, a high rate of chromosome mis-segregation due to mutations in genes required to ensure accurate chromosome segregation (Holland and Cleveland, 2012). We will refer to aneuploid cells derived from chromosomal instability as *acute random aneuploidies* because they are generated spontaneously as the cell divides and the identity of the mis-segregated chromosome(s) varies with each non-disjunction event. In cells with CIN, it can be difficult to clearly separate the effects of aneuploidy from other CIN-associated phenotypes such as structural chromosomal aberrations, or from additional potential functions of the genes that are mutated to induce chromosome mis-segregation. In addition, populations of cells with CIN continuously spawn new heterogeneous karyotypes, allowing for increased adaptive potential to various selective pressures (Foijer et al., 2008). Nevertheless, general phenotypes emerge in many CIN models despite this complexity, providing insight into the cellular response to the induction of aneuploidy.

Studies of the effects of aneuploidy on cells and organisms have analyzed whether gene expression is correlated with gene copy number in aneuploid cells or whether mechanisms exist that compensate for the gain or loss of chromosomes. In yeast and mammals, gene expression seems to correlate with gene copy number, at least in the case of chromosome gains. An increase in genomic material is generally accompanied by a corresponding increase in the transcription of those genes in excess, as observed in yeast cells with an extra chromosome, trisomic mouse cells, human cells with trisomy 21 (Prandini et al., 2007; Torres et al., 2008; Williams et al., 2008), yeast cells with complex aneuploid karyotypes (Pavelka et al., 2010) and in aneuploid human cell lines created by microcell-mediated chromosome transfer (Stingele et al., 2012; Upender et al., 2004). Whether mechanisms that compensate for the loss of an entire autosome exist remains unknown. However, a study that examined the effects of heterozygous deletions on protein expression levels in budding yeast showed that gene expression compensatory mechanisms are rare in this organism (Springer et al., 2010). Consistent with this conclusion, when monosomy of a chromosome is induced in diploid yeast strains, endoreplication to duplicate the remaining chromosome or non-disjunction occurs, rebalancing the genome from 2N-1 to 2N (Reid et al., 2008).

The correlation between gene copy number and gene expression levels does not seem to be universal. Mechanisms that compensate on the transcriptional level for changes in chromosome copy number have been described in Drosophila and plants (Birchler et al., 2001; Birchler, 2010), suggesting that species-specific differences may exist in the ability to respond to gene copy number variations on the transcriptional level.

Chronic defined aneuploidies

Analyses of chronic defined aneuploid cells have provided insight into the consequences of changing the gene expression pattern of entire chromosomes in organisms that do not have

prevalent compensatory mechanisms. An initial, systematic analysis of the effects of chronic defined aneuploidies in *Saccharomyces cerevisiae* strains showed that strains harboring an additional chromosome (known as "disomes") have —in addition to chromosome-specific effects— an "aneuploidy stress response" manifested as defects in cell growth, altered metabolic properties, and increased sensitivity to inhibitors of protein synthesis and folding (Torres et al., 2007)Figure 2).

Further characterization of these phenotypes has yielded insight into the causes of the aneuploidy stress response in yeast cells. With regard to cell growth, proliferation and DNA replication, disomic yeast have decreased growth rates during G1 and delayed entry into the cell cycle (Thorburn et al., 2013). Further, these cells experience increased sensitivity to DNA damage and genotoxic stress (Sheltzer et al., 2011) and increased DNA damage during DNA replication (Blank et al., 2015). Disomic yeast strains and aneuploid strains obtained as progeny of triploid meioses also have phenotypes indicative of proteotoxic stress, such as temperature sensitivity, sensitivity to protein folding and degradation inhibitors and protein aggregate formation (Oromendia et al., 2012; Torres et al., 2007; 2010). In addition, evolution experiments showed that a mutation in *UBP6*, a ubiquitin-specific protease that antagonizes proteasome function, confers tolerance to some disomies in yeast (Torres et al., 2010), indicating that protein imbalance is a major problem for aneuploid yeast cells. Thus, disomic yeast cells seem to partially compensate for altered gene dosage at the protein level by selectively degrading approximately 20% of proteins present in excess that are found in complexes (Dephoure et al., 2014; Torres et al., 2007) and aggregating extra proteins that are improperly folded (Oromendia et al., 2012).

Chronic defined aneuploidies also adversely impact mammalian cells. Trisomic mouse embryonic fibroblasts (MEFs) have growth defects, altered metabolism and differential kinetics of spontaneous immortalization in culture (Williams et al., 2008). Skin fibroblasts isolated from Down Syndrome individuals (containing three copies of chromosome 21) also proliferate more slowly in culture (Segal and McCoy, 1974). Human cell lines transformed with one or two extra chromosomes by micronucleus-mediated chromosome transfer also show proliferation defects due to delays in G1 (Stingele et al., 2012). Furthermore, comparative cytogenetic analysis of early and late stage human blastocysts derived from *in vitro* fertilizations that contain some aneuploid cells revealed that the percentage of aneuploid cells in the preimplantation embryo decreases with subsequent cell divisions, suggesting that in aneuploid/euploid mosaic embryos, euploid cells outcompete the aneuploid cells and could ultimately "normalize" the embryo (Fragouli et al., 2008).

Similar to the disomic yeast strains, trisomic MEFs exhibit signs of energy and proteotoxic stress (Tang et al., 2011; Figure 2). Specifically, they have increased sensitivity to the Hsp90 inhibitor 17-AAG and the autophagy inhibitor chloroquine, basal activation of the autophagy pathway and express higher levels of the inducible chaperone Hsp72, which prevents protein aggregation (Tang et al., 2011). However, increased sensitivity to proteasome inhibitors was not observed in these aneuploid MEFs (Tang et al., 2011), suggesting that proteasomal degradation is not rate-limiting in these cells. Rather it appears that aneuploid mammalian cells are more reliant on autophagy to cope with changes in protein homeostasis caused by aneuploidy. Autophagy is upregulated in trisomic human cell lines (Stingele et al., 2012) and human cell lines that are acutely aneuploid after treatment with a chemical inhibitor of the spindle assembly checkpoint (S. Santaguida, unpublished results).

Chronic defined aneuploidies are a major genetic perturbation, and collectively, these studies suggest that aneuploidy causes —among other detrimental outcomes— a set of shared phenotypes that is independent of the specific set of genes amplified on the additional chromosome and indicative of energy and proteotoxic stress. To further support this idea, a systematic study of combined amplification of chromosome-specific dosage sensitive genes in yeast cells revealed that the amplification of a few dosage sensitive genes is generally neither necessary nor sufficient to account for the cell proliferation defects observed in the disomes (Bonney et al., 2015). While amplification of specific genes can sometimes be attributed to particular phenotypes– e.g. the co-expression of *DSCAM* and *COL6A2* are sufficient to cause congenital heart defects frequently seen in DS in mice (Grossman et al., 2011)—general phenotypes are also observed in aneuploid cells that are elicited by the amplification of individual genes and/or combinations of a small number of genes present on the aneuploid chromosome.

Acute random aneuploidies

Cells that contain acute random aneuploidies due to CIN also exhibit proliferation defects and features of cellular stress. This was first noted in human cells using live cell imaging and clonal cell analyses, which showed that chemically-induced chromosome mis-segregation compromises the proliferation of cells (Thompson and Compton, 2008). Subsequent studies in these cells showed that cells that become aneuploid as a result of chromosome mis-segregation activate p53, inducing cell cycle arrest and apoptosis (Thompson and Compton, 2010). MEFs that mis-segregate their chromosomes also undergo apoptosis (Jeganathan et al., 2007),which is consistent with these observations.

Other studies of the immediate cellular response to CIN-induced aneuploidy revealed similar phenotypes. Knock-down of the spindle assembly checkpoint (SAC) components

BUB1B or MAD2 or of the centromere-associated kinesin CENP-E in near diploid HCT116 cells causes a p53 response (Li et al., 2010). MEFs with a mutation in the SAC component Bub1, or with a mutation that renders the checkpoint effector Cdc20 unresponsive to the checkpoint signal activate p53 during the genesis of aneuploidy. This activation depends on the DNA damage checkpoint kinase ATM but not Chk1/Chk2 (Li et al., 2010). Interestingly, activation of ATM is not caused by DNA damage but by increased reactive oxygen species (ROS) levels, as the cell cycle arrest observed in these aneuploid cells could be suppressed by treating the cells with ROS scavengers (Figure 2). Furthermore, the cellular response to CIN-induced aneuploidy differs depending on the degree of aneuploidy and leads to an enrichment of aneuploid cells, suggesting that massive chromosome mis-segregation induces p53-mediated apoptosis, whereas low levels of chromosome mis-segregation induce a p53-mediated cell cycle arrest (Li et al., 2010). The graded response to the genesis of aneuploidy led to the proposal of an "aneuploidy checkpoint" mediated by p53 and ROS (Figure 2). The increased energy demands generated by increased genomic content, the subsequently induced energy stress and/or proteotoxic stress, and/or some other aspect of the aneuploid condition could trigger the production of ROS, which activate ATM (Guo et al., 2010) in a DNA damage-independent manner. This in turn activates p53. Thus, this mechanism would limit the proliferation of aneuploid cells, and the degree of aneuploidy toggles the strength of the cell cycle arrest and induction of apoptosis so that the cell needs to reach a ROS threshold to activate p53 (Fang and Zhang, 2011).

The prominent role of p53 in promoting cell death or cell cycle arrest in response to aneuploidy was suggested previously. Embryos lacking both copies of the gene encoding the spindle assembly checkpoint component MAD2 die by embryonic day 7.5, but deletion of p53 allows these embryos to survive until embryonic day 10.5 (Burds et al., 2005). However, other

studies suggested that p53 activation as a consequence of chromosome instability may not be due to the genesis of aneuploidy *per se* but could be a consequence of CIN. Chromosome mis-segregation leads to lagging chromosomes, which become damaged upon cytokinesis and trigger a DNA damage response (Figure 2). Defects in chromosome attachment to the mitotic spindle after treatment with Monastrol —which inhibits the kinesin Eg5, arresting cells in mitosis—cause chromosomes to linger in the center of the spindle (Janssen et al., 2011). The ensuing cytokinesis results in chromosome breaks on the lagging chromosomes, which elicit an ATM/Chk2-mediated p53 DNA damage response, characterized by increased levels of H2A.X foci and expression of a p53 reporter. Therefore, DNA damage could also serve as a cellular sensor for detecting CIN and the ensuing aneuploidies. Errors in chromosome segregation could lead to anaphase bridges and/or chromosomes remaining in the spindle midzone, which will break during cytokinesis and thus trigger a p53 response (Figure 2).

Several other studies in mice with an altered spindle assembly checkpoint (discussed below) showed an increase in the frequency of lagging chromosomes during anaphase (Baker et al., 2004; Jeganathan et al., 2007; van Ree et al., 2010; Ricke et al., 2011). However, the DNA damage response in these cells has not yet been characterized. Additionally, cells heterozygous for a deletion in CENP-E do not have increased H2A.X expression upon induction of aneuploidy as a result of chromosome mis-segregation (Weaver et al., 2007). Therefore, further analysis is required to better understand the role of DNA damage in the induction of a response to CIN and the aneuploidy that accompanies it.

Several studies have implicated p53 in the response to the aneuploid state, but aneuploidy clearly also interferes with cell proliferation through other mechanisms. Cells harboring single constitutional chromosomal aneuploidies (trisomic MEFs) do not mount a p53 response yet

exhibit proliferation defects (Tang et al., 2011)Figure 2). In addition, p53 inactivation does not suppress the proliferation defect of trisomic MEFs when compared to wild type controls. Although increased proliferation is observed in trisomic MEFs treated with a p53 shRNA, the fold change in proliferation is similar to that seen in wild type cells treated with a p53 shRNA: there is no additional effect in trisomic cells (S. Santaguida, unpublished data). Furthermore, human HCT116 cells in which p53 is disrupted maintain a near diploid karyotype in continuous growth in culture for many passages (Bunz et al., 2002). Thus, mechanisms other than p53 must be preventing aneuploid cells from accumulating in these cell populations. Indeed, there is recent evidence implicating Hippo pathway activation in inhibiting cell proliferation upon induction of tetraploidy by cytokinesis failure in RPE-1 and mouse liver cells (Ganem et al., 2014). Together, these data suggest that aneuploidy interferes with cell proliferation in both a p53-dependent and p53-independent manner. Identifying these additional effectors of the aneuploidy-induced cellular response could provide critical new targets in cancer therapy.

Cells with CIN caused by chemically-induced chromosome mis-segregation, by gain of function alleles of Cdc20 (Li et al., 2010), or loss of function alleles of Bub1B (Baker et al., 2004), or by overexpressing the checkpoint factor Mad2 (Sotillo et al., 2007) proliferate poorly. However, not all cells with CIN-induced aneuploidy have been reported to have proliferation defects. Cells heterozygous for deletions in the SAC genes BUB3 or RAE1 (Babu et al., 2003), cells heterozygous for deletions in CENP-E (Weaver et al., 2007), and cells that overexpress the ubiquitin conjugating enzyme UbcH10 (van Ree et al., 2010) become aneuploid *in vitro* but do not seem to slow cell proliferation. This apparent inconsequence of aneuploidy on cell proliferation could be due to several reasons. As observed in Bub1 deficient MEFs, perhaps the gene that is mutated is itself involved in promoting cell cycle arrest and apoptosis when mis-

segregation events occur (Jeganathan et al., 2007). Thus, even if cells acquire aneuploidies, they are not eliminated. It is also possible that in these mouse models of aneuploidy, only a subset of

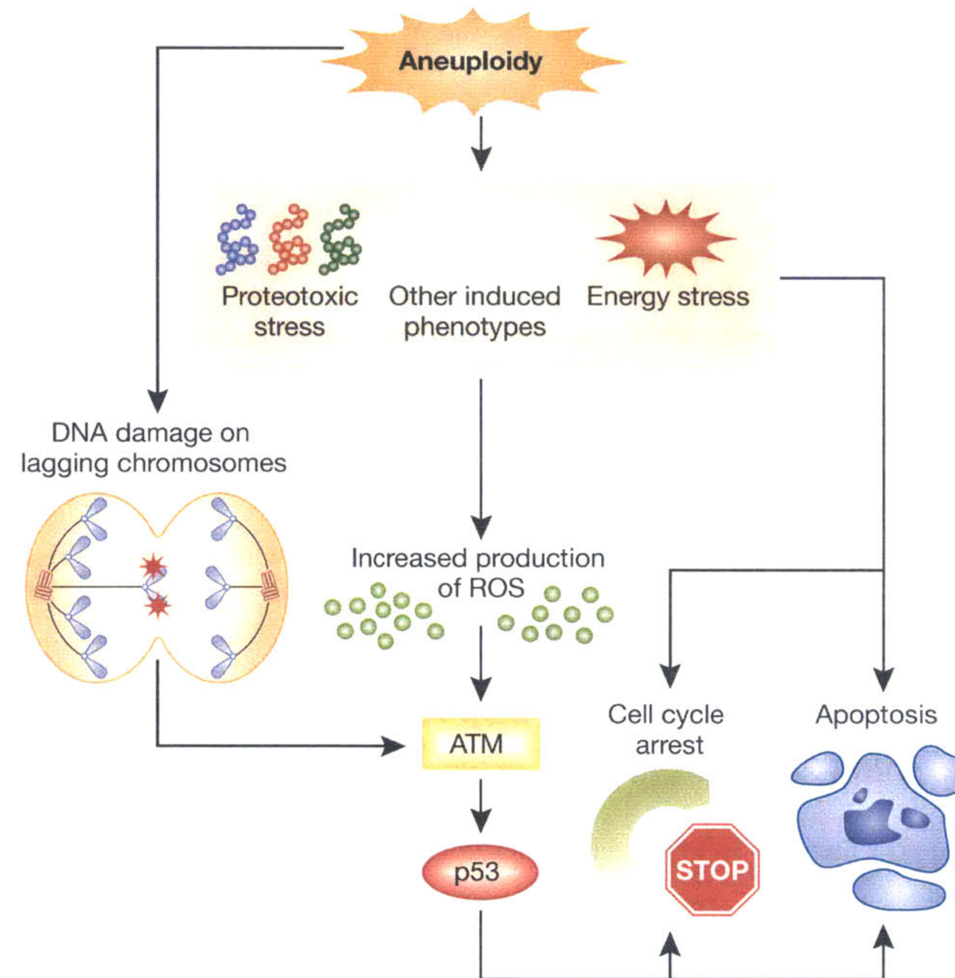

Figure 2. *Effects of aneuploidy on cell physiology*

The generation of aneuploidy after chromosome missegregation has been proposed to trigger a checkpoint-like cellular response. Energy, proteotoxic and other aneuploidy-associated stresses have been proposed to increase the production of reactive oxygen species, which activate p53 through ATM (Li et al., 2010). DNA damage on lagging chromosomes during aberrant mitoses triggers a p53-response through ATM (Janssen et al., 2011). Depending on the level of aneuploidy, this p53 response can either trigger a cell-cycle arrest or promote apoptosis. Aneuploidy can also interfere with cell proliferation by p53-independent mechanisms, as trisomic MEFs do not mount a p53 response but have proliferation defects. ATM, ataxia telangiectasia mutated; MEF, mouse embryonic fibroblast; ROS, reactive oxgen species.

cells in the population acquire low-grade aneuploidies. Growth defects or death of a small fraction of the cell population could go unnoticed in population doubling measurements. Live-cell analysis may be needed to detect proliferation defects of individual aneuploid cells.

Aneuploidy and cancer

In vitro characterization of chronic defined and acute random aneuploid cells has determined that whole chromosomal aneuploidy is generally detrimental to cellular fitness. The genetic imbalances induced by aneuploidy cause —among other deleterious outcomes— a disruption in protein and energy homeostasis and proliferation defects. However, aneuploidy has been postulated to be involved in tumorigenesis for over 100 years (Boveri, 2008), and the prevalence of aneuploidy in human tumors (Weaver and Cleveland, 2006) argues that something about the aneuploid cell condition is advantageous to cancer cells. The complex effects of aneuploidy are exemplified in its role in tumorigenesis.

Several approaches have been taken to examine the impact of aneuploidy and CIN on tumorigenesis. In asking how aneuploidy *per se* influences tumorigenesis, we are limited by the fact that only a very small subset of aneuploidies is viable in mammals: two constitutional trisomies can survive infancy in humans and none survive embryonic development in the mouse. However, cancer karyotypes are complex and have high degrees of aneuploidy, calling into question the relevance of studies of single chromosomal aneuploidies in understanding the role of aneuploidy in tumorigenesis. Mouse models of CIN have been developed that recapitulate the more complex aneuploidies seen in cancer but, as discussed above, additional aspects of chromosomal instability and/or additional effects of the mutations that cause CIN make it difficult to unambiguously determine how aneuploidy *per se* affects tumorigenesis. Therefore, the combined analysis of both models, chronic defined aneuploidies and CIN-induced random

acute aneuploidies, is critical to unravel the role of aneuploidy in tumorigenesis. In what follows, we will discuss studies of chronic defined aneuploidies and CIN in both humans and mice that shed light on the role of aneuploidy in cancer.

Constitutional aneuploidy and cancer

In humans, individuals trisomic for either autosome 13, 18 or 21 survive to birth in appreciable frequencies in the population. As in other organisms, constitutional trisomy in humans leads to developmental defects and increased risk of specific pathologies. Trisomy 18 — also known as Edwards syndrome— has a prevalence between 1 in 3,000 and 1 in 8,000 live births, and only about 10% of these individuals survive the first year of life, generally due to severe cardiovascular and brain defects (Lin et al., 2006). Trisomy 13 —or Patau syndrome— is the least frequently observed constitutional autosomal trisomy. Individuals with Patau syndrome have a number of severe developmental abnormalities at birth, making their survival after the first year of life extremely rare, although a few individuals have been reported to live into their teens (Hsu and Hou, 2007). Trisomy 21 —or Down Syndrome (DS)— is observed with an incidence of about 1 in 700 live births, making it the most commonly observed constitutional autosomal trisomy (Hassold and Jacobs, 1984). The phenotypic manifestation of DS is complex and variable, but is commonly associated with mental retardation, heart defects, early onset of Alzheimer disease (Roizen and Patterson, 2003) and reduced life expectancy (Glasson et al., 2002).

Evaluating the tumor spectra of individuals with DS and Edwards syndrome provides a means for observing the effect of chronic aneuploidy on tumorigenesis in humans. Because DS is the most prevalent chromosome abnormality in the population and affords the longest life expectancy of all autosomal trisomies, there are substantially more data on the tumor profile of people with trisomy 21 than with trisomy 18. Individuals with trisomy 21 have an increased risk

for acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) —particularly acute megakaryocytic leukemia (AMKL)— which is especially high in the first few years of life. In addition, lymphomas and germ cell tumors are more frequently observed in DS individuals than in the general population. However, they have a decreased risk of developing solid tumors throughout life (Satge et al., 1998). Although there are fewer individuals to evaluate, case studies reveal that Edwards syndrome predisposes to an increased risk of Wilms tumors and hepatoblastomas, when compared to age-matched controls (Ganmore et al., 2009).

It seems that DS or Edwards syndrome increase the risk of developing childhood cancers (Ganmore et al., 2009), which might be explained by chromosome-specific effects. Consistent with this idea is that the chromosome constitutionally trisomic in DS and Edwards Syndrome is also found to be trisomic in sporadic cases of the same types of cancer. For example, acquired trisomy 21 is a prominent cytogenetic characteristic in many hematologic neoplasms (Cheng et al., 2009; Mitelman et al., 1990)—notably in many non-DS cases of AMKL (Hama et al., 2008)— suggesting that it could be an oncogenic event that promotes the development of acute leukemias. Additionally, although trisomy 18 is not observed in non-Edwards syndrome hepatoblastomas (Sainati et al., 2002), it is frequently observed in non-Edwards syndrome Wilms tumors (Betts et al., 1997).

In contrast to these childhood cancers, the incidence of solid tumors is decreased in DS (due to the short life expectancy, tumor incidence of non-childhood solid tumors can not be well-examined in the other viable trisomies). Because there are only data regarding the incidence of solid tumors in adulthood from one constitutional trisomy, it is difficult to distinguish chromosome-specific effects from general aneuploidy effects. Several tumor suppressors are encoded on human chromosome 21, which could account for the tumor protective effect

observed in DS. However, although some DS phenotypes can be explained by amplification of specific genes or sets of genes, others cannot (Korbel et al., 2009). Therefore, the decreased incidence of solid tumors in DS individuals could also be reflective of decreased cellular fitness associated with constitutional trisomy 21, and the aneuploid state could provide tumor protection from childhood through adulthood. However, cancer is largely considered a disease of aging and environment, and it has been suggested that the decreased incidence of solid tumors observed in the DS population is due to the decreased life expectancy of these individuals or environmental biases (Satge et al., 1998).

Several mouse models of DS have been used to analyze the effects of constitutional trisomy 21 on cancer incidence (summarized in Table 1). Most studies were conducted in the Ts65Dn and Tc1 mouse models. The Ts65Dn mouse harbors 3 copies of those genes that are homologous to those encoded in human chromosome 21 which are found on mouse chromosome 16 (Reeves et al., 1995). This amplification includes all genes found in the "Down Syndrome Critical Region" (DSCR), including *DSCR1* or *RCAN1*, which is a regulator of VEGF-calcineurin signaling in endothelial tissues that decreases tumor growth and angiogenesis upon overexpression (Minami et al., 2004; Ryeom et al., 2008). The Tc1 mouse is a trans-chromosomal mouse model that contains ~90% of all genes on human chromosome 21 (O'Doherty et al., 2005), but does not contain the portion of the "Down Syndrome Critical Region" that contains *DSCR1*.

Two studies of these mice demonstrate that tumor angiogenesis is reduced in transplantable, subcutaneous lung carcinoma and melanoma tumor models (Baek et al., 2009; Reynolds et al., 2010). Ts65Dn mice exhibit decreased tumor burden —which is largely dependent on the expression of *DSCR1* in a dose-dependent manner— and the tumors that arise

have decreased microvessel density. Similar results were obtained with the Tc1 mouse (Reynolds et al., 2010). However, *DSCR1* is not contained in the amplified genomic region of the Tc1 mouse, suggesting that the decreased tumor angiogenesis observed is caused by altered dosage of other genes.

The effects of trisomy were also examined in mouse models of human cancer. The Apc^{Min} model of small intestine and colon cancer (Su et al., 1992) was examined in the Ts65Dn mouse (Sussan et al., 2008). Incidence of tumor formation and tumor size are reduced in Ts65Dn mice compared to their euploid littermates, an effect partially mediated by specific genes amplified in the DSCR, notably the transcription factor *ETS2*. Triplication of *ETS2* largely, but not completely, accounts for the decreased tumor incidence. However, the Ts65Dn model of DS does not affect tumor growth in the aggressive $Nf1^{+/-}$ $TP53^{+/-}$ (NPcis) neurofibromatosis type 1 cancer model (Yang and Reeves, 2011). NPcis mice develop lymphomas, sarcomas, or carcinomas with 100% penetrance due to loss of heterozygosity of the normal allele (Cichowski et al., 1999; Vogel et al., 1999). Trisomy does not decrease the incidence of tumor formation or reduce tumor size, but Ts65Dn mice have increased survival after tumor induction, an effect the authors hypothesize is due to a shift in the observed tumor spectrum, from largely sarcomas to adrenal and brain tumors and lymphomas. However, the increased survival time is not attributable to *ETS2* dosage. Furthermore, Ts65Dn NPcis mice do not have reduced tumor angiogenesis. It thus appears that the effects of trisomy of the genes amplified in these two mouse models have highly context-specific effects on tumorigenesis, but in both tumor models, the trisomy inhibits rather than promotes tumorigenesis.

CIN and cancer

Studies in constitutionally aneuploid humans and mice describe how a specific chromosome affects tumorigenesis. Most human tumors, however, become aneuploid by a

spontaneous aneuploidizing event, and often harbor a complex and diverse assortment of chromosomes and experience continuous changes in karyotype due to CIN. In humans, there is only one known heritable syndrome —mosaic variegated aneuploidy (MVA)— with increased levels of random cellular aneuploidies. MVA results from biallelic loss of function mutations in the spindle checkpoint component BubR1, which leads to premature sister chromatid separation and frequent mitotic nondisjunction (Hanks et al., 2004), or from biallelic mutations in Cep57, a centrosomal protein involved in nucleating and stabilizing microtubules and therefore ensuring correct chromosomal division during mitosis (Snape et al., 2011). Individuals with MVA exhibit mental retardation and other developmental defects and have cancer predisposition (Callier et al., 2005; Plaja et al., 2001b). Although this condition is extremely rare and few individuals with MVA have been reported to live past childhood, case studies reveal that rhabdomyosarcoma and Wilms tumors are frequent early in life (Plaja et al., 2001a). As with DS and Edwards syndrome, the increased incidence of these childhood cancers is probably the result of abnormal embryonic development due to changes in the gene dosage of specific gene products. Because the life expectancy of individuals with MVA is very short, it is difficult to determine whether having constitutional premature sister chromatid separation would lead to cancer predisposition later in life. In the one reported case where an individual survived beyond childhood (Plaja et al., 2001b), the generation of sporadic tumors later in life was not dramatically increased —as in DS— even though the aneuploidies generated in MVA are random and, thus, an appropriate gene combination that promotes tumor growth could be selected for. The individual developed and died from AML, suggesting there could be a bias towards hematologic cancers as in DS. However, many more additional case studies are necessary to draw any definitive conclusions about the role of MVA-induced aneuploidy in tumorigenesis in adults.

Although BubR1 is mutated in MVA, mutations in spindle assembly checkpoint (SAC) and chromosome segregation factor encoding genes are rarely observed in sporadic cancers (Schvartzman et al., 2010). However, a recent study found that a diverse range of tumor types contain deletions or inactivating mutations in *STAG2*, a gene located on the X chromosome that encodes a subunit of one of the mammalian cohesin complexes (Solomon et al., 2011). Because cohesin complexes hold sister chromatids together, their proper function is critical for accurate chromosome segregation (Jessberger, 2012; Nasmyth and Haering, 2009). Indeed, inactivation of *STAG2* in diploid cell lines leads to significant aneuploidy, suggesting that aneuploidy promotes tumorigenesis. A mouse model of cohesion deficiency further lends support to the idea that cohesins are critical for preventing tumor formation. Mice with a heterozygous deletion in the gene encoding the cohesin subunit SA1 lack cohesion at telomeres, leading to increased levels of aneuploidy and decreased cellular proliferation. Remarkably, however, these mice show an increased incidence of spontaneous tumors (Remeseiro et al., 2012). Nevertheless, it is important to bear in mind that cohesins do much more than ensure accurate chromosome segregation. During interphase, they have a critical role in gene expression and repair of DNA damage (Onn et al., 2008). Thus it is also possible that the role of cohesins in these cellular processes, in addition to their roles in ensuring accurate chromosome segregation, contributes to their tumor suppressive function.

To understand the role of spontaneous whole-chromosomal aneuploidy in tumorigenesis, several mouse models with decreased chromosome segregation fidelity have been generated (summarized in Table 1). Such models of CIN use genetic alterations that decrease chromosome segregation fidelity, either by interfering with the chromosome segregation machinery itself or with SAC function. Although it is difficult to determine whether the genetic alterations used to

induce chromosome mis-segregation, CIN itself and/or other potential genetic alterations resulting from chromosomal instability lead to a particular effect on tumorigenesis, these models provide invaluable insights into tumorigenesis and the effects that a potentially continuously changing genome has on disease progression.

Deficiency of BubR1 (encoded by the BUB1B gene) — an essential component of the SAC (Musacchio and Salmon, 2007)— has been analyzed in mice at the cellular and organismal levels (Baker et al., 2004) by using hypomorphic alleles. MEFs derived from BubR1-deficient mice have increased levels of chromosomal aneuploidy and an increased frequency of cellular senescence, and mice with decreased levels of BubR1 exhibit early aging and infertility phenotypes, without a significant increase in tumor formation. Thus, reduced expression of the BubR1 protein does not seem to result in cancer predisposition in mice. Rather, BubR1 deficient mice exhibit decreases in cellular and organismal fitness. Additionally, overexpression of BubR1 confers increased lifespan, decreased levels of endogenous aneuploidy and decreased risk for developing cancer in mice (Baker et al., 2012).

Mice with a heterozygous deletion of CENP-E —which encodes an essential, centromere-associated, kinesin motor— are largely normal, despite the presence of aneuploid cells throughout the body (Weaver et al., 2007). They have an increased incidence of tumorigenesis in some tissues, such as the lungs and lymphoid cells, but a decreased incidence and reduced size of liver tumors. However, haploinsufficiency of CENP-E in cells confers a transformed phenotype in soft agar and increased tumorigenicity in xenograft assays. Thus, tissue-specific effects seem to modulate the consequences of reduced levels of CENP-E.

Cdc20 is the mitotic activator of APC/C, an E3 ubiquitin ligase that targets key mitotic substrates —notably cyclin B1 and securin— for degradation, allowing anaphase onset. The

SAC inhibits the ability of Cdc20 to activate APC/C when chromosomes are not correctly attached to the mitotic spindle, but the $Cdc20^{AAA}$ allele cannot be inhibited by the SAC and therefore induces premature sister chromatid separation and subsequent aneuploidies (Li et al., 2009). This allele increases late-onset tumorigenesis in mice: heterozygous animals exhibit an increased tumor incidence by 24 months of age, with 50% of the $Cdc20^{+/AAA}$ mice developing tumors compared to 10% of the wild type control, and an altered tumor spectrum, as hepatomas are observed in the mutant but not the control population.

Mad2, a key component of the spindle assembly checkpoint, has been both overexpressed to hyperactivate the SAC and deleted to weaken its activity. Mad2 inhibits APC/C-Cdc20 and is thus the lynchpin of the SAC (Minami et al., 2004). Deletion of one copy of MAD2 induces aneuploidy *in vitro* and *in vivo* and leads to a high frequency of mice with papillary lung adenocarcinomas, a tumor that is extremely rare in wild type mice (Michel et al., 2001). However, a much more dramatic phenotype is observed when Mad2 is overexpressed. Overexpression of Mad2 delays rather than accelerates anaphase entry and results in a greatly increased incidence of spontaneous tumors with a wide ranging spectrum (Sotillo et al., 2007). This oncogenic effect of a spindle assembly checkpoint component in mice is consistent with what is observed in human tumors. Loss-of-function mutations of SAC components are rarely observed in cancer cells, but overexpression is frequently seen. Loss of function of the tumor suppressor *RB1* has been shown to lead to increased basal activation of the mitotic checkpoint (Hernando et al., 2004). Decreased Rb levels relieve inhibition of the transcription factor E2F, leading to overexpression of its direct target, Mad2. Mad2 inhibits APC/C-Cdc20, leading to

Table 1. *Mouse models of CIN or aneuploidy and cancer*

Affected Gene	Study	Karyotype effect	Effect on Cell Proliferation	Effect on Tumorigenesis
BUB1	(Jeganathan et al., 2007)	CIN	None reported	$Bub1^{+/-}$ mice have decreased tumor incidence; $Bub1^{+/H}$ and $Bub1^{H/H}$ mice have increased tumor incidence and altered tumor spectrum
BUB1 overexpression	(Ricke et al., 2011)	CIN	None reported	Increased tumor incidence and altered tumor spectrum
BUB1B +/- (aka BUBR1)	(Baker et al., 2004)	CIN	$Bub1b^{H/H}$ MEFs proliferate more slowly that WT by proliferation assays; $Bub1b^{-/H}$ MEFs proliferate even more slowly	$Bub1b^{H/H}$ mice have early aging and infertility phenotypes, no significant increase in tumor formation; $Bub1b^{-/H}$ mice die a few hours after birth
BUB3 +/-	(Babu et al., 2003)	CIN	Growth rate not different from WT by proliferation assays	Increased incidence of tumors after carcinogen treatment
$CDC20^{AAA}$	(Li et al., 2009)	CIN	Cells proliferate more slowly than WT	Increased incidence of tumors and altered tumor spectrum
CENP-E	(Weaver et al., 2007)	CIN	Growth rate not different from WT by proliferation assays	Altered spontaneous tumor spectrum and reduced tumor incidence after treatment with carcinogens
Mad2 +/-	(Michel et al., 2001)	CIN	None reported	Increased tumor incidence and altered tumor spectrum
Mad2 overexpression	(Sotillo et al., 2007)	CIN	Cells proliferate more slowly than WT	Greatly increased incidence of spontaneous tumors, wide-ranging tumor spectrum
Rae1+/-	(Babu et al., 2003)	CIN	Growth rate not different from WT by proliferation assays	Increased incidence of tumors after carcinogen treatment
SA1+/-	(Remeseiro et al., 2012)	CIN	Cells proliferate more slowly than WT	Increased incidence of tumors and altered tumor spectrum
UbcH10 overexpression	(van Ree et al., 2010)	CIN	Growth rate not different from WT by proliferation assays	Increased incidence of spontaneous tumors and tumors after carcinogen treatment
CENP-E+/- $p19^{Arf}$-/-	(Weaver et al., 2007)	CIN	None reported	Decreased tumor incidence and size
Mad2 overexpression $Kras^{G129D}$	(Sotillo et al., 2010)	CIN	None reported	Larger, more aggressive tumors produced that are prone to relapse
Ts65Dn xenograft	(Baek et al., 2009)	DS	None reported	Decreased tumor burden and reduced tumor angiogenesis
Tc1 xenograft	(Reynolds et al., 2010)	DS	None reported	Decreased tumor burden and decreased tumor angiogenesis
Ts65Dn Apc^{Min}	(Sussan et al., 2008)	DS	None reported	Decreased tumor incidence and size
Ts65Dn NPcis	(Yang and Reeves, 2011)	DS	None reported	Altered tumor spectrum and increased survival after tumor induction

prolonged metaphase arrest and consequent mitotic slippage, whereby cells exit from mitosis without undergoing chromosome segregation and become tetraploid or mis-segregate chromosomes (Schvartzman et al., 2010). Similar results are observed when the SAC is hyperactivated by overexpression of the outer kinetochore component Hec1 (Diaz-Rodriguez et al., 2008) or by overexpressing the SAC kinase Bub1 (Ricke et al., 2011). Both result in aneuploidies *in vitro* and cause an increase in tumor incidence and alteration of tumor spectra *in vivo*, although this increase is not as dramatic as that observed upon Mad2 overexpression.

In summary, both weakening and hyperactivating the SAC is sufficient to generate aneuploidy and to induce tumorigenesis. However, although tumorigenesis is elevated, this increase is somewhat modest in many cases, particularly in mice with loss-of-function mutations in SAC genes. It has been suggested that —just as cells with defects in chromosome cohesion may have more dramatic phenotypes because cohesins have various functions— the range in severity of the phenotypes observed in cells with CIN differs depending on the number of processes that will be affected when such a mutation is incurred. If mutating a factor only affects one cellular process that promotes tumorigenesis, the effect will be less severe than if multiple tumor-promoting pathways are affected by a single alteration (Ricke et al., 2008).

The situation changes when these chromosome instability-inducing mutations are combined with p53 loss or other oncogenic mutations. Crossing mice with mutated SAC components into mice homozygous for a p53 deletion has dramatic effects. $p53^{-/-}/Cdc20^{+/AAA}$ and $p53^{-/-}/Bub1^{H/H}$ mice have increased tumorigenesis and decreased survival compared to either mutation alone (Li et al., 2010). The combined overexpression of Mad2 and an inducible $Kras^{G12D}$ model also has dramatic effects, generating larger tumors that are more aggressive and exhibit higher grade aneuploidy than tumors generated with oncogenic Kras expression alone

(Sotillo et al., 2010). Furthermore, these mice have ~50% tumor recurrence after repression of Mad2 and $Kras^{G12D}$ expression, a phenotype which was never observed in mice with oncogenic Kras expression alone (Sotillo et al., 2010). These results not only indicate that sustained overexpression of Mad2 is not required for tumor progression once tumors have developed, but also suggest that Mad2 overexpression leads to increased chromosomal instability, which overcomes addiction to the $Kras^{G12D}$ oncogene. In contrast, other oncogenic mutations do not lead to increased tumor formation in mice with increased chromosome mis-segregation frequencies. For example, deletion of the tumor suppressor $p19^{Arf}$ in CENP-$E^{+/-}$ mice leads to decreased tumor incidence and a reduction in tumor size (Weaver et al., 2007). Perhaps loss of a tumor suppressor such as p53 is a prerequisite for the development of aneuploidy in human tumors or an event required immediately after aneuploidy induction to promote tolerance to the aneuploid state. Combined models of inducible aneuploidy and inducible loss of tumor suppressors such as the one described in (Sotillo et al., 2010) could be used to fully dissect this relationship.

Together, these results demonstrate that similar to the range of tumorigenesis phenotypes observed in mouse models of CIN, introduction of CIN into mouse models of cancer has tumor-promoting and tumor-suppressive effects. However, when CIN inducing mutations are combined with the loss of p53, more aggressive disease is consistently observed. Thus, in the absence of the gene that limits the proliferation of aneuploid cells, the tumorigenesis-promoting effects of CIN seem to reach their full potential. Exactly how CIN primes cells for tumorigenesis has not yet been elucidated. Below, we propose a model for how this may occur and discuss how CIN and aneuploidy could both promote and suppress tumorigenesis.

Aneuploidy and CIN in tumorigenesis – a model

The study of the effect of CIN and chromosomal trisomies on tumor formation in mouse models has revealed complex interactions between the aneuploid state and tumorigenesis. Aneuploidy seems to promote a dual cellular state: generally the presence of an unbalanced genome induces a cellular stress response and slow growth, whereas in rare selective circumstances —or in the presence of aneuploidy-tolerating mutations— aneuploidy can be beneficial and lead to increased cellular proliferation and cancer. We believe that the following general themes provide a working model for how CIN and aneuploidy impact tumorigenesis (Figure 3A).

A. Aneuploidy hinders cell proliferation in most cases. This anti-proliferative effect can be mitigated by genetic alterations that allow cells to tolerate the adverse effects of aneuploidy and/or by mutating genes that restrict proliferation of aneuploid cells, such as p53.
B. Chromosome mis-segregation is at least sometimes accompanied by DNA damage, due to events such as chromosome breakage during cytokinesis. The aneuploid state itself can also induce genome instability.
C. Under specific selective pressures, aneuploidy can provide a survival advantage.

The results discussed above obtained in various mouse models clearly indicate that not all chromosomal instability-inducing mutations have the same effect. Mutations that cause defects in sister chromatid cohesion prior to mitosis are highly tumorigenic, as evidenced by the mice expressing a hypermorphic allele of Cdc20 (Li et al., 2009), mice heterozygous for SA1 (Remeseiro et al., 2012), and the *STAG2* loss-of-function found in many human cancers (Solomon et al., 2011). SAC genes, the primary function of which is to ensure accurate chromosome segregation —and the inactivation of which has modest effects on sister chromatid cohesion before mitosis—are rarely found mutated in human cancers. Mouse models with loss-of-function mutations in SAC genes generally have a somewhat modest increase in tumor incidence.

The first conclusion we can draw from these observations is that CIN caused by the mutations that affect sister chromatid cohesion is not the only reason for the significant tumor-promoting effects of these mutations, and that the role of cohesin in controlling gene expression and DNA damage repair is probably also critical for its tumor suppressive functions. The second conclusion we can draw is that CIN and the aneuploidies produced upon chromosome mis-segregation have only a moderate positive impact on tumorigenesis on their own. In the case of trisomy 21, aneuploidy even has a tumor protective function (the tumors that are seen early in life are caused by developmental abnormalities due to imbalances in the dosage of specific genes). This is not surprising in the light of point (A), that aneuploidy generally interferes with cell proliferation (Figure 3A).

However, the picture changes when the anti-proliferative effects of CIN and aneuploidy are mitigated through the inactivation of p53. As mentioned above, mice with mutations that cause increased chromosome mis-segregation combined with loss of function p53 mutations show a dramatic increase in tumorigenesis. Mutations that improve the proliferation of aneuploid cells have been described in yeast (Torres et al., 2010). Aneuploidy-tolerating mutations in mammalian cells could similarly increase the proliferative potential of aneuploid cells. Once the adverse effects of aneuploidy have been suppressed or ameliorated, the potential tumorigenesis-promoting effects of the condition could come into play (Figure 3B).

Aneuploidy and cellular adaptability

One hypothesis is that aneuploidy contributes to cancer by conferring cells with a fitness advantage in extreme environments. This idea is supported by work in yeast that has demonstrated that aneuploidy can be a short-term solution for cells under extreme stress. For example, yeast cells often gain a copy of chromosome 3 to tolerate continuous heat shock (Yona et al., 2012); yeast grown continuously in the presence of radicicol select for amplification of

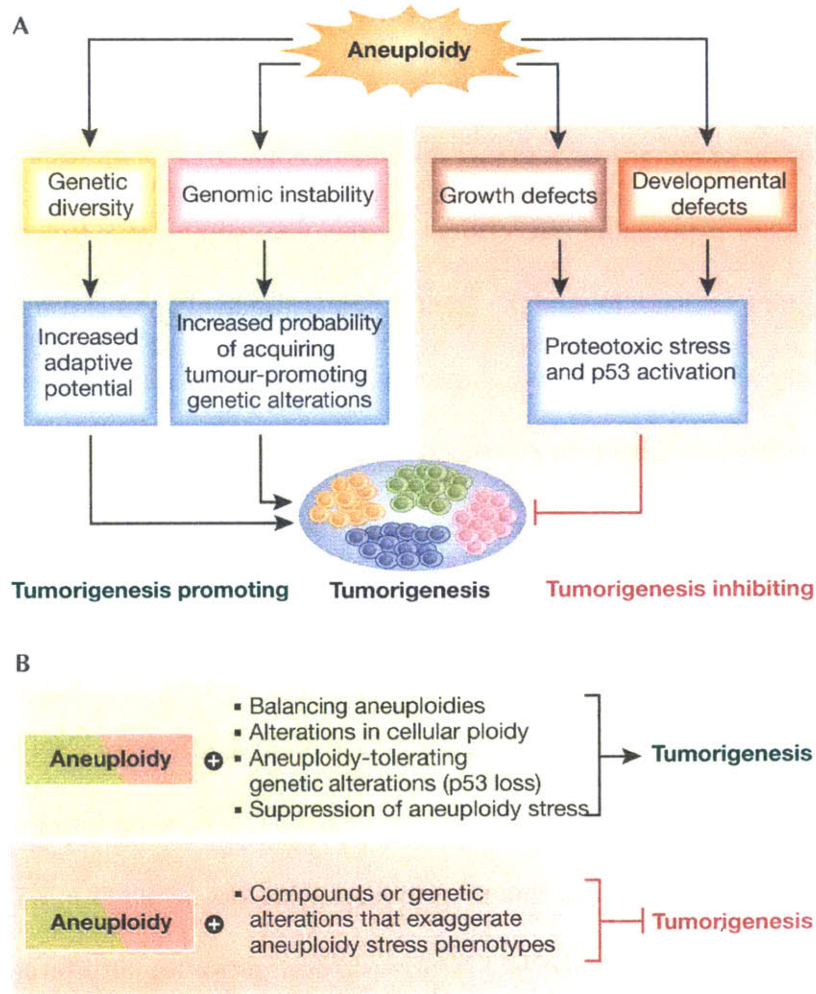

Figure 3. *A model for the role of aneuploidy in tumorigenesis*

(**A**) Aneuploidy is most frequently associated with characteristic phenotypes, such as defects in cell proliferation and developmental defects in whole organisms. These phenotypes are often accompanied by particular cellular responses, such as increased proteotoxic stress and activation of p53. Generally, these adverse effects of aneuploidy serve to inhibit tumorigenesis. Aneuploidy can also generate genetic diversity, which can provide cells with increased adaptive potential when challenged and thus could be a means for promoting special aspects of tumorigenesis, such as metastasis. Finally, aneuploidy is also commonly associated with genomic instability, increasing the probability of acquiring tumour-promoting genetic alterations and thereby significantly contributing to tumorigenesis. (**B**) The adverse effects of aneuploidy can impair tumorigenesis, but in the presence of aneuploidy-tolerating mutations, increased ploidy or balancing aneuploidies, this anti-tumorigenic effect is lessened and the potential tumorigenesis-promoting effects of aneuploidy reach their full potential. Conversely, compounds or genetic alterations that enhance the adverse effects of aneuploidy could shift the equilibrium towards its anti-proliferative effects, thus preventing the growth of aneuploid cancer cells.

chromosome 15 (Chen et al., 2012); *Candida albicans* strains isolated from patients treated with fluconazole acquired segmental trisomy of chromosome 3 or 5 to develop resistance to drug treatment (Ford et al., 2015; Selmecki et al., 2006); and yeast grown under continuous nutrient selection conditions acquire reproducible, sub-chromosomal aneuploidies and rearrangements (Dunham et al., 2002). A more rigorous exploration of the role of aneuploidy in adaptation to various stresses has identified aneuploidy as a common adaptive solution for yeast cells under a variety of nutrient-limiting growth conditions (Sunshine et al., 2015). This hypothesis is further supported by the finding that aneuploid yeast cells show improved growth in some conditions that are sub-optimal for euploid yeast cells (Pavelka et al., 2010). Additionally, tetraploidy – a genomic state often proposed to be a key step in tumorigenesis in a number of cancers (Ganem et al., 2007) – has been shown to increase the rate of adaptability when yeast strains are challenged to grow in poor nutrient conditions by allowing cells to more extensively explore the fitness landscape by gaining and/or losing a number of chromosomes (Selmecki et al., 2015).

It is thought that acquisition of aneuploidy in these instances selects for gains of key genes that promote survival in each particular environment. For example, the growth advantage of disomy 15 in yeast grown in radicicol can be attributed to the acquisition of extra copies of the pleiotropic drug pump *PDR5* and the protein chaperone *STI1* (Chen et al., 2012). There is also evidence for the role of aneuploidy in mammalian cellular adaptation. A similar mechanism of evolution as seen in yeast was observed in a mouse model of liver injury (Duncan et al., 2012). Mice that lose expression of homogentisic acid dioxygenase *(HgdI)* in the fumarylacetoacetate hydrolase *(Fah)* deficient mouse model of liver disease are protected from disease. *Fah* deficient mice that were heterozygous for a loss of function *HgdI* allele were exposed to chronic liver injury. Injury-resistant nodules appeared in the liver, and karyotypic analysis of these cells

revealed that 50% of resistant nodules did not express Hgd1 protein because they had lost chromosome 16, where the *Hgd1* gene is located.

Aneuploidy and genomic instability

In the context of cancer, aneuploidy may allow cells to define and modify their functional capacities. If cancer cells use karyotype changes in a similar manner to evolving yeast strains, aneuploidy could provide a convenient way to alter the dosage of specific beneficial genes. Such alterations could promote the development of cancer cell phenotypes such as escape from growth control. However, such changes in karyotype, when selected in the right combination, could also lead to the acquisition of new functions, such as the ability to migrate or seed distant metastatic sites. This idea is further supported by additional adaptation experiments in yeast: yeast acquire whole-chromosome aneuploidies when challenged to evolve new traits (Rancati et al., 2008). Mammalian cell lines can also acquire new cellular functions upon acquisition of aneuploidy: MEF cell lines often become aneuploid when adapting to growth in culture (Todaro and Green, 1963); p53$^{-/-}$ mouse mammary epithelial cells chemically induced to become tetraploid become chromosomally unstable and acquire new functions, such as the ability to form tumors in a xenograft assay (Fujiwara et al., 2005); and primary human fibroblasts harboring an additional copy of chromosome 8 have transformed cell phenotypes, such as loss of contact inhibition (Nawata et al., 2011).

In addition to generated cellular phenotypic diversity, aneuploidy has also been hypothesized to contribute to cancer cell phenotypes by further increasing genomic instability. Indeed, the genome instability-inducing effects of aneuploidy and chromosome mis-segregation recently described in yeast (Sheltzer et al., 2011) and mammals (Crasta et al., 2012; Janssen et al., 2011) respectively, probably have a major impact on disease progression. Chromosome mis-segregation has been shown to cause DNA damage during the subsequent cytokinesis (Janssen et

al., 2011). Increased double strand breaks, as a result of inadequate replication in micronuclei, could also be a significant source of oncogenic mutations. Recent studies in mammalian cell lines demonstrate that micronuclei that are generated after chromosome mis-segregation undergo substantial DNA damage during replication (Crasta et al., 2012; Hatch et al., 2013). Cells that contain these micronuclei only persist when p53 function is absent, further emphasizing the importance of p53 in preventing genome instability (Crasta et al., 2012). DNA from micronuclei can reincorporate into the nucleus, providing a mechanism by which a micronucleus could contribute to a number of simultaneous mutations and alterations in chromosomal composition and copy number (Zhang et al., 2015). This mechanism of transient sequestration of chromosomes in micronuclei could also explain the recently discovered process of chromothripsis, where hundreds of genomic rearrangements occur within one or few chromosomes (Stephens et al., 2011), as is observed in 2-3% of all cancers (for example medulloblastoma (Rausch et al., 2012)). In addition to chromosome mis-segregation leading to further aneuploidy, the aneuploid state itself has been shown to cause multiple forms of genomic instability in budding yeast, with increased number of double strand breaks observed in many different aneuploid strains as well as aneuploid fission yeast cells (Sheltzer et al., 2011). DNA damage has a critical role in tumor evolution (Halazonetis et al., 2008), and we speculate that the DNA damage-inducing features of whole-chromosome mis-segregation and/or the aneuploid state itself could be a critical aspect of the tumor-promoting effects of aneuploidy (Figure 3A).

In summary, we propose that CIN and aneuploidy have modest tumor-promoting abilities conferred through their associated genome instability and their potential for generating new traits. However, these tumorigenic traits are offset by the antiproliferative effects associated with aneuploidy. When these anti-proliferative effects are suppressed —through aneuploidy tolerating

mutations, increased ploidy or balancing aneuploidies— the full tumorigenic potential of the condition is unleashed (Figure 3B). This hypothesis predicts that mutations that suppress the antiproliferative effects of CIN and/or aneuploidy are especially critical factors in tumorigenesis (Figure 3B). Indeed, p53 is one of the most frequently mutated genes in human cancers, and the familial form of p53 loss —known as Li-Fraumeni syndrome— predisposes affected individuals to a wide spectrum of cancers (Kleihues et al., 1997). p53 may be special in that it seems to protect cells not only from numerical chromosomal abnormalities such as aneuploidy (Fang and Zhang, 2011) and tetraploidy (Ganem et al., 2007; Storchova and Pellman, 2004; Stukenberg, 2004) , but also from structural aberrations through its central function in the DNA damage checkpoint pathway (Dalton et al., 2010).

Cancer cells also seem to evolve karyotypes in which aneuploidies are mitigated by polyploidy and/or additional aneuploidies. Chromosome gains often co-occur with other chromosome gains, and likewise chromosome losses often co-occur with other chromosome losses, suggesting that these extra gain/loss events are a compensatory mechanism that attempt to balance alterations in gene dosage caused by chromosomal instability (Ozery-Flato et al., 2011). Other aneuploidy-tolerating effects may also be important. Disomic yeast cells also try to compensate for their altered gene dosage by degrading the excess of some proteins, especially of proteins found in large molecular complexes such as the ribosome (Torres et al., 2007; 2010). Evolution experiments uncovered aneuploidy-tolerating mutations in proteins such as *UBP6,* a ubiquitin-specific protease that antagonizes the degradation of a number of proteasome substrates in yeast (Torres et al., 2010). Identifying the genetic alterations that allow unbalanced, aneuploid mammalian cells to restore balance and tolerate aneuploidy could provide key insights into tumorigenesis and new targets for the development of cancer therapeutics.

Aneuploidy as a therapeutic target

Initial approaches to cancer treatment targeted a phenotype common to all cancer cells: increased proliferation. Cancer is a disease of uncontrolled proliferation, and therefore, chemotherapeutics —which kill all rapidly dividing cells by interfering with DNA synthesis and chromosome segregation— are effective anti-cancer agents. However, cancer cells have a nearly relentless ability to adapt to their environments, mutate and survive in response to treatments (Hanahan and Weinberg, 2011). Therefore, patients treated with chemotherapeutics often relapse due to the acquisition of chemotherapeutic resistance (Gottesman, 2002) or the presence of dormant tumor cells (Goss and Chambers, 2010), and their cancers metastasize.

Subsequent approaches in cancer treatment targeted single gene products to which cancer cells are addicted. Imatinib (Gleevec) (Druker et al., 2001; Tuveson et al., 2001), which targets the hybrid kinase BCR-ABL and trastuzumab (Herceptin) (Baselga et al., 1998), which targets the EGFR family member HER2, are among the most successful examples in this category of cancer therapeutics. However, even these targeted treatments eventually cease to be effective, as cells develop additional mutations to acquire resistance to these drugs (Knight et al., 2010). Combination therapies that eliminate all tumor cells by targeting both specific genetic lesions and general cancer cell characteristics early during treatment currently seem like the most promising method for exacting a cure. Thus, identifying as many differences between normal and tumor cells, and developing agents that selectively target as many of these differences as possible simultaneously could prove to be a potent means of eliminating cancer cells.

Because adaptability is so important to cancer cell survival and higher degrees of aneuploidy are frequently associated with poor prognosis (Rajagopalan and Lengauer, 2004), aneuploidy should be considered as a therapeutic target. A proof-of-principle small-scale screen for compounds that preferentially impair proliferation of trisomic MEFs compared to euploid

MEFs, identified the energy-stress inducing compound AICAR, the autophagy inhibitor chloroquine, and the Hsp90 inhibitor 17-AAG as aneuploid selective drugs (Tang et al., 2011). Autophagy and Hsp90 are both required for eliminating protein aggregates and maintaining proteostasis, and their protein quality control functions appear to be rate-limiting in aneuploid cells. In contrast, proteasome inhibitors were not identified in this screen suggesting that, unlike in aneuploid yeast, proteasome activity is not limiting in trisomic MEFs. Interestingly, AICAR and 17-AAG also inhibit the growth of highly aneuploid colon cancer cell lines that have chromosomal instability. AICAR has not yet been tested in clinical trials, but treatment of cancer patients with 17-AAG in phase II clinical trials either did not yield anti-cancer properties, despite activation of Hsp70 (Solit et al., 2008) or needed to be stopped due to adverse side effects (Heath et al., 2008). Therefore, as with many cancer drug candidates, although these compounds seem to be effective *in vitro*, they may be ineffective in a therapeutic setting. Nevertheless, targeting aneuploidy for cancer therapy is worthy of further exploration and large-scale screens could identify novel unanticipated sensitivities of aneuploid cells (Figure 3B).

In addition to identifying pan-aneuploidy inhibitors, the isolation of compounds that selectively impair the proliferation of cells harboring specific aneuploidies should also be explored. Many cancers frequently show gain or loss of a specific chromosome. For example, trisomy 8 is frequently observed in patients with AML, and its presence is associated with poor survival when combined with other genetic aberrations (Wolman et al., 2002). Developing compounds that selectively eliminate cells with this particular aneuploidy could also provide an additional way in which to target specific cancers. Furthermore, proof of principal experiments in yeast have provided a potential therapeutic path for targeting chromosomally unstable cancer cells (Chen et al., 2015). By predicting the particular aneuploidies cells tend to adapt upon

exposure to specific drugs or stresses and then targeting simultaneously the stress and the characteristic aneuploidy that occurs as a response to that stress could be an effective way to eliminate aneuploid cells from a population.

In summary, aneuploidy has a complicated but significant role in tumorigenesis. Finding treatments that exacerbate the phenotypes exhibited by aneuploid cells and selectively kill them could prove a fruitful endeavor with regards to cancer treatments.

Towards a cellular system to study aneuploidy *in vivo*

Studies of aneuploid cells *in vitro* and mouse models of Down syndrome and CIN *in vivo* have shed some light on the role of aneuploidy in tumorigenesis. However, the precise role of aneuploidy in cancer still remains unclear. Because targeting aneuploidy-associated phenotypes has demonstrated potential as a therapeutic strategy, it is important to develop systems to better dissect the relationship of aneuploidy to cancer. Part of the problem may be the model systems that have been used to evaluate this complex relationship: *in vitro* systems do not fully recapitulate the complex environmental conditions cancer cells experience *in vivo*; and, as described above, mouse models of DS and CIN are generally not particularly tumor prone, in part due to tumor microenvironment differences. Thus, we sought develop a cellular model to characterize the effects of aneuploidy *per se* on cell physiology *in vivo*. To develop this system, we chose to utilize hematopoietic stem cells (HSCs). This model is both convenient for studying aneuploidy *in vivo* and relevant to DS, as DS individuals exhibit a number of hematopoietic phenotypes, including an increased risk of leukemia (Satge et al., 1998). Furthermore, it offers a unique model for studying aneuploid cells in the context of an otherwise euploid environment, a setting more typical of tumorigenesis *in vivo*.

Blood and hematopoietic stem cells

The blood is a liquid organ found throughout the body. While a variety of cell types comprise the blood (Figure 4), there are two main lineages, the myeloid and the lymphoid lineages (Alberts et al., 2002). Myeloid cells include red blood cells, which deliver oxygen throughout the body; megakaryotyes, which give rise to platelets, small portions of megakaryocyte cytoplasm that permit blood clotting; and cells that are the major components of

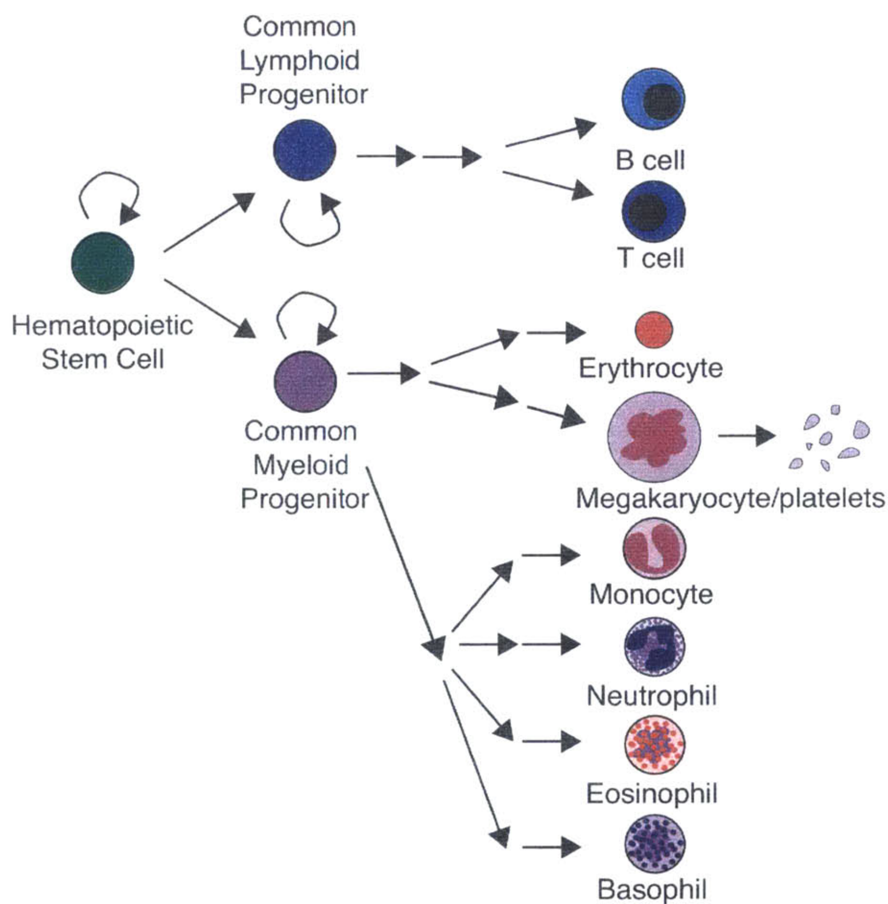

Figure 4. *Hematopoiesis*

Hematopoiesis is initiated by a hematopoietic stem cell (HSC) that either divides to self-renew or to differentiate into a cell that will become a common lymphoid progenitor (CLP) or a common myeloid progenitor (CMP). These common progenitors further divide to self-renew and differentiate into different, committed progenitor cells that will ultimately give rise to all the differentiated cell types in the blood.

the innate immune system including monocytes, the precursors to macrophages – cells that survey tissues for infections; and granulocytes, granule-containing comprised of neutrophils, basophils and eosinophils that combat infections. Lymphoid cells are the major components of the adaptive immune system and are comprised of B cells, which produce antibodies and become memory cells after infection; and T cells, which assist other immune cells, destroy virus-infected or tumor cells, become memory cells after infection and are important for developing immunological tolerance.

Blood cells can be distinguished by their expression of cell type-specific cell surface antigens, which allow cells to recognize one another and potentially stimulate immune responses through cell-cell interactions. Antibodies raised against these specific cell surface markers identified specific "clusters of differentiation" and permitted initial identification of different cell types by immunophentoyping (Bernard and Boumsell, 1984). These specific antibodies remain a convenient research tool for identifying different blood cell types by their expression of unique cell surface markers or combinations of cell surface markers.

While the blood contains many cells types that perform numerous functions, all blood cells are derived from a single cell type, the hematopoietic stem cell (HSC). Hematopoiesis, the process by which blood is made, is a hierarchical differentiation process whereby hematopoietic stem cells divide asymmetrically to generate progenitor cells, which subsequently divide to amplify and also differentiate to give rise to all downstream cell types (Figure 4). HSCs are found in the bone marrow in adult mice and humans, where they are mostly quiescent (Busch et al., 2015; Sun et al., 2015); however, HSCs undergo several migrations during embryonic development to reach this final destination (Figure 5).

Primitive hematopoiesis is the process of forming blood cells that help sustain the

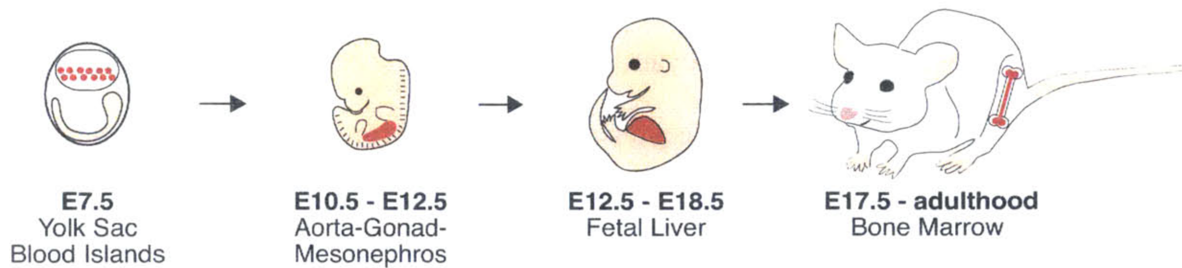

Figure 5. *Ontogeny of the hematopoietic stem cell in mice*

Primitive HSCs arise around E7.5 in yolk sac blood islands. These cells do not contribute to the adult HSC pool. Definitive HSCs are largely formed around E10.5 in the aorta-gonad-mesonephros (AGM) region. These cells migrate to the fetal liver, where they rapidly proliferate for several days before reaching the bone marrow during late embryonic development. HSCs largely remain in the bone marrow throughout adulthood.

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embryo but do not ultimately contribute to the adult organism. Primitive hematopoiesis begins around embryonic day 7.5 (E7.5) with the emergence of yolk sac blood islands that produce primarily primitive erythroid cells (Baron et al., 2012; Mikkola and Orkin, 2006). This production of red blood cells allows for proper oxygenization of embryonic tissues during a period of rapid growth (Orkin and Zon, 2008). Definitive hematopoiesis is the process of forming blood cells that both sustains the developing embryo and contributes to adult hematopoiesis after development. While there is evidence that HSCs in the yolk sac can also contribute to definitive hematopoiesis (Moore and Metcalf, 1970; Palis et al., 1999; Samokhvalov et al., 2007), the majority of definitive HSCs are thought to arise from the aorta-gonad-mesonephros (AGM) region around E10.5 (Müller et al., 1994). These cells then migrate to the developing fetal liver around E11.5, where they rapidly expand for several days (Morrison et al., 1995) before gradually entering circulation to seed the bone marrow starting around E15.5 (Christensen et al., 2004). HSCs enter circulation and then "home" to the bone marrow by

entering the bone marrow niche, where they interact with niche cells via cell adhesion molecules and colonize (Lapidot, 2005).

Much of what has been learned about hematopoietic stem cells has been gleaned from transplantation experiments. By administering whole body irradiation, one can eliminate HSC function by inducing apoptosis (Meng et al., 2003) or senescence (Wang et al., 2006) of HSCs. Then by injecting cell suspensions containing potential hematopoietic stem cells into the bloodstream of isogenic mice, one can assess the cell type of interest by viability of the recipient mice and long-term contribution of the donor cells to hematopoiesis. Donor- and recipient-derived blood cells can be distinguished by using the pan-leukocyte cell surface marker CD45, which is present in two isoforms in laboratory mice that can be distinguished by isoform-specific antibodies (Morse et al., 1987). This system has not only been useful for identifying HSCs, but also for evaluating the fitness of HSCs derived from different genetic models, for example as in (Park et al., 2003).

Using a combination of antibodies against cell surface markers on blood cells and transplantation experiments, an immunophenotypic identification scheme has been delineated for identifying HSCs. One initial scheme for identifying HSCs demonstrated that there was a defined cell type that could be isolated by expression of cell surface markers that led to long-term reconstitution of lethally irradiated recipients with cells derived from the bone marrow (Morrison and Weissman, 1994) and the fetal liver (Morrison et al., 1995). Further gene expression profiling of these cells revealed the unique expression patterns of SLAM receptors on the surface of HSCs (Kiel et al., 2005; Kim et al., 2006; Yilmaz et al., 2006). These purification schemes greatly enrich for HSCs in bone marrow and fetal liver cell suspensions and also emphasize the

rarity of this cell type (approximately 0.001% of bone marrow cells and 0.04% of fetal liver cells).

Taken together, hematopoietic stem cells provide a tractable cellular system to study the effects of aneuploidy *in vivo* for several reasons. First, HSCs can be isolated from both embryos and adults from the fetal liver and the bone marrow, respectively, allowing for a range of aneuploid models to be tested. Additionally, HSCs can be transplanted into lethally irradiated recipient mice. These radiation chimeras will be otherwise euploid mice in which the blood is contributed in part or entirely by aneuploid HSCs. This *in vivo* model system is most similar to the situation observed in sporadic human cancers. Finally, by evaluating engraftment, comparing survival and performing immunophenotyping analysis of the peripheral blood of aneuploid radiation chimeras, we can assess the fitness of aneuploid HSCs in hematopoietic reconstitution experiments.

Mouse models of aneuploidy in this study

For establishing this experimental system, we chose to characterize both models of chronic defined aneuploidy and acute random aneuploidy. This allows for comparison between the two major types of model systems for generating aneuploid cells. To study chronic defined aneuploidy, we utilized Robertsonian translocations to generate constitutional whole chromosomal trisomy in mice. To study acute random aneuploidy, we analyzed a mouse model harboring a hypomorphic allele of the spindle assembly checkpoint component BubR1.

Trisomy in mice is generated by using Robertsonian translocations as described previously (Williams et al., 2008; Figure 6). However, to minimize immune effects due to graft-versus-host disease – which are due to both histoincompatibility and other genetic factors (Allen et al., 1999)—we first backcrossed these Robertsonian lines into the C57BL/6J inbred background. Once this backcrossing was complete, C57BL/6 mice homozygous for a

Robertsonian translocation of two chromosomes (*e.g.* Rb 13.16) could be crossed to mice homozygous for another Robertsonian translocation of one chromosome common to the first Robertsonian translocation and another chromosome (*e.g.* Rb 16.17). Male mice that are heterozygous for these Robertsonian translocations—"compound heterozygotes"—give rise to trisomic embryos upon mating with wild type C57BL/6J females when meiotic nondisjunction occurs in the male germline.

We decided to focus on generating two trisomic strains: trisomy 16, the closest whole-chromosomal mouse model for human trisomy 21, and trisomy 19, trisomy of the smallest mouse autosome. Although this mouse model is embryonic lethal, both of these mice survive until at least E14.5 (Gropp, 1982), and thus contain fetal liver HSCs. Furthermore, a previous study demonstrated that HSCs derived from mouse embryos trisomic for six different chromosomes are capable of repopulating the bone marrow of lethally irradiated mice (Herbst and Winking, 1991). This study revealed that mice irradiated and reconstituted with trisomy 16 fetal liver cells show anemia and leukopenia, decreased counts of red and white blood cells, respectively, in the peripheral blood, and decreased survival whereas mice reconstituted with trisomy 19 fetal liver cells only show leukopenia. These phenotypes were both determined by semi-quantitative blood cell counts from blood films. However, this study utilized non-backcrossed animals and was thus potentially affected by delayed graft-versus-host disease (Gropp et al., 1983). While backcrossing into an inbred line reduces fitness (see Appendix), lethality was an issue in the initial study, as only 70% of control animals survived after 9 weeks (Herbst and Winking, 1991). These issues make differences in survival and peripheral blood statistics difficult to interpret. We decided to take advantage of improved adoptive transfer methods—including our backcrossed animals— and flow cytometric analysis of blood lineages to quantitatively assess

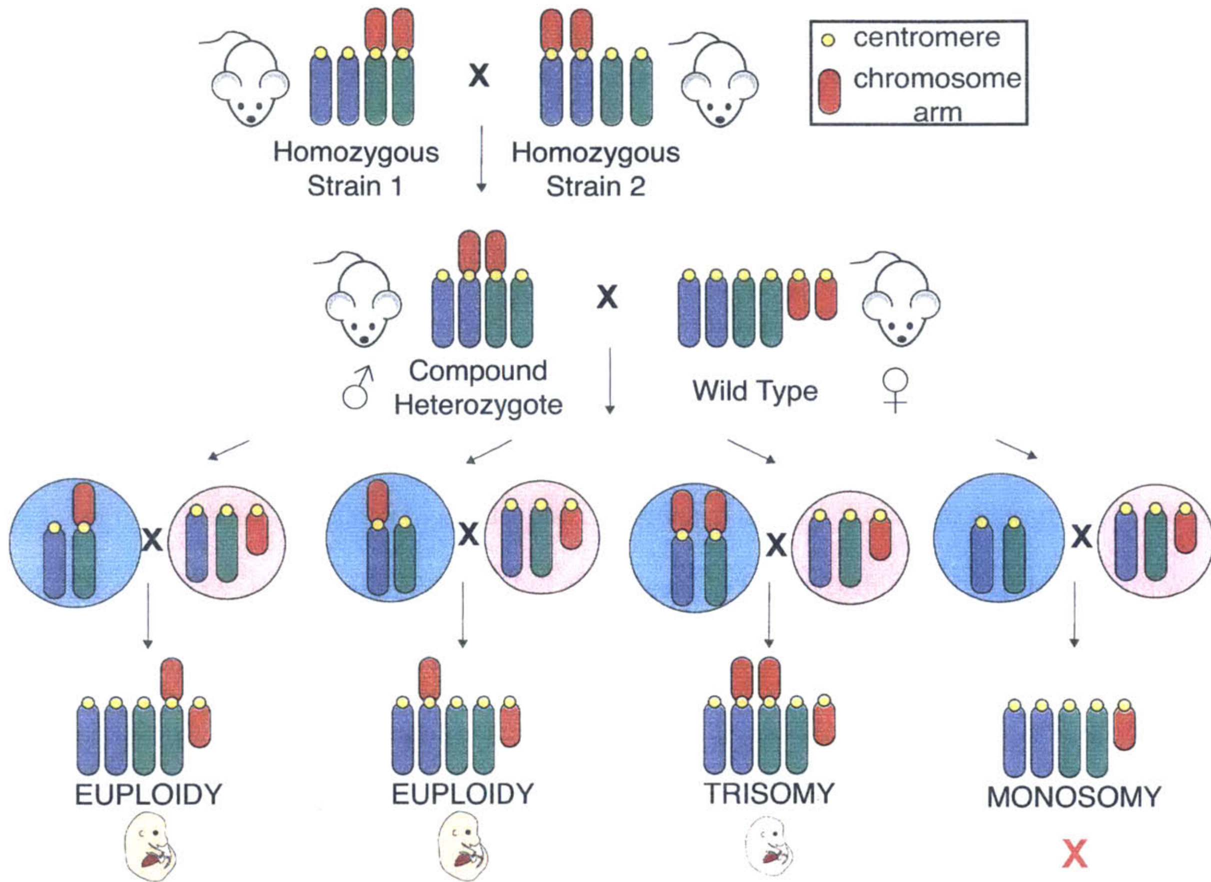

Figure 6. *Generation of trisomy 16 mice using Robertsonian translocations*

Mice homozygous for a Robertsonian translocation of two chromosomes are crossed to mice homozygous for a different Robertsonian translocation. Importantly, one chromosome is shared in each of the two Robertsonian translocations. The resulting mice, which are heterozygous for both of these Robertsonian translocations, are called "compound heterozygotes." When meiotic nondisjunction occurs in the germline of compound heterozygotes and both Robertsonian chromosomes are segregated into the same gamete, a trisomic embryo is produced upon fertilization. Male compound heterozygotes were utilized in our studies to permit repeated matings. Potential male gametes are shown in blue, and potential female gametes are shown in pink. Monosomic embryos are not observed, as they die in a very early stage of embryonic development.

defects in reconstitution in recipient mice over an extended period of time *in vivo*. We also aimed to utilize improved HSC characterization methods to better understand the source of these potential hematopoietic defects.

In addition to utilizing improved tools and a pure mouse background, we also aimed to complement our studies of chronic defined aneuploid HSCs with evaluation of acute random aneuploid HSCs. The mouse model we employed to characterize cells with CIN in the hematopoietic lineage harbors a hypomorphic allele of the spindle assembly checkpoint protein BubR1 ($BubR1^{H/H}$; Baker et al., 2004). The spindle assembly checkpoint ensures proper chromosome segregation. Thus, when BubR1 function is compromised by this loss-of-function allele, chromosomes mis-segregate more frequently, resulting in a randomly aneuploid cell population mixed with euploid cells. The BubR1 hypomorphic allele is better tolerated than constitutional trisomy, as these mice survive to adulthood with a mean life expectancy of 6 months; however these mice have a progeroid phenotype and become progressively more aneuploid with age as evaluated by metaphase spreads of stimulated splenocytes (Baker et al., 2004). This model is a good orthogonal method for evaluating aneuploid HSCs. Additionally, use of $BubR1^{H/H}$ cells enables differentiation between chromosome-specific effects and aneuploidy-specific effects, as the aneuploid cell population derived from cells harboring this allele contains a variety of aneuploid chromosomes due to random mis-segregation events. Furthermore, because this mouse model survives to adulthood, both fetal liver and bone marrow HSCs can be evaluated from $BubR1^{H/H}$ mice.

Hematopoietic phenotypes in DS and mouse models of DS

As described above, individuals with DS are at an elevated risk for developing leukemia in the first few years of life. However, most individuals who develop cancer show perturbed hematopoiesis before the onset of the disease. The majority of DS individuals show some sort of hematologic abnormality at birth. Neutrophilia – increased neutrophil counts – thrombocytopenia – decreased platelet counts – and polycythemia – increased hemoglobin concentration in the peripheral blood – manifest in 80, 66 and 34% of DS newborns, respectively (Choi, 2008; Henry

et al., 2007). These aberrations can also be detected in human trisomy 21 fetal liver cells: human trisomy 21 fetal livers have increased relative numbers of HSCs and megakaryocyte-erythroid progenitors and decreased numbers of pre-pro-B cell progenitors (Chou et al., 2008; Roy et al., 2012). Similar perturbations in the myeloid lineage were observed in HSCs derived from trisomy 21 induced pluripotent cells (Chou et al., 2012; Maclean et al., 2012). Furthermore, about 10% of DS infants have a transient myeloproliferative disorder (TMD), a condition caused by increased proliferation in the myeloid lineage that is apparent usually within the first few weeks of life and generally spontaneously resolves within 3 months (Choi, 2008). This alteration in the myeloid lineage can be attributed to both trisomy 21 and mutations in the transcription factor GATA1 that occur during embryonic development (Mundschau, 2003). In some cases, this TMD will progress to acute myeloid leukemia, but in others it will remain in remission (Alford et al., 2011).

Similar perturbations in the myeloid lineage have been observed in mouse models of DS. Additionally, while not present at birth (Henry et al., 2007), macrocytosis – increased cell volume –of red blood cells has been observed in children with DS analyzed between the ages of 2 and 15 (David et al., 1996). This phenotype is also observed in mouse models of DS. The Ts65Dn mouse model – which harbors an extra copy of 104 genes present on human chromosome 21 – show sustained macrocytic anemia and increased numbers of megakaryocytes and common myeloid progenitors in the bone marrow (Kirsammer et al., 2008). The Ts1Cje mouse model – which contains about two-thirds of the genes amplified in the Ts65Dn mouse model – also identified macrocytic anemia throughout life; however, no differences in the myeloid lineage were observed, even after a loss-of-function allele of GATA1 was introduced into the line (Carmichael et al., 2009). Further analysis of the HSCs in this mouse model and

theTs65Dn mouse model revealed that the Ts65Dn mice have fewer HSCs in the bone marrow while the Ts1Cje mouse model have normal levels of HSCs (Adorno et al., 2013). Additionally, while bone marrow cells from the Ts1Cje mouse model could contribute to the peripheral blood of a recipient mouse upon bone marrow transfer, Ts65Dn bone marrow cells fail to engraft recipients (Adorno et al., 2013), demonstrating that trisomy has an effect on HSC fitness. The Tc1 mouse model – which harbors an exogenous copy of 80% of human chromosome 21– also showed macrocytic anemia throughout life (Alford et al., 2010). Introduction of a mutant, truncated form of GATA1 induced increased megakaryopoiesis in this model, but was not sufficient to induce leukemia in these animals (Alford et al., 2010).

Thus, there is evidence for perturbations of the hematopoietic lineages in both humans with DS and mouse models of DS. The altered myeloid proliferation phenotype was observed in one mouse model without further alteration, in one mouse model upon expression of a truncated form of GATA1, and was not observed in another model, even after introduction of a loss-of-function form of GATA1. While the phenotypes observed are variable, there are some common observations in all systems. For example, macrocytosis is observed in both individuals with DS and in all DS mouse models studied. Notably, increased cell volume is also a feature exhibited by aneuploid cells in general (Torres et al., 2007; Williams et al., 2008). Further study of the effect of aneuploidy on HSCs using our mouse models of trisomy and CIN will permit evaluation of these phenotypes in the context of a different chromosomal amplifications to determine whether these phenotypes are specific to the particular amplification of the set of genes present on human chromosome 21 or if some of these phenotypes can be attributed to a general cellular response to aneuploidy in the blood.

Concluding Remarks

Aneuploidy is a generally detrimental cellular state that has a complicated relationship with cancer. This thesis will describe work performed to establish a cellular system to better understand this relationship *in vivo* in the context of the hematopoietic stem cell. I have characterized the fitness and potential of HSCs trisomic for mouse chromosomes 16 or 19 or harboring a hypomorphic allele of BubR1 that results in chromosomal instability. I have found that these three models show a range of phenotypes *in vivo* that scale with the size of the genomic amplification present. Additionally, while mice reconstituted with trisomy 16 HSCs show severe defects, trisomy 19 seems to be well-tolerated in the hematopoietic lineage. BubR1$^{H/H}$ HSCs show decreased fitness upon serial transplantation; however, karyotype analysis of the peripheral blood of these recipient mice indicates that an aneuploidy-selective mechanism may be employed by the hematopoietic system to eliminate aneuploid cells.

This work reveals how an organ system *in vivo* either rejects or tolerates aneuploid cells, even in the presence of numerous defects. This variability is consistent with the phenotypic heterogeneity observed in DS individuals and mouse models. Furthermore, establishment and characterization of this system permits future evaluation of the role of aneuploidy in tumorigenesis through the activation of oncogenes before transplantation. In summary, this work has shed light on the impact of aneuploidy on cellular growth and physiology *in vivo* and has helped develop new tools to dissect the impact of aneuploidy on tumorigenesis in hematologic cancers. We hope that our findings and future work using this system help clarify the effects of aneuploidy in Down syndrome and provide insight into the precise role of aneuploidy in cancer.

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Chapter 2: Effects of aneuploidy on hematopoietic stem cell fitness

Introduction

In 1914, Theodor Boveri proposed that aneuploidy, an imbalanced karyotype characterized by whole chromosome gains and/or losses, causes cancer (Boveri, 2008). Implicit to this hypothesis is that aneuploidy provides a fitness advantage to cancer cells, endowing them with the increased proliferative potential that defines the disease. However, studies of the fitness of aneuploid cells have not been able to provide decisive evidence for or against this hypothesis. Cell culture studies of primary human (Segal and McCoy, 1974; Stingele et al., 2012) or mouse (Williams et al., 2008) cells harboring one extra chromosome demonstrated that aneuploid cells exhibit decreased proliferative potential. Primary cells obtained from mice harboring mutations that increase chromosome mis-segregation and hence generate progeny with random aneuploidies have been reported to either proliferate normally (Babu et al., 2003; Jeganathan et al., 2007; van Ree et al., 2010; Weaver et al., 2007) or more slowly (Baker et al., 2004; Li et al., 2009; Remeseiro et al., 2012; Sotillo et al., 2007) in cell culture, indicating that aneuploidy is, at best, fitness neutral in these *in vitro* cellular systems.

Analyses of individuals with trisomy 21, or Down syndrome (DS), provide evidence that aneuploidy can both impair and improve cellular fitness depending on the cell lineage *in vivo*, in particular the hematopoietic lineage. 10-20% of newborns with DS are diagnosed with a transient myeloproliferative disorder (TMD), a premalignant condition characterized by the clonal expansion of blast cells with erythoid and megakaryocytic features (Gamis and Smith, 2012). In most cases, TMD will spontaneously resolve and remain in remission; however, in 20-30% of cases, TMD will progress into AML (Dixon et al., 2006). While somatic truncation mutations in the gene encoding the hematopoietic transcription factor GATA1 are found in all cases of TMD (Hitzler, 2003; Mundschau et al., 2003), trisomy 21 alone is sufficient to bias differentiation in the human fetal liver leading to a relative expansion of myeloid cells (Chou et al., 2008;

Tunstall-Pedoe et al., 2008), and classification of different mutation types of GATA1 is not predictive of whether TMD will progress to AML in DS individuals (Alford et al., 2011). Thus, in this context trisomy 21 can provide a relative proliferative advantage to specific hematopoietic lineages in the context of an aneuploid karyotype. However, DS newborns also often present with thrombocytopenia (Choi, 2008) – decreased platelet counts – and DS children between ages 2 and 15 generally present with macrocytosis (David et al., 1996) – increased red blood cell volume associated with decreased proliferation. These observations indicate that trisomy 21 can also cause decreased fitness in the hematopoietic lineage. It is thus remains fundamentally unknown how an aneuploid karyotype affects mammalian cell fitness and proliferative potential.

The reason for why, more than 100 years after the "chromosomal theory of cancer" was first proposed, we still do not understand whether aneuploidy improves or impairs cellular fitness is due to a lack of tractable and comprehensive *in vivo* model systems. Evaluation of the effects of aneuploidy on cellular fitness *in vivo* is difficult because most autosomal aneuploidies are embryonic lethal in mammals (Gropp et al., 1983; Hassold and Jacobs, 1984), thus precluding the direct comparison of euploid and isogenic aneuploid animals. Here, we circumvent this difficulty by performing transplantation experiments with hematopoietic stem cells (HSCs) derived from aneuploid embryos. Using this approach we examined the impact of constitutional aneuploidy (trisomy) and chromosome instability (CIN) on HSC fitness. We compared the fitness of aneuploid hematopoietic stem cells (HSCs) with that of isogenic euploid control HSCs with *in vivo* competition assays. These studies revealed a decrease in fitness of aneuploid HSCs that correlates with the degree of aneuploidy. This fitness decrease was also observed in reconstitutions with HSCs derived from each aneuploid line individually. Our results demonstrate that aneuploidy causes a decrease in the proliferative potential of stem cells *in vivo*

in the context of a euploid organism, arguing against aneuploidy driving tumorigenesis by increasing proliferative capacity and cellular fitness.

Results

Aneuploidy decreases HSC competitive fitness in vivo *in a dose-dependent manner*

To compare the fitness of aneuploid and euploid control HSCs, we chose three mouse models of aneuploidy: trisomy 16, trisomy 19 and mice harboring a hypomorphic allele of the gene *BUB1B*, which encodes the spindle assembly checkpoint protein BubR1 (BubR1$^{H/H}$; Baker et al., 2004). This model of chromosomal instability produces aneuploid cells with mostly single chromosome gains or losses (Baker et al., 2004; Fig. 1f).

Although trisomy is embryonic lethal in mice (Gropp, 1982), HSCs are present in the fetal liver between embryonic stage E12.5 and E15.5 (Orkin and Zon, 2008). We therefore isolated fetal livers from aneuploid embryos and euploid wild type littermate E14.5 embryos (Figure 1a). Fetal livers from a common euploid competitor were isolated at the same embryonic age from a separate timed mating. Aneuploid or wild type control fetal liver cells were then co-transferred with an equal number of fetal liver cells from the common euploid competitor into a lethally irradiated euploid recipient. Each of the donors was tracked using a different isoform of the CD45 pan-leukocyte cell surface marker. Aneuploid donors and their wild type littermates expressed the CD45.2 isoform, whereas the common euploid donor and the recipient mice expressed the CD45.1 isoform. The percentage of the peripheral blood contributed by HSCs from each donor was measured by flow cytometry using isoform-specific antibodies.

Trisomy 16 fetal liver HSCs were much less fit than fetal liver HSCs derived from their wild type littermates and were nearly completely outcompeted by the common euploid donor within 3 weeks of transfer to the recipient (Figure 1b). Trisomy 19 fetal liver HSCs are of similar fitness as the common euploid donor; however, previous studies have shown that CD45.1

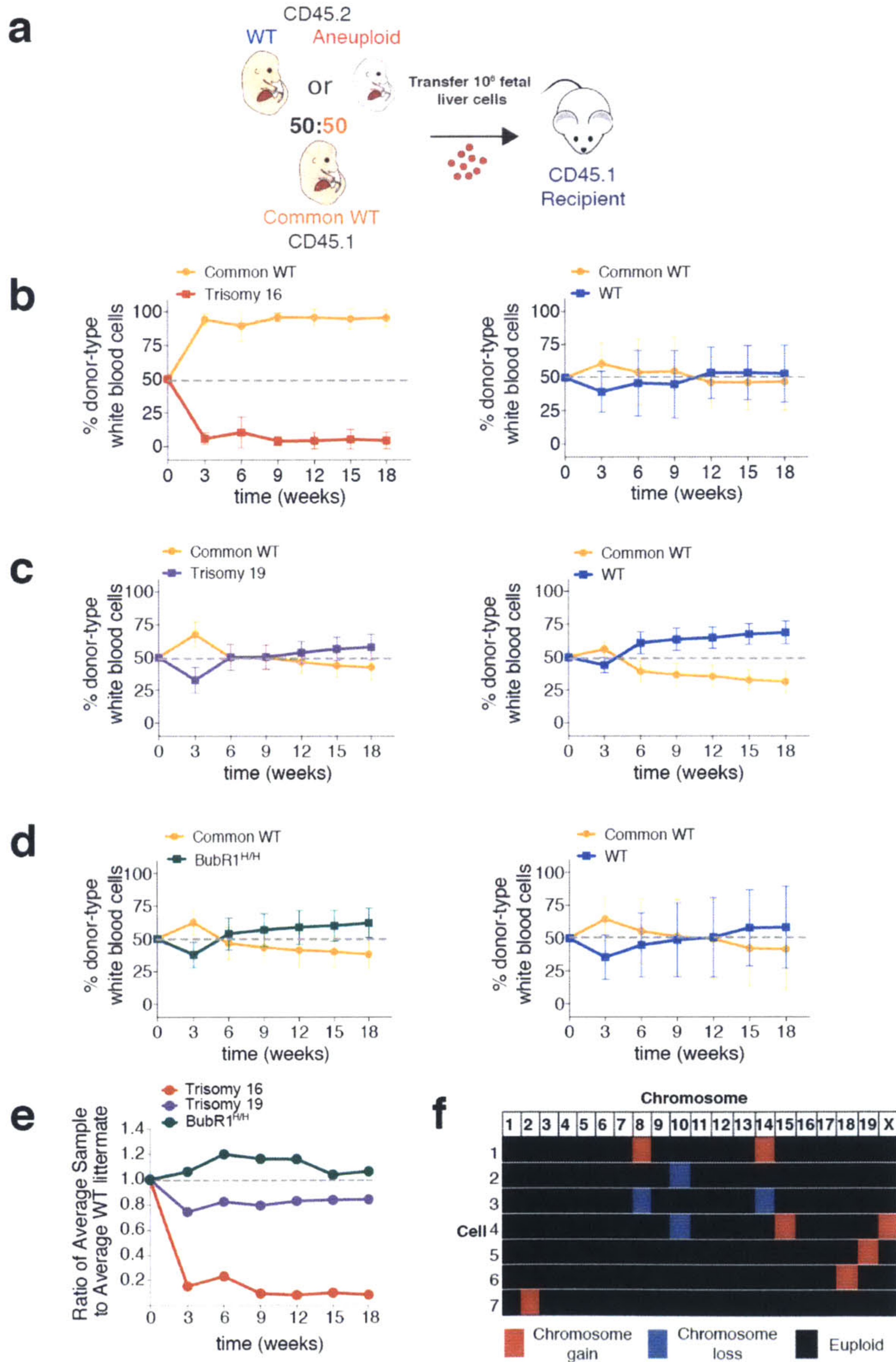

a
CD45.2
WT
Aneuploid
or
50:50
Transfer 10⁶ fetal liver cells
CD45.1 Recipient
Common WT
CD45.1
b
Common WT
Trisomy 16
WT
% donor-type white blood cells
time (weeks)
c
Trisomy 19
d
BubR1 H/H
e
Ratio of Average Sample to Average WT littermate
f
Chromosome
Cell
Chromosome gain
Chromosome loss
Euploid

Figure 1. *Aneuploidy decreases HSC competitive fitness* in vivo *in a dose-dependent manner*

(a) Schematic of competitive reconstitution experiments. (b – d) CD42.5 fetal liver cells from wild type or aneuploid E14.5 littermates were co-injected into a lethally irradiated CD45.1 recipient with an equal number of fetal liver cells from a CD45.1 common WT donor of the same age derived from a separate mating. Peripheral blood was sampled at indicated times. The percentage of the white blood cell population contributed by each donor was quantified by flow cytometry with isoform-specific antibodies against CD45.1 and CD45.2 for recipients of common WT cells and trisomy 16 fetal liver cells (b, left graph, n=17), trisomy 19 fetal liver cells (c, left graph, n=10) and BubR1$^{H/H}$ fetal liver cells (d, left graph, n=10). The contribution of WT littermates when competed against the common WT for all aneuploidies was quantified at the same time (b-d; right graphs). The mean value with standard deviation of the experiments is shown for each time point. (e) Ratios of the average percentage of the peripheral blood reconstituted by the aneuploid fetal liver cells to the average percentage of the peripheral blood reconstituted by wild type littermate fetal liver cells at the indicated times are shown.
(f) Single cell sequencing of white blood cells from a mouse competitively reconstituted with CD45.2 BubR1$^{H/H}$ and CD45.1 euploid FL-HSCs (Fig 1d.) revealed that 7 of 18 CD45.2 BubR1$^{H/H}$ cells analyzed (~39%) are aneuploid. Karyotypes of the 7 aneuploid cells are shown with chromosome gains in red, chromosome losses in blue, and euploidy shown in black. Segmentation plots of all sequenced cells are shown in Appendix Figure 2.

HSCs show lower fitness than CD45.2 HSCs in competition assays (Waterstrat et al., 2010). When directly compared to cells derived from their wild type littermates, trisomy 19 fetal liver cells have slightly impaired fitness (Figure 1c). BubR1$^{H/H}$ HSCs showed no fitness defects (Figure 1d); however, this was not due to the absence of aneuploidy in blood cells. We karyotyped peripheral white blood cells derived from the BubR1$^{H/H}$ fetal liver HSCs in one of the mice from the *in vivo* competition assay. Cells were sorted by FACS based on their expression of the CD45.2 surface antigen, then karyotype was determined by single cell sequencing (Knouse et al., 2014). Analysis of 18 cells revealed 7 to be aneuploid (~38.89%; Figure 1f), indicating that the lack of fitness defects in this model is not simply due to the absence of aneuploid cells. In summary, when compared to the average value of their wild type littermates after 18 weeks, the average relative fitness of trisomy 16, trisomy 19, and BubR1$^{H/H}$ fetal liver HSCs is 0.08, 0.84 and 1.06 respectively (Figure 1e).

We conclude that in this experimental setting, some aneuploidies confer a fitness decrease whereas others appear fitness neutral. This finding indicates that either only certain aneuploidies impact HSC fitness or that in mice (or in this experimental setup) a certain level of HSC aneuploidy is well tolerated. Consistent with the latter possibility, we find that when compared with their wild type littermates after 18 weeks, the average relative fitness of trisomy 16, trisomy 19, and BubR1$^{H/H}$ fetal liver HSCs correlated well with the degree of aneuploidy that is present in each cell ($R^2 = 0.8922$, Figure 2a).

Defects in aneuploid HSC fitness are not due to decreased HSC number or reduced homing efficiency but decreased proliferation

Next we investigated why trisomy 16 and 19 HSCs exhibited decreased fitness in competitive reconstitution assays. Because the defect was so severe in trisomy 16 HSCs, we hypothesized that these fetal livers could contain a lower concentration of HSCs than their euploid littermates. To address this possibility we quantified HSC levels in trisomy 16 fetal livers. This analysis revealed no difference in the levels of HSCs in trisomy 16 fetal livers (Figure 2b). The same is true of trisomy 19 fetal livers (Figure 2b), indicating that lower HSC levels in our transplantation experiments were not responsible for the decreased contribution of trisomy 16 or trisomy 19 to the blood lineages in the reconstitution experiments.

When HSCs are injected into recipient animals they need to home to the bone marrow niche to be able to repopulate the hematopoietic lineages of the lethally irradiated host animal. To determine whether aneuploid HSCs are defective in homing to this stem cell niche we labeled fetal liver cells with a fluorescent cell surface dye, injected them into irradiated recipients and quantified the number of labeled cells in the bone marrow after 24 hours. We found no significant decrease in homing efficiency between aneuploid and euploid HSCs (Figure 2c).

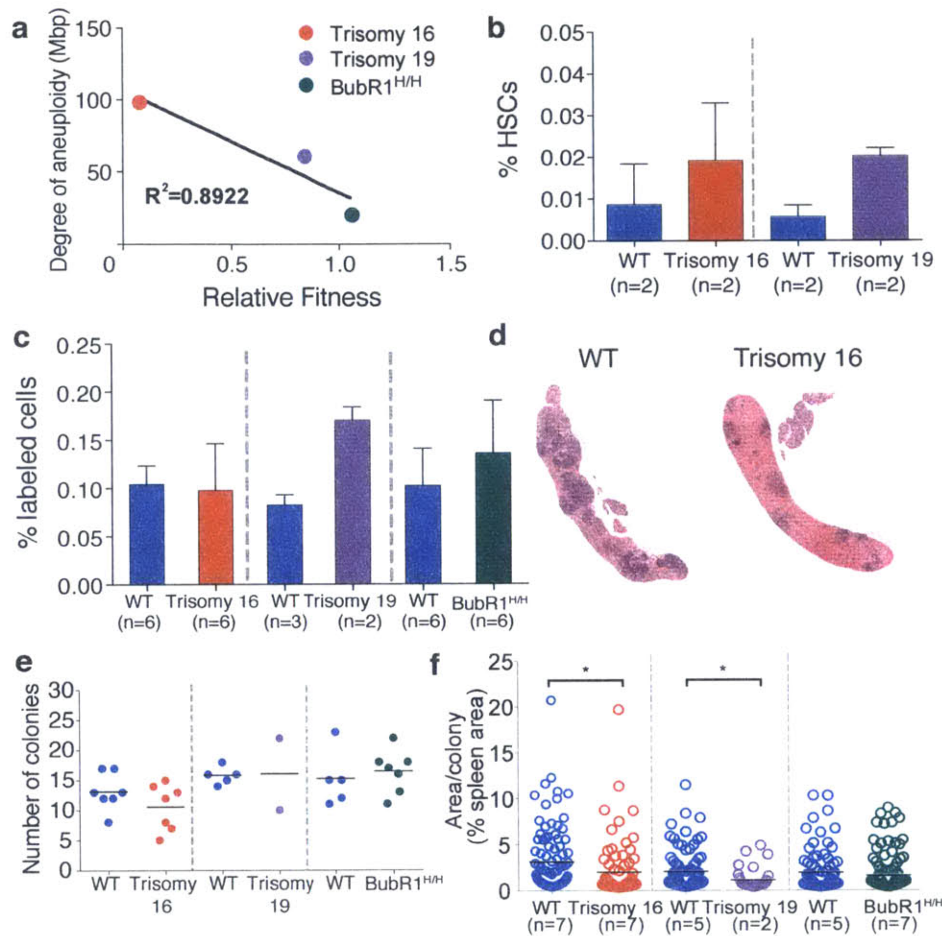


Figure 2. *HSC number and homing ability of aneuploid cells are normal but proliferation is reduced in trisomy 16 and trisomy 19 HSCs*

(a) Plot of relative fitness of aneuploid cells versus degree of aneuploidy present in cells. Relative fitness was determined as the ratio of % aneuploid cells to % wild type littermate cells at 18 weeks from Fig 1e. Degree of aneuploidy for BubR1$^{H/H}$ peripheral blood cells was calculated from Fig 1f. (b) The percentage of HSCs (CD150^{+} CD48^{-} Sca-1^{+} lin^{-} cells) found in trisomy 16 and 19 fetal livers was quantified by flow cytometry. (c) DiI-labeled fetal liver cells were injected into irradiated recipient mice. The percentage of DiI-positive cells in the bone marrow of recipient mice was measured 24 hours after injection. (d) Representative images of sections of spleens isolated from mice transferred with WT or trisomy 16 fetal liver cells 8 days after reconstitution. (e) Quantification of CFU-S colonies from spleen sections of recipient mice of trisomy 16 or wild type littermate fetal liver cells 8 days after injection and trisomy 19, BubR1$^{H/H}$ or wild type littermate fetal liver cells 7 days after injection. (f) Quantification of the average size of each colony in Fig. 2e as determined by percent of total spleen area. Populations were compared by t-test; * indicates $p<0.05$.

We next examined whether proliferation of HSCs was impaired by determining whether short-term reconstituting cells were affected by an aneuploid karyotype. To test this possibility, we performed colony forming unit spleen (CFU-S) assays. This assay reflects the short term ability of early engrafting cells to repopulate the hematopoietic system during the first several weeks after irradiation (Purton and Scadden, 2007). Irradiated mice were injected with equal numbers of fetal liver cells and spleens are analyzed 7 to 8 days later (Till and McCulloch, 1961; Figure 2d). We found that aneuploid and euploid fetal liver cells formed similar numbers of colonies in the spleen (Figure 2e). However, the average size of colonies produced by trisomy 16 and 19 donor cells was reduced when compared to colonies formed by their wild type littermates (Figure 2f). The average size of BubR1$^{H/H}$ CFU-S colonies was similar to those formed by wild type littermate fetal liver cells (Fig. 2f). These results indicate that aneuploid hematopoietic cells isolated from the fetal liver can home effectively but show impaired proliferative potential in the case of trisomy 16 and 19. We conclude that this decreased proliferative capacity of aneuploid hematopoietic cells is at least in part responsible for the fitness defects of FL-HSCs observed in competitive reconstitutions.

Trisomy 16 primary recipients show peripheral blood defects and decreased survival

While trisomy 16 HSCs exhibited a severe fitness defect in competitive reconstitution experiments, the fitness decrease was more subtle for trisomy 19 HSCs and not evident for BubR1$^{H/H}$ HSCs. This finding raised the possibility that either low levels of aneuploidy do not impair stem cell fitness or that HSCs were not challenged sufficiently to reveal a decrease in cellular fitness. To distinguish between these possibilities we evaluated the long-term fitness of HSCs from each aneuploid line individually by serially transferring HSCs from one recipient to another (Figure 3). Serial transfer poses a significant challenge to the replicative potential of HSCs, causing the eventual exhaustion of even wild type HSCs after about 5 transfers

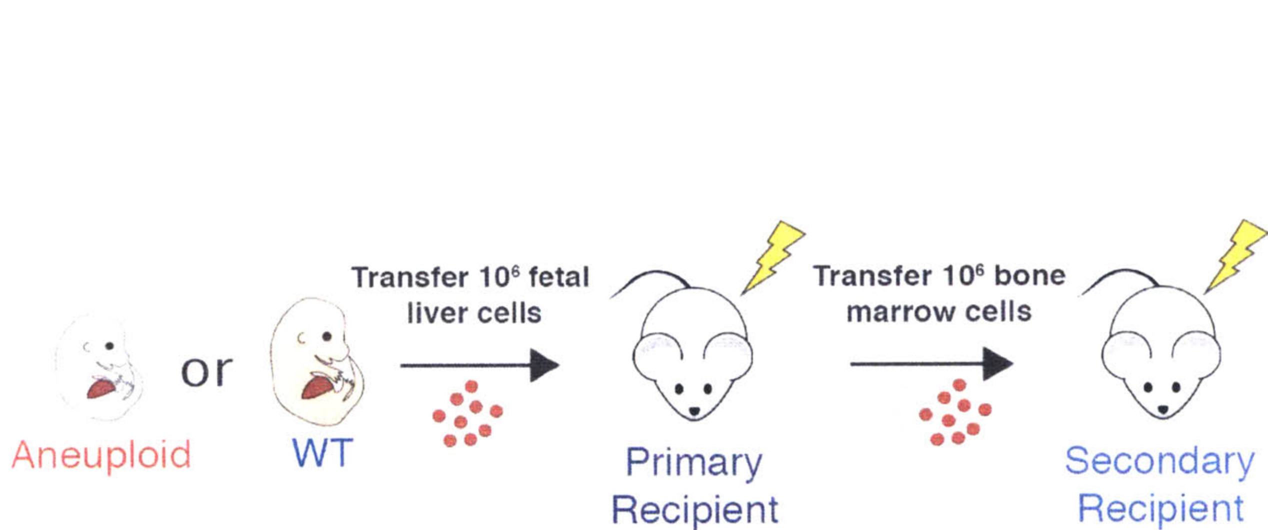


Figure 3. *Adoptive transfer of aneuploid HSCs*

For primary reconstitutions, fetal liver cells from a trisomic embryo or its wild type littermate were injected into lethally irradiated recipients. Bone marrow cells from primary recipients were transferred into secondary recipients to assess serial repopulation capacity.

(Harrison and Astle, 1982). The trisomic HSCs we analyzed have been evaluated previously (Herbst and Winking, 1991); however, previous studies were not performed on an isogenic background, and therefore it is difficult to determine whether observed phenotypes were due to aneuploidy or other factors such as graft-versus-host disease (Gropp et al., 1983).

Trisomy 16 fetal liver cells were transferred into lethally irradiated CD45.1 primary recipient mice (Figure 3). As a control, fetal liver cells from a wild type littermate were transferred at the same time into other lethally irradiated CD45.1 mice. Peripheral blood was analyzed periodically between 4 weeks and 16 weeks. Analysis of these primary recipients revealed that trisomy 16 HSCs showed significant fitness defects when transferred individually, similar to what was observed in the *in vivo* competition assay. This fitness decrease seems to be largely due to decreased proliferation. Trisomy 16 HSCs contributed many fewer peripheral white blood cells (Figure 4a) and in some animals contributing virtually no cells. Furthermore, trisomy 16 primary recipients exhibited leukopenia – reduced white blood cell counts – when compared to radiation chimeras reconstituted with cells from their wild type littermates (Figure 4b), especially at 4 weeks after transfer (Figure 5i). This seemed to be largely due to low numbers of B cells (Figure 5a). Complete blood cell counts further showed that trisomy 16 radiation chimeras have comparatively fewer lymphocytes and a macrocytic anemia (Figure 4c; 5c-g; 6a), a reduced red blood cell count accompanied by increased red blood cell volume. These results are in line with a previous *in vivo* characterization of trisomy 16 HSCs (Herbst and Winking, 1991). In addition to peripheral blood defects, trisomy 16 primary recipients exhibited decreased survival when compared to primary recipient mice reconstituted with HSCs from their wild type littermates (Figure 4d). As the ultimate test of HSC potential, we performed secondary transplantations of bone marrow cells isolated from trisomy 16 primary recipients that survived

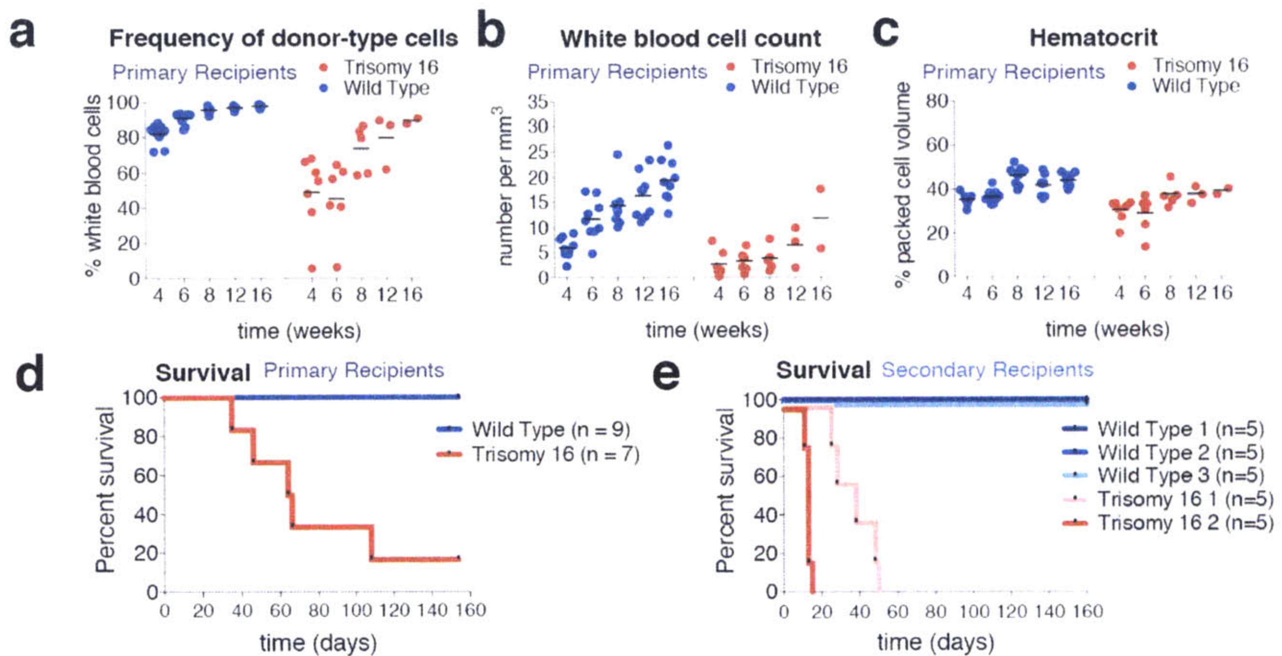


Figure 4. *Trisomy 16 primary recipients show peripheral blood defects and decreased survival*

Peripheral blood of primary recipients of trisomic fetal liver cells or their wild type littermates was sampled at the indicated times. The percentage of CD45.2 positive cells in the blood of trisomy 16 or wild type primary recipients was determined by flow cytometry (a) and white blood cell count (b) and hematocrit (c) were determined by complete blood cell counts.
(d) Survival of recipients of trisomy 16 or wild type fetal liver cells after transfer. (e) Survival of secondary recipients of trisomy 16 or wild type bone marrow cells from primary recipients.

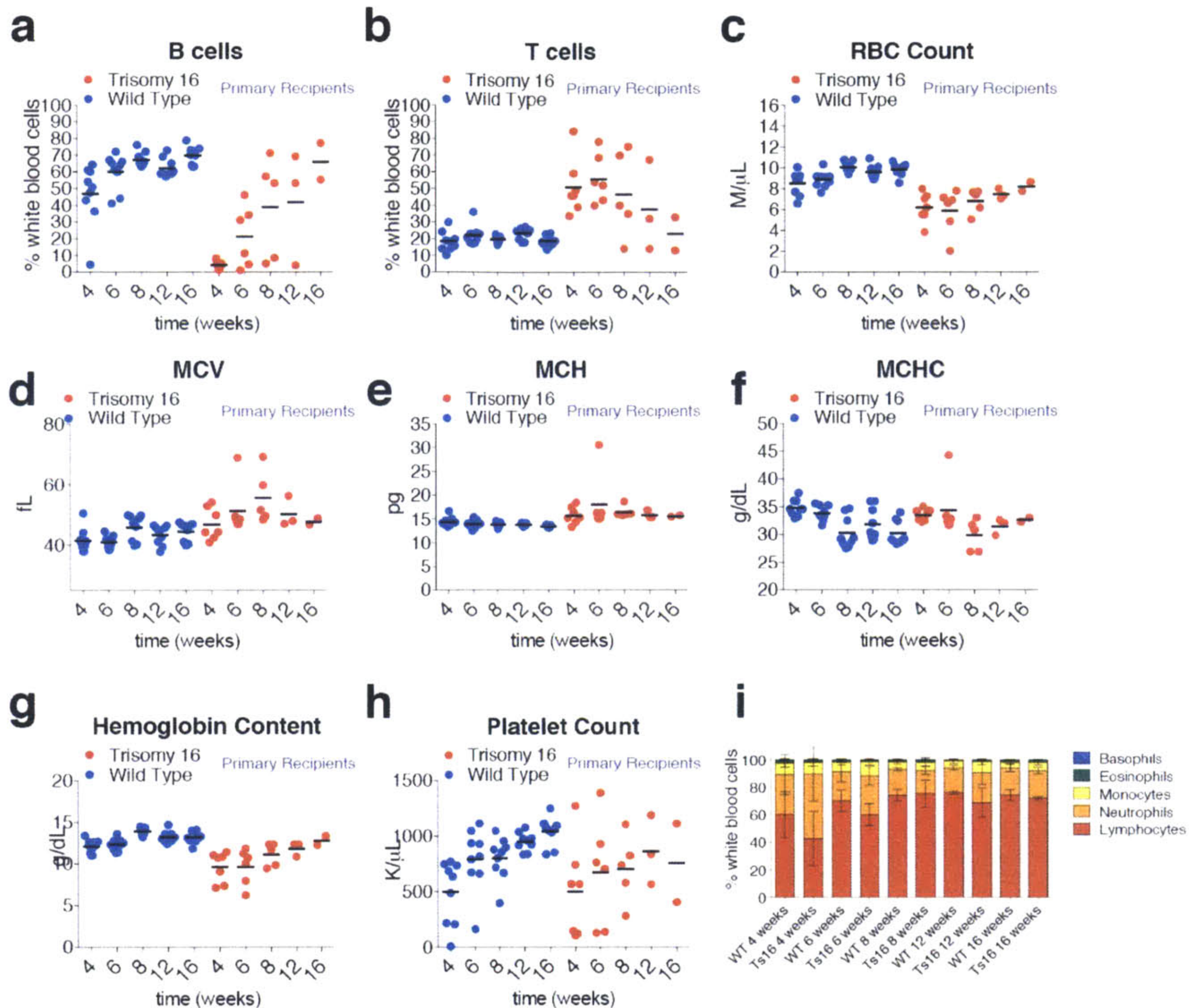


Figure 5. *Peripheral blood analyses of trisomy 16 primary recipients*

Flow cytometry of peripheral blood from trisomy 16 and wild type primary recipients was performed to quantify the percentage of B220-positive B cells (a) and Thy1.2-positive T cells (b) in the peripheral blood. Complete blood cell counts of peripheral blood from trisomy 16 and wild type primary recipients was performed to determine red blood cell count (c), mean corpuscular volume (MCV) (d), mean corpuscular hemoglobin (MCH) (e), mean corpuscular hemoglobin concentration (MCHC) (f), hemoglobin content (g) and platelet counts (h). The composition of the leukocyte population was evaluated by automated differential for trisomy 16 and wild type primary recipients (i).

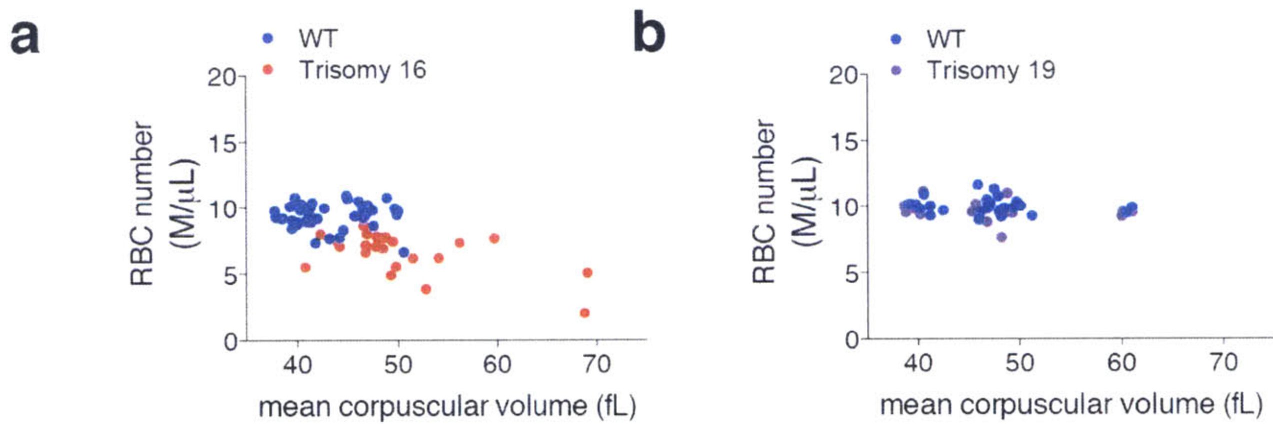


Figure 6. *Macrocytic anemia in trisomy 16 primary recipients*

Plots of red blood cell number versus mean corpuscular volume (MCV) of all trisomy 16 primary recipients and wild type primary recipients (a) and all trisomy 19 and wild type primary recipients (b) at all times evaluated in Figure 5.

to the end of the initial experiment. Trisomy 16 bone marrow HSCs failed to reconstitute secondary recipients (Figure 4e). We conclude that trisomy 16 HSCs have a substantial fitness defect that is at least in part due to a decreased ability to proliferate.

Trisomy 19 primary and secondary recipients show few phenotypes

Analysis of animals reconstituted with trisomy 19 fetal liver cells revealed that trisomy 19 HSCs contributed most of the white blood cells observed in the primary recipients (Figure 7a). However, complete blood cell counts indicated that trisomy 19 primary recipients also exhibit leukopenia when compared to radiation chimeras reconstituted with cells from their wild type littermates (Figure 7b). In contrast to trisomy 16 reconstituted animals, trisomy 19 primary recipients had a normal hematocrit (Figure 7c) and no red blood cell defects (Figure 8c-g), did not show macrocytic anemia (Figure 6b) but harbored fewer platelets compared to primary recipients reconstituted with wild type HSCs (Figure 8h). Although white blood cell counts were reduced in trisomy 19 primary recipients, the proportion of the blood comprised of each white blood cell type was not significantly perturbed (Figure 8 a,b,i). Despite these minor defects in specific hematopoietic lineages, mice transplanted with trisomy 19 HSCs showed no difference in survival in either the primary transfer of fetal liver cells or the secondary transfer of bone marrow cells isolated from the primary recipients (data not shown). Furthermore, secondary recipient mice showed no obvious peripheral blood defects (Figure 7d-f; Figure 9), except for a slightly weaker initial engraftment after 4 weeks (Figure 7d). We confirmed the trisomic karyotype of these peripheral blood cells after secondary transfer by metaphase spreads (data not shown) to ensure that the aneuploid donor cells remained trisomic. Thus, unlike trisomy 16, trisomy 19 is well-tolerated in the hematopoietic lineage, with no difference in survival and little difference in peripheral blood cell counts in both primary and secondary recipient mice.

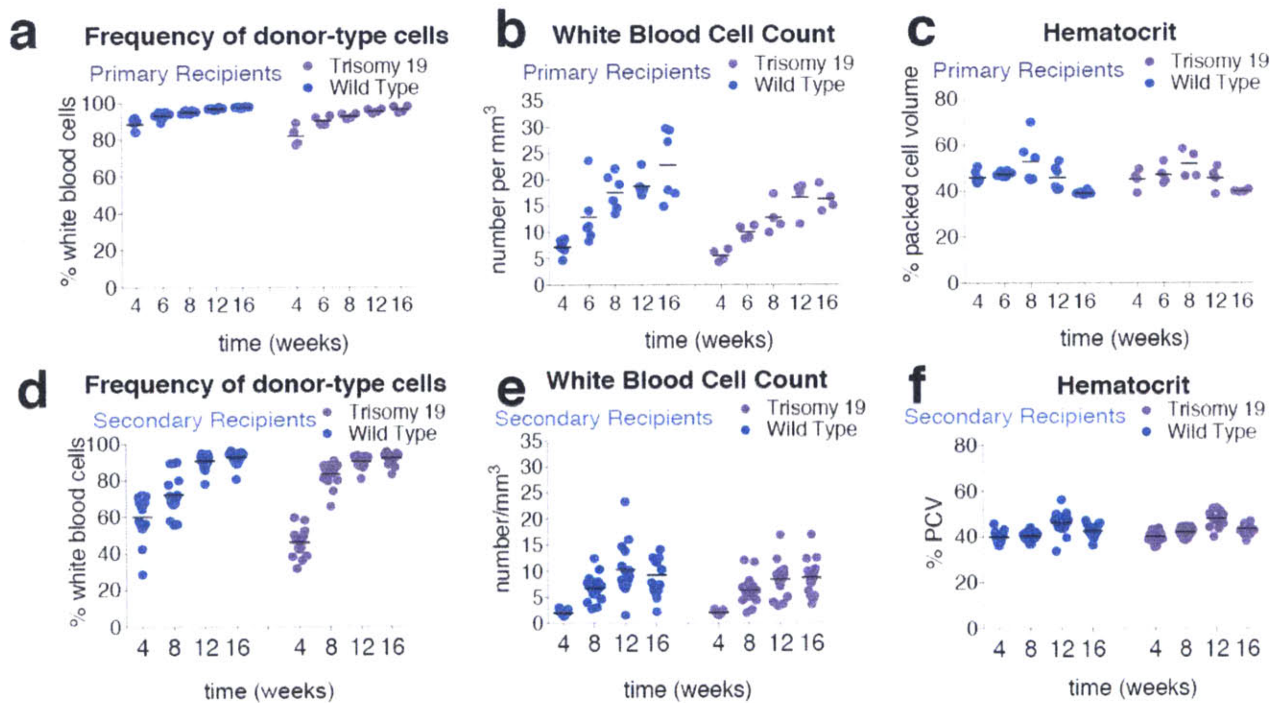


Figure 7. *Peripheral blood analyses of trisomy 19 primary and secondary recipients*

For trisomy 19 or wild type primary recipients, the percentage of CD45.2 positive cells in the peripheral blood (a), white blood cell count (b) and hematocrit (c) was determined. For trisomy 19 or wild type secondary recipients, the percentage of CD45.2 positive cells in the peripheral blood (d), white blood cell count (e) and hematocrit (f) was determined.

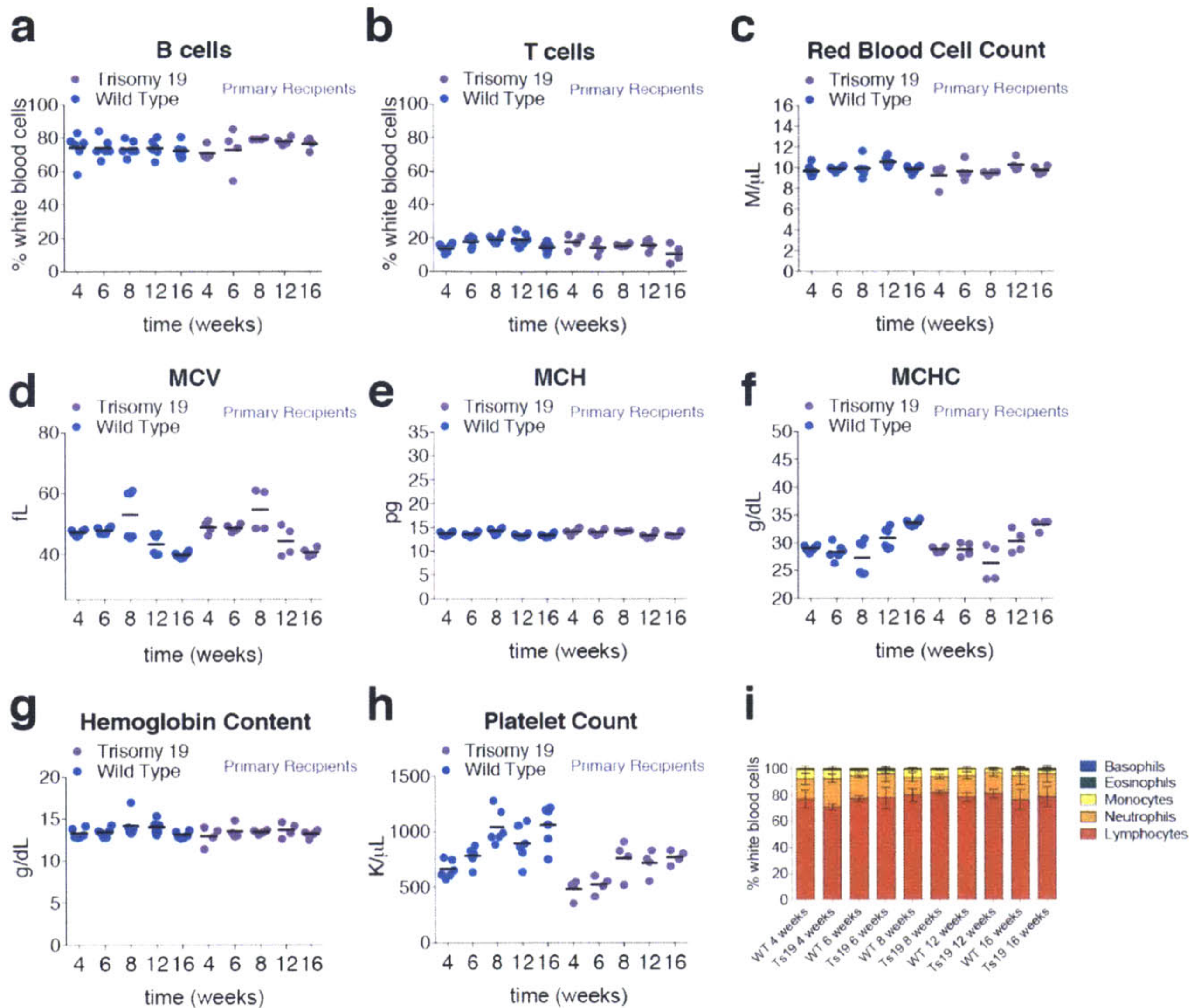


Figure 8. *Peripheral blood cell counts of trisomy 19 primary recipients*

Flow cytometry of peripheral blood from trisomy 19 and wild type primary recipients was performed to quantify the percentage B220-positive B cells (a) and Thy1.2-positive T cells (b) in the peripheral blood. Complete blood cell counts of peripheral blood from trisomy 19 and wild type primary recipients was performed to determine red blood cell counts (c), mean corpuscular volume (MCV) (d), mean corpuscular hemoglobin (MCH) (e), mean corpuscular hemoglobin concentration (MCHC) (f), hemoglobin content (g) and platelet counts (h). The composition of the leukocyte population was evaluated by automated differential for trisomy 19 and wild type primary recipients (i).

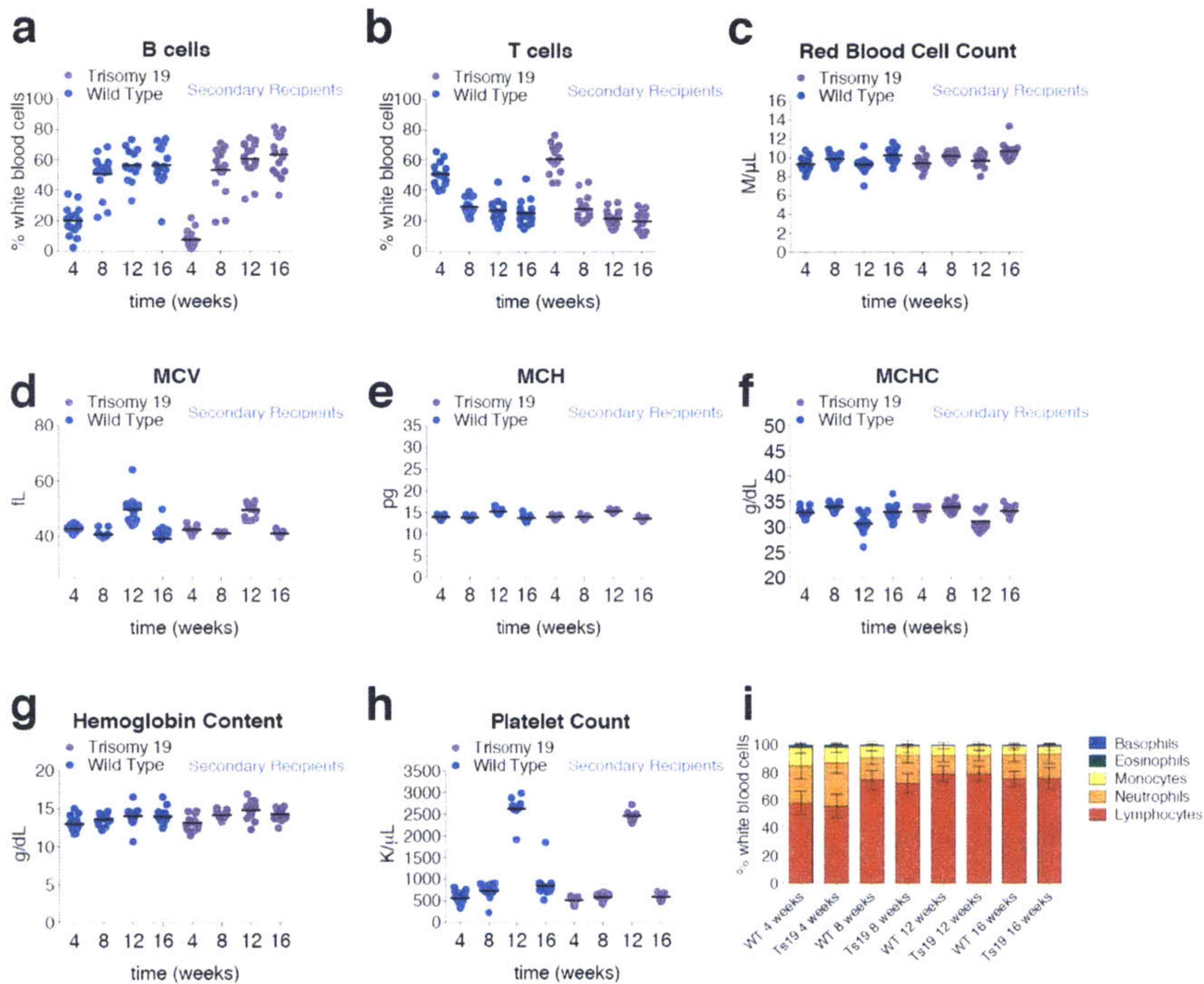


Figure 9. *Peripheral blood cell counts of trisomy 19 secondary recipients*

Flow cytometry of peripheral blood from trisomy 19 and wild type secondary recipients was performed to quantify the percentage of B220-positive B cells (a) and Thy1.2-positive T cells (b) in the peripheral blood. Complete blood cell counts of peripheral blood from trisomy 19 and wild type secondary recipients was performed to determine red blood cell counts (c), mean corpuscular volume (MCV) (d), mean corpuscular hemoglobin (MCH) (e), mean corpuscular hemoglobin concentration (MHCH) (f), hemoglobin content (g) and platelet counts (h). The composition of the leukocyte population was evaluated by automated differential for trisomy 19 and wild type secondary recipients (i).

$BubR1^{H/H}$ HSCs undergo stem cell exhaustion upon serial transplantation

To evaluate the long-term regenerative potential of $BubR1^{H/H}$ HSCs, we utilized bone marrow HSCs, as $BubR1^{H/H}$ mice survive to adulthood (Baker et al., 2004). Like $BubR1^{H/H}$ fetal liver HSCs, $BubR1^{H/H}$ bone marrow HSCs show no fitness defects in *in vivo* competition assays and are found at similar numbers to their wild type littermates (Figure 10). As these cells have already migrated from the fetal liver to populate the bone marrow of an adult mouse, we considered this to be a more significant proliferative challenge than transfer of fetal liver cells. Additionally, fetal liver HSCs have been shown to repopulate irradiated recipients more effectively than bone marrow HSCs (Harrison et al., 1997; Morrison et al., 1995). $BubR1^{H/H}$ bone marrow HSCs contributed most of the peripheral white blood cells in primary recipient mice (Figure 11b). The animals were mildly leukopenic but exhibited no other blood defects (Figure 12).

To further challenge $BubR1^{H/H}$ HSCs we performed a serial bone marrow transfer (Figure 11a). Transfer of bone marrow cells from a primary $BubR1^{H/H}$ bone marrow recipient into secondary lethally irradiated recipients yielded a range of phenotypes (Figure 11c). While the donor contributed most of the peripheral white blood cells in mice transferred with wild type cells (on average 83.5% at 12 weeks, n=5), some secondary recipient mice reconstituted with $BubR1^{H/H}$ HSCs had strong donor contribution (86% at 12 weeks), some mice had weak donor contribution (18% at 12 weeks) and some mice had an intermediate level of donor contribution (66% at 12 weeks). Interestingly, secondary recipient mice with weak $BubR1^{H/H}$ donor contribution were quite healthy, having the highest white blood cell count and the highest levels of B cells amongst $BubR1^{H/H}$ secondary recipients (Figure 13). Peripheral blood analysis provided an explanation for this observation. The blood of animals with weak contribution of

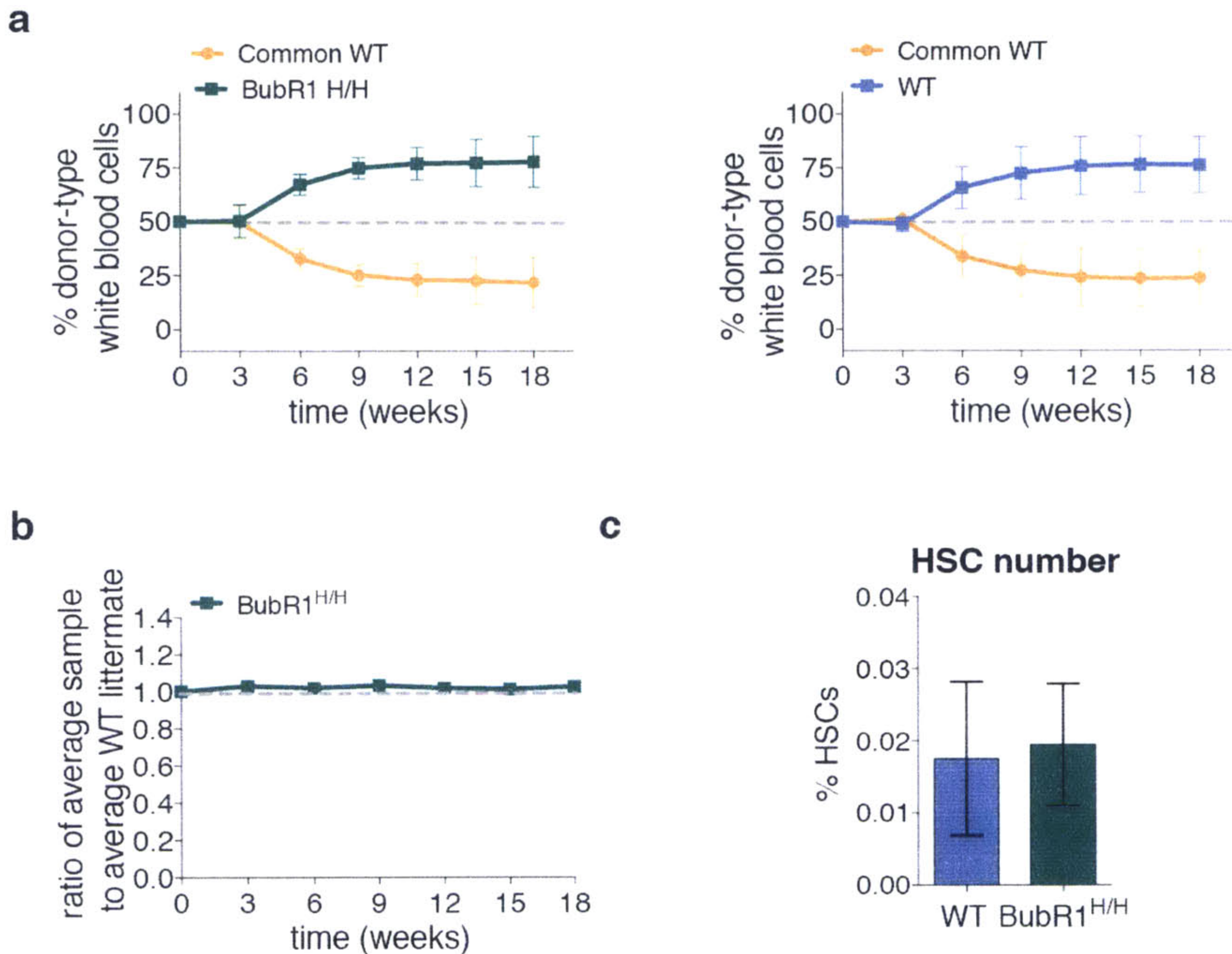


Figure 10. *BubR1$^{H/H}$ adult bone marrow HSCs show no fitness defects*

CD45.2 BubR1$^{H/H}$ or wild type littermate bone marrow cells were co-transferred with an equal number of bone marrow cells from a common CD45.1 donor mouse of the same age into a lethally irradiated CD45.1 recipient. Peripheral blood was sampled at indicated times and the percentage of the white blood cell population contributed by each donor was quantified by flow cytometry with antibodies against CD45.1 and CD45.2 (a). Ratios of the average percentage of the peripheral blood reconstituted by the BubR1$^{H/H}$ bone marrow cells to the average percentage of the peripheral blood reconstituted by wild type littermate bone marrow cells at all indicated times (b). (c) Quantification of the percentage of CD150^{+} Sca-1^{+}, CD117^{+}, CD48^{-} cells in the bone marrow of adult BubR1$^{H/H}$ mice and wild type littermates (n=3 for each).

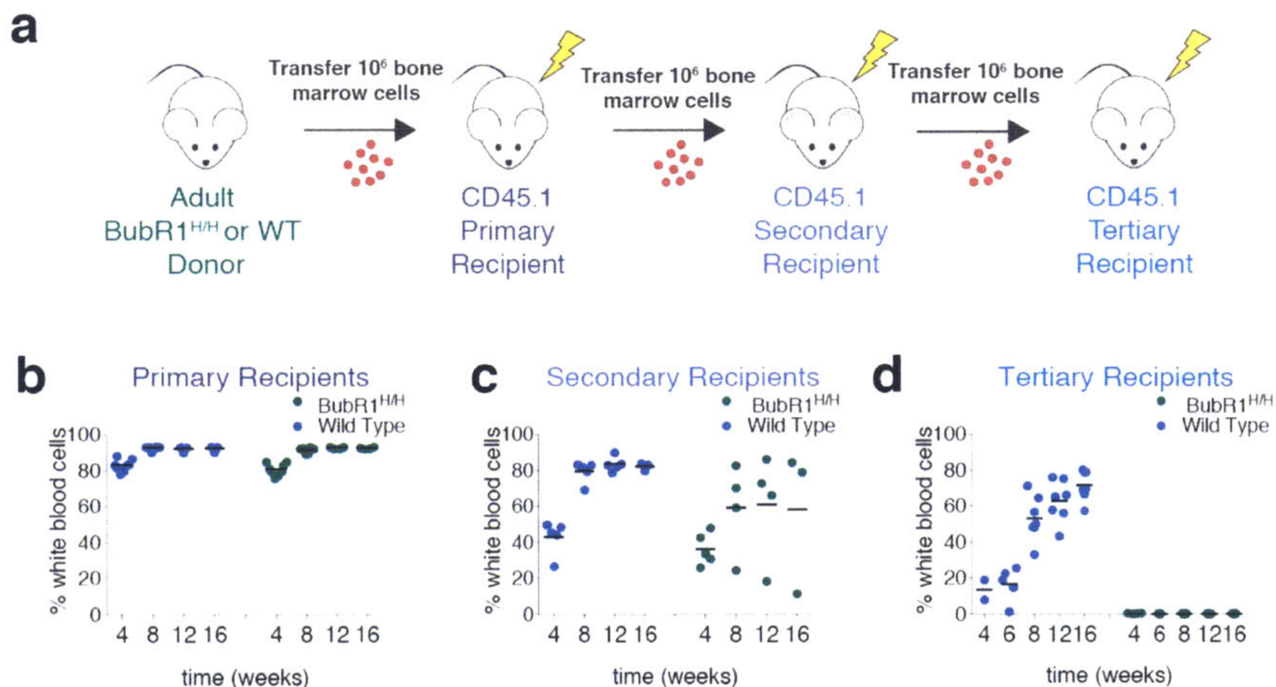
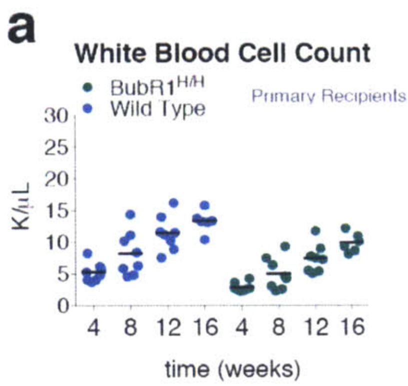


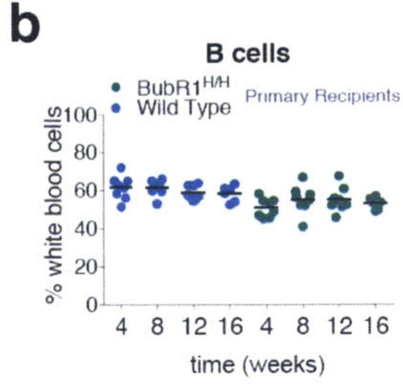
Figure 11. *$BubR1^{H/H}$ bone marrow HSCs undergo stem cell exhaustion upon serial transplantation*

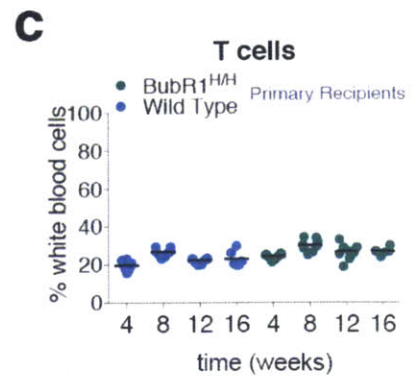
(a) Serial transplantation of $BubR1^{H/H}$ bone marrow cells to primary, secondary and tertiary recipients. (b – d) CD45.2 adult $BubR1^{H/H}$ bone marrow cells were serially transplanted into lethally irradiated primary, secondary and tertiary CD45.1 recipients. The percentage of the peripheral blood that is CD45.2 positive was determined in primary (b), secondary (c) and tertiary (d) recipients at the indicated times.

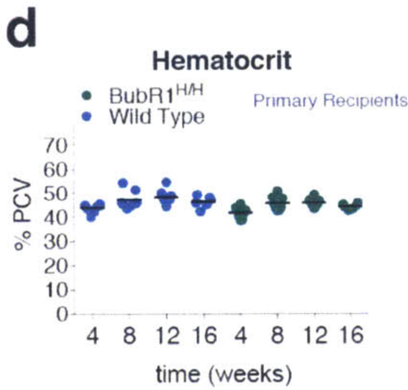
BubR1$^{H/H}$ HSCs was comprised of CD45.1 positive cells, presumably descendants from rare recipient HSCs that survived irradiation. There are several reasons why BubR1$^{H/H}$ HSCs could be losing their ability to reconstitute the hematopoietic lineages upon serial transfer. First, the proliferative potential of these HSCs could be impaired: perhaps these cells do not have the same serial repopulation potential as wild type cells. Another possibility is that that BubR1$^{H/H}$ HSC pool could be becoming progressively more aneuploid as the number of cell divisions it performs increases, such that the level of aneuploidy in the HSCs and/or the descendants of the HSCs reaches such high levels that these cells are no longer compatible with effective proliferation. To distinguish between these possibilities, we performed single cell sequencing of peripheral blood cells to determine the karyotype of the peripheral blood of one BubR1$^{H/H}$ secondary recipients. All 17 cells sequenced were euploid, indicating that the phenotypes we observed may not be due to an increase in aneuploidy in the cells derived from BubR1$^{H/H}$ HSCs. While more cells should be sequenced to confirm this finding, these data suggest that aneuploid cells produced by BubR1$^{H/H}$ HSCs are unable to effectively contribute to the peripheral blood of secondary recipients. This indicates that BubR1$^{H/H}$ HSCs are less fit than wild type HSCs in this context, although if this decrease in fitness is due to an increase in aneuploidy in HSCs that decreases their proliferative potential or some other molecular cause remains to be determined. However, this decreased replicative potential is consistent with the progeroid nature of the BubR1$^{H/H}$ mouse model (Baker et al., 2004).

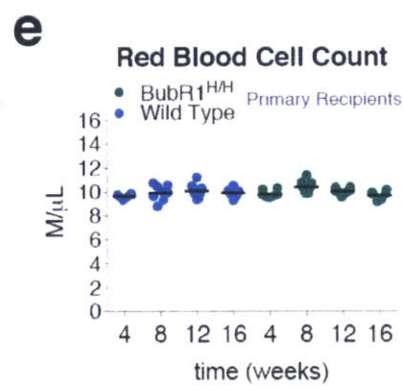
The results of the secondary transplantations indicate that BubR1$^{H/H}$ HSCs lose their regenerative potential with time. This would allow the rare euploid CD45.1 recipient HSC that survived irradiation to repopulate the hematopoietic system. To test this, we performed a tertiary transfer with bone marrow cells derived from secondary recipients with a high, intermediate and

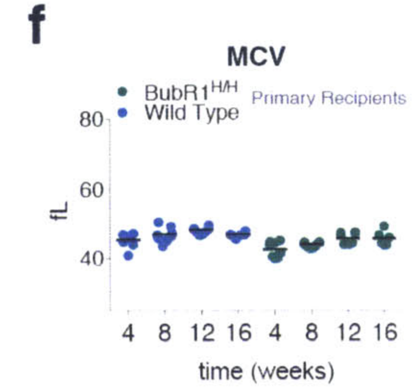

a
White Blood Cell Count
BubR1H/H
Wild Type
Primary Recipients
K/μL
time (weeks)

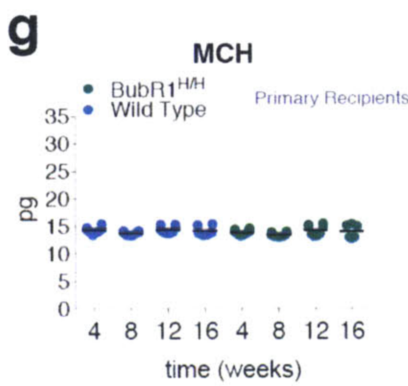

b
B cells
BubR1H/H
Wild Type
Primary Recipients
% white blood cells
time (weeks)

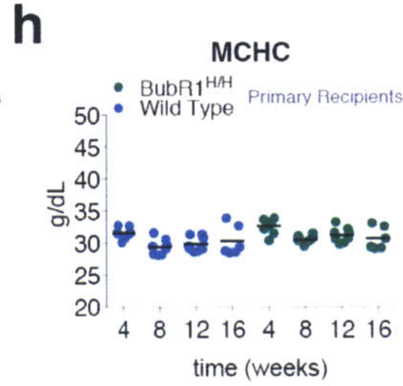

c
T cells
BubR1H/H
Wild Type
Primary Recipients
% white blood cells
time (weeks)

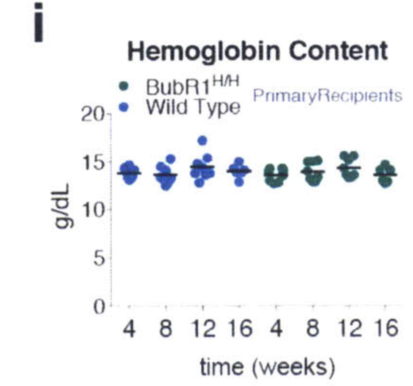

d
Hematocrit
BubR1H/H
Wild Type
Primary Recipients
% PCV
time (weeks)

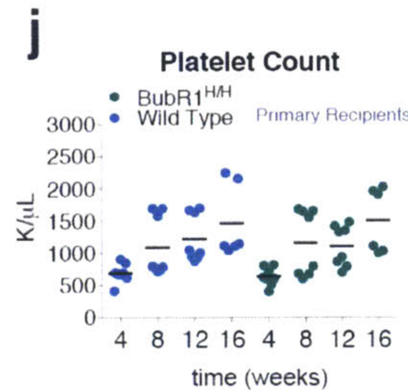

e
Red Blood Cell Count
BubR1H/H
Wild Type
Primary Recipients
M/μL
time (weeks)


f
MCV
BubR1H/H
Wild Type
Primary Recipients
fL
time (weeks)


g
MCH
BubR1H/H
Wild Type
Primary Recipients
pg
time (weeks)


h
MCHC
BubR1H/H
Wild Type
Primary Recipients
g/dL
time (weeks)


i
Hemoglobin Content
BubR1H/H
Wild Type
PrimaryRecipients
g/dL
time (weeks)


j
Platelet Count
BubR1H/H
Wild Type
Primary Recipients
K/μL
time (weeks)

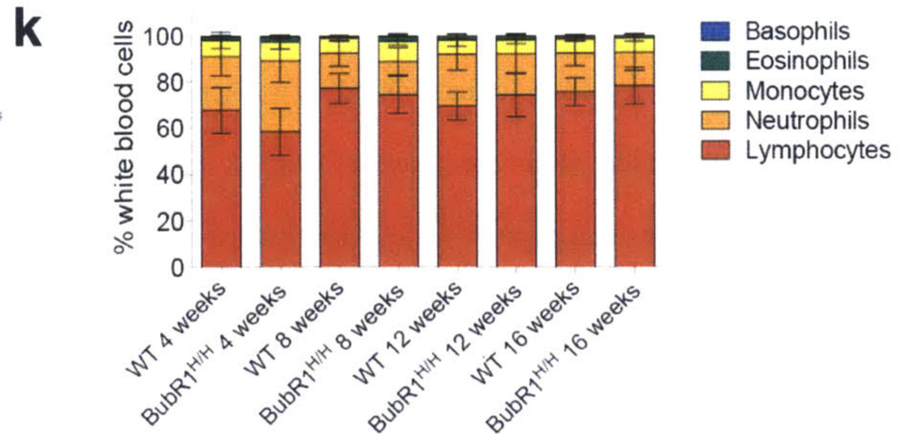

k
% white blood cells
Basophils
Eosinophils
Monocytes
Neutrophils
Lymphocytes
WT 4 weeks
BubR1H/H 4 weeks
WT 8 weeks
BubR1H/H 8 weeks
WT 12 weeks
BubR1H/H 12 weeks
WT 16 weeks
BubR1H/H 16 weeks

Figure 12. *Peripheral blood analyses of BubR1$^{H/H}$ primary recipients*

Complete blood cell counts of peripheral blood from BubR1$^{H/H}$ and wild type primary bone marrow recipients were performed to determine white blood cell counts (a). Flow cytometry was performed to quantify the percentage B220-positive B cells (b) and Thy1.2-positive T cells (c) in the peripheral blood of primary recipients. Complete blood cell counts further determined hematocrit (d), red blood cell counts (e), mean corpuscular volume (MCV) (f), mean corpuscular hemoglobin (MCH) (g), mean corpuscular hemoglobin concentration (MCHC) (h), hemoglobin content (i) and platelet counts (j). The composition of the leukocyte population was evaluated by automated differential for BubR1$^{H/H}$ and wild type primary recipients (k).

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low contribution of BubR1$^{H/H}$ HSCs to the animal's peripheral blood. Mice that received bone marrow from secondary recipient donors with strong BubR1$^{H/H}$ peripheral blood contribution did not survive the tertiary transfer (0% survival after 4 weeks, n=5). Mice that received bone marrow from donors with weak or intermediate BubR1$^{H/H}$ peripheral blood contribution showed increased survival (60% and 80%, respectively after 16 weeks, n=5 for each condition); however, peripheral blood analysis of all mice that survived the tertiary transfer revealed that virtually no cells were derived from the CD45.2 BubR1$^{H/H}$ donor, but rather were derived from some surviving CD45.1 HSCs from a recipient (Figure 11d). This is in contrast to the tertiary recipients that received bone marrow from secondary recipients with wild type bone marrow, which had on average a 71.6% contribution from the wild type donor after 16 weeks (n=7). This finding demonstrates that all types of aneuploidy evaluated, both constitutional trisomy and randomly generated aneuploidies caused by an increase in chromosome mis-segregation frequency, adversely affect the fitness of HSCs. Lineage-specific developmental defects contribute to this phenotype in some cases that are likely due to chromosome-specific effects, for example the B cell defect observed with trisomy 16 HSCs. However, proliferation defects are at least in part responsible for this decrease in stem cell fitness.

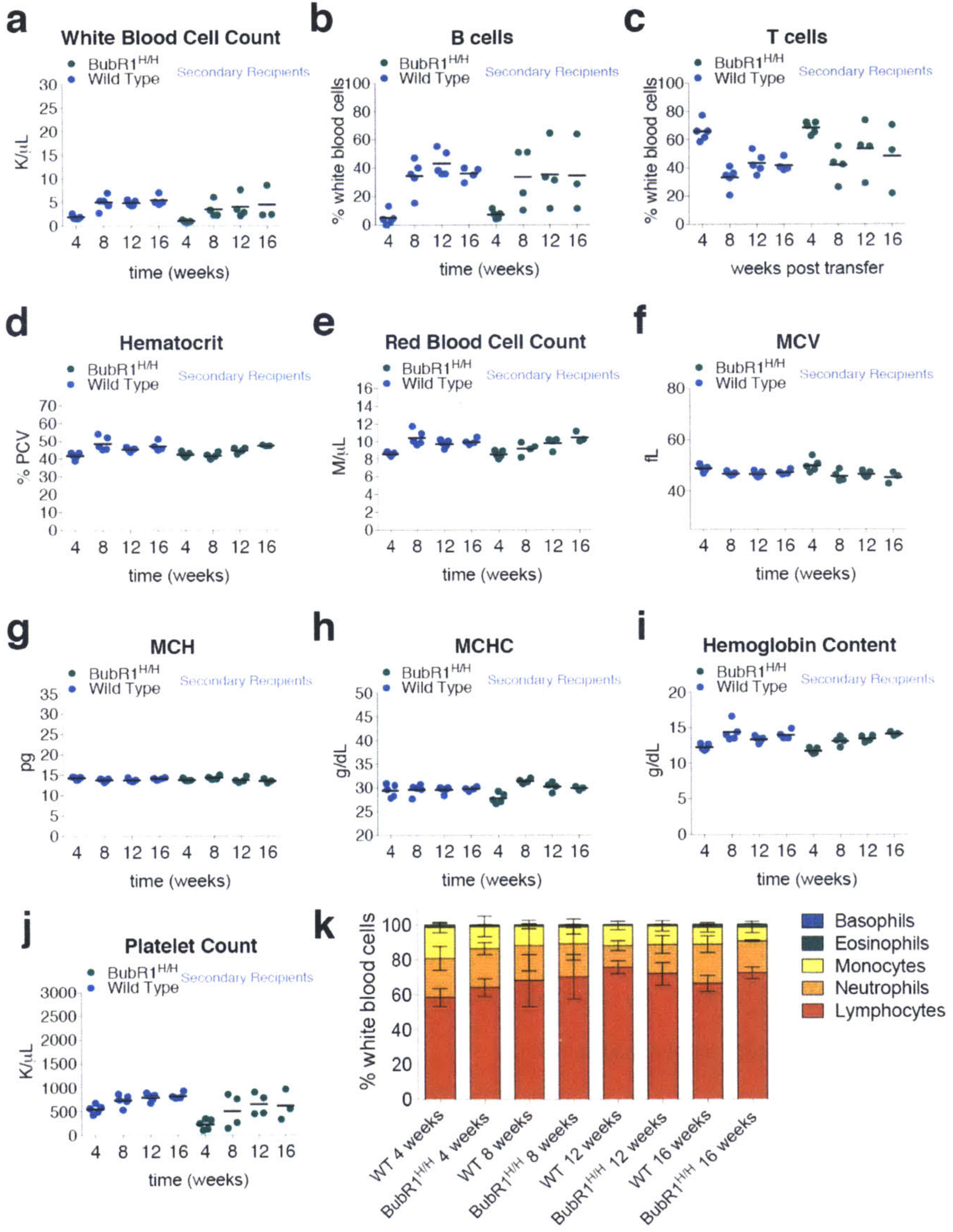
a
White Blood Cell Count
BubR1$^{H/H}$
Wild Type
Secondary Recipients
K/μL
time (weeks)
b
B cells
% white blood cells
c
T cells
weeks post transfer
d
Hematocrit
% PCV
e
Red Blood Cell Count
M/μL
f
MCV
fL
g
MCH
pg
h
MCHC
g/dL
i
Hemoglobin Content
j
Platelet Count
k
Basophils
Eosinophils
Monocytes
Neutrophils
Lymphocytes
WT 4 weeks
BubR1$^{H/H}$ 4 weeks
WT 8 weeks
BubR1$^{H/H}$ 8 weeks
WT 12 weeks
BubR1$^{H/H}$ 12 weeks
WT 16 weeks
BubR1$^{H/H}$ 16 weeks

Figure 13. *Peripheral blood analyses of BubR1$^{H/H}$ secondary recipients*

Complete blood cell counts of peripheral blood from BubR1$^{H/H}$ and wild type secondary bone marrow recipients were performed to determine white blood cell counts (a). Flow cytometry was performed to quantify the percentage B220-positive B cells (b) and Thy1.2-positive T cells (c) in the peripheral blood of secondary recipients. Complete blood cell counts further determined hematocrit (d), red blood cell counts (e), mean corpuscular volume (MCV) (f), mean corpuscular hemoglobin (MCH) (g), mean corpuscular hemoglobin concentration (MCHC) (h), hemoglobin content (i) and platelet counts (j). The composition of the leukocyte population was evaluated by automated differential for BubR1$^{H/H}$ and wild type secondary recipients (k).

Discussion

Here we have tested Boveri's "chromosome theory of tumorigenesis" hypothesis *in vivo* to determine whether aneuploid karyotypes provide a fitness advantage to cells that allows them to hyperproliferate and thus leads to cancer. We set up a transplantation system that allowed us to directly compare the fitness of HSCs harboring either constitutional aneuploidies or a mutation that leads to the generation of cells harboring random aneuploidies with isogenic euploid controls.

We found that trisomy 16 fetal liver HSCs are much less fit than euploid HSCs and show phenotypes characteristic of proliferation defects when challenged to reconstitute irradiated euploid recipients. In contrast, lower levels of aneuploidy are better tolerated in the hematopoietic lineage, namely cells derived from trisomy 19 and BubR1$^{H/H}$ HSCs. In fact, we observed a correlation between the severity of fitness defects and the level of aneuploidy present in the blood. Furthermore, mice reconstituted with trisomy 19 HSCs showed very mild phenotypes, even after serial transplantation.

However, the relatively fit BubR1$^{H/H}$ HSCs begin to show more severe phenotypes upon repeated proliferative challenge. Recent studies have shown that murine HSCs divide very infrequently and utilize numerous, more differentiated progenitors cells to sustain long-term

hematopoiesis (Busch et al., 2015; Sun et al., 2015), providing a potential explanation as to why repeated challenges are needed to reveal the fitness defects of aneuploidy in an HSC population in which only a subpopulation of cells are aneuploid. In the same vein, perhaps further serial transplantation of trisomy 19 HSCs will make a proliferative defect apparent. Thus, our *in vivo* fitness analysis of primary aneuploid cells does not support Boveri's hypothesis. Aneuploidy *per se* in the hematopoietic lineage is not a driver of proliferation and does not increase cellular fitness. Rarely, it is, at best, a fitness neutral condition.

Mouse chromosome 16 is the closest whole chromosome mouse homolog to human chromosome 21. Trisomy 16 has a dramatic effect on mouse HSC fitness, as it affects all hematopoietic lineages, particularly the B cell lineage. In light of this finding, we speculate that the increased numbers of megakaryotcyte and erythroid progenitors observed in DS fetal livers and individuals with TMD is not because this lineage is favored by trisomy 21 but rather because the negative fitness effect of trisomy 21 is less pronounced on this lineage than on others, i.e. the precursors to the B cell lineage. However, it remains possible that triplication of some genes on chromosome 21 do contribute to the hyperproliferation of certain hematopoietic lineages. For example, triplication of HMGN1 has been implicated in B cell transformation (Lane et al., 2014). Trisomy 21 is also observed in non-DS leukemia (Mitelman et al., 1990; Cheng et al., 2009; Hama et al., 2008). It will be interesting to determine whether particular aneuploidies can contribute to tumorigenesis in specific oncogenic contexts or in the presence of aneuploidy-tolerating mutations. The model system we have established here will permit effective molecular dissection of the effect of aneuploidy on the development of leukemias and lymphomas.

Materials and Methods

Mouse strains

The mouse strains utilized in (Williams et al., 2008)were backcrossed for at least 10 generations into the C57BL/6J background from Jackson Laboratory to generate congenic strains. Strains used to generate trisomic embryos were B6.Cg-Rb(6.16)24Lub/JAmonJ or B6.Cg-Rb(13.16)1Mpl/JAmonJ and B6.Cg-Rb(16.17)7Bnr/JAmonJ (Trisomy 16) and B6.Cg-Rb(5.19)1Wh/JAmonJ and B6Ei.Cg-Rb(9.19)163H/J (Trisomy 19). All male compound heterozygous mice were mated with C57BL/6J females to generate trisomic animals. $BubR1^{H/H}$ mice were a generous gift from Dr. J.M. van Deursen. Embryos from all strains were collected at embryonic days E13.5 – E15.5 by timed matings (See Appendix). Recipient mice were 6-8 week old B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ (CD45.1) mice from Jackson Laboratory. All animal studies and procedures were approved by the MIT Institutional Animal Care and Use Committee.

Sample preparation and karyotyping

Fetal livers were removed from embryos by dissection. Samples were homogenized by pipetting and passage through a 70 µm mesh filter, frozen down in FBS + 5% dimethylsulfoxide and stored in liquid nitrogen. For $BubR1^{H/H}$ embryos and adult mice, tissue samples were sent to Transnetyx (Cordova, TN) for genotyping using the protocol described previously (Baker et al., 2004). For trisomic embryos, mouse embryonic fibroblasts (MEFs) were derived, and karyotype was determined by metaphase spreads of MEFs as described previously (Williams et al., 2008). Karytoype was confirmed by qPCR of genomic DNA derived from MEF cell pellets. Briefly, cells were digested 4-16 hours at 55°C in 10 mM Tris pH 8.0, 100 mM NaCl, 10 mM EDTA pH 8.0, 0.5% SDS solution with 0.4 mg/ml proteinase K. Genomic DNA was precipitated with isopropanol, washed with 70% ethanol, resuspended in double deionized water, and treated at 95°C for 5 minutes. qPCR was performed on DNA samples by mixing Takara SYBR *Premix Ex*

Taq II (Tli RNase H Plus, ClonTech, Mountain View, CA) master mix with the primers described in Table 1, and amplified using a LightCycler 480 II (Roche). Samples were normalized to the copy number-invariant reference gene *Glucagon* (GCG) as described in (Ballester et al., 2013). Genes amplified were selected based on low propensity for copy number variation (She et al., 2008) and chromosomal location, and primers were designed to amplify across intron-exon boundaries.

Hematopoietic reconstitutions

For all reconstitution experiments, mice were closely monitored for signs of bone marrow failure and rapid weight loss. All protocols for treating irradiated mice were outlined by MIT's Division of Comparative Medicine. Whole body irradiation was performed using a 137^{Cs} irradiator (γ cell 40) at a dose rate of ~ 100 cGy/min.

For competition assays and hematopoietic reconstitution experiments, recipient mice were irradiated with a 12 Gy total dose administered as a split dose of 8 Gy followed by a second dose of 4 Gy 3 hours later. Fetal liver cells were thawed in Iscove's modified Dulbecco's medium supplemented with 2% FBS and counted on a Cellometer Auto T4 automated hemacytometer (Nexelcom). Viability was assessed by propidium iodide exclusion using a FACSCalibur flow cytometer (Becton Dickinson). 10^6 total live cells were injected intravenously in Hank's Balanced Salt Solution.

Recipient mice for colony forming unit spleen assays were irradiated with 9.5 Gy administered as a single dose. Fetal liver cells were then prepared and injected as described above. 7-8 days after injection, spleens were harvested from recipients and then fixed overnight in Bouin's fixative. Spleens were sectioned into 5 μm and stained with hematoxylin and eosin. Slides were then scanned on a Leica Aperio slide scanner, and area was measured using ImageJ. To control

for small residual white blood cell nodules in the spleen, sections of spleens from irradiated mice that were not transferred with cells were also analyzed. We determined the average background colony size to be 0.336% total spleen area, and colonies larger than this average were considered CFU-S colonies.

Recipient mice for homing assays were irradiated with 8.5 Gy administered as a single dose. After thawing, fetal liver cells were labeled with CM-DiI (Life Technologies) according to the manufacturer's instructions, and then evaluated for degree of labeling and viability by propidium iodide exclusion with an LSR II flow cytometer (Becton Dickinson). $2x10^6$ live cells were injected intravenously, and bone marrow was harvested by flushing the long bones 24 hours after injection. The proportion of labeled cells in the bone marrow was evaluated on an LSR II flow cytometer (Becton Dickinson).

Recipient mice for bone marrow transfers were irradiated with 9.5 Gy, administered as a single dose. Bone marrow cells were flushed from the donor's long bones with IMDM + 2% fetal bovine serum. Red blood cells were lysed in ACK Lysing Buffer, then white blood cells were counted. Mice were then reconstituted by intravenous injection of 10^6 cells in Hank's Balanced Salt Solution.

Peripheral blood analysis

For complete blood cell count, peripheral blood was collected with heparinized capillary tubes into EDTA-coated Microvette 100 tubes (Sarstedt). Blood was then analyzed on a HemaVet 950FS (Drew Scientific). Peripheral blood for flow cytometry analysis was collected with heparinized capillary tubes into sodium heparin diluted in PBS. Red blood cells were lysed in ACK Lysing Buffer, then washed in Hank's Balanced Salt Solution + 2% fetal bovine serum. Cells were then incubated with antibodies according to the manufacturer's specifications, and

then analyzed with a FACSCalibur or LSR II flow cytometer (Becton Dickinson). Antibodies used are listed below and were all obtained from BioLegend: CD45.1 (A20), CD45.2 (104), CD45R/B220 (RA3-6B2), CD90.2/Thy-1.2 (53-2.1).

HSC quantification

Fetal livers and bone marrow cells were harvested, genotyped or karyotyped, processed and counted as described above. Fetal liver HSCs were quantified as described previously (Kim et al., 2006). Bone marrow HSCs were quantified as described previously (Kiel et al., 2005). Antibodies used are listed below and were all obtained from BioLegend: CD150 (TC15-12F12.2), CD48 (HM48-1), Sca-1 (E13-161.7), CD117 (2B8), CD3ε (145-2C11), Ly-6G/Ly-6C (Gr-1) (RB6-8C5), B220/CD45R (RA3-6B2), Ter-119, CD5 (53-7.3), CD8a (53-6.7).

Single cell sequencing

Peripheral blood was collected from one transplantation chimera reconstituted with FL-HSCs derived from a BubR1$^{H/H}$ embryo and a common euploid CD45.1 donor. Blood was processed as described above, and BubR1$^{H/H}$ white blood cells were sorted using a MoFlo cell sorter (Beckman-Coulter) using a CD45.2 antibody. CD45.1 cells were excluded using a CD45.1 antibody. Single cells were then picked, prepared, sequenced and analyzed as described in (Knouse et al., 2014).

Acknowledgements

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Jianzhu Chen, Ömer Yilmaz, and members of the Amon lab for critical reading of this manuscript. This work was supported by the Kathy and the Curt Marble Cancer Research Fund and in part by the Koch Institute Support Grant P30-CA14051. S.J.P. was supported by an NSF Graduate Research Fellowship. A.A is an investigator of the Howard Hughes Medical Institute and the Glenn Foundation for Biomedical Research.

Table 1. *Primers used for karyotyping qPCR*

Chromosome	Gene	Forward Primer	Reverse Primer	Source
2	Glucagon (*GCG*)	5′-AACATTGCCAAACGTCATGATG-3′	5′-GCCTTCCTCGGCCTTTCA-3′	Ballester et al., 2013
X	Glycoprotein M6B (*GM6B*)	5′-CTCTTCCACCAGCTGATCTACATG-3′	5′-TCCCGACTCTTAAACTTCAAAACC-3′	Ballester et al., 2013
16	Runt-related transcription factor 1 (*RUNX1*)	5′-CAGGTATACCTTGGATCAGTGC-3′	5′-CAACACAGCATCTTCTGATGGC-3′	This study
16	Eph receptor A3 (*EPHA3*)	5′-AGGAATCATCCCAGCAACACAC-3′	5′-GAGAGCAATCTAGTATTGTTCTGGG-3′	This study
16	Oxysterol binding protein-like 11 (*OSBPL11*)	5′-CCCAATTAAGTGCATACCCAGC-3′	5′-CAAGAGACAGTCAGCAAACACGG-3′	This study
16	Epithelial membrane protein 2 (*EMP2*)	5′-CTCTGTTCTCATGAATGAGCCTG-3′	5′-CAGAAAGAATCGAAGGGAGATTG-3′	This study
19	Bestrophin 1 (*BEST1*)	5′-CAGGGCAGAGGTCATGGTTC-3′	5′-CTGGTGCTCAAGGCAGACCT-3′	This study
19	Ankyrin repeat domain 1 (cardiac muscle) (*ANKRD1*)	5′-GTGCACATGGAAATGACTGG-3′	5′-TGGGCCACAACTCAATGTTA-3′	This study
19	Oligonucleotide/ oligosaccharide-binding fold containing 1 (*OBFC1*)	5′-CTGCACGAAACCTTGCATGA-3′	5′-GCCCCGGCTGATCTTAATCT-3′	This study
19	Caspase 7 (CASP7)	5′-CAATCTGCCACTCTGCAACC-3′	5′-CAGCAACATTGAACAGGCT- 3′	This study

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Chapter 3: Conclusions and Future Directions

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Summary of Key Conclusions

In recent years, research evaluating the effects of aneuploidy on cell physiology and investigating its role in cancer have greatly increased understanding of this complicated cellular state. A number of model systems have been developed to study the role of chromosomal instability and aneuploidy in tumorigenesis. Yet, the results are surprisingly complex (as discussed in Chapter 1), with the conditions sometimes promoting and sometimes inhibiting tumor formation.

By studying aneuploidy *per se*, we have learned that aneuploidy has a profound effect on cells and organisms. By introducing such a large genomic perturbation, a multitude of cellular processes are altered, from transcription and translation, to protein folding and degradation mechanisms. Cells respond with a characteristic stress response, proliferating slowly and upregulating protein degradation pathways. When present in a multicellular organism, this response is often lethal, and the particular genomic doses that are tolerated result in individuals with developmental defects.

To date, much of the work performed to characterize aneuploid cells how been performed *in vitro* or in animals models of Down syndrome. While these systems have greatly increased our understanding of the aneuploid cell condition, they do not provide a complete picture of the general effects of aneuploidy on cell physiology *in vivo*: cell culture systems, while permitting a wide range of aneuploid cell types to be tested, do not recapitulate the complex environment of an organism; and mouse models of DS only permit study of the amplification of genes present on human chromosome 21, making it difficult to distinguish phenotypes due to aneuploidy *per se* from phenotypes due to the amplification of chromosome 21-specific genes.

In this thesis, I sought to address this by setting up an experimental system to study aneuploid cells *in vivo*. By utilizing aneuploid hematopoietic stem cells, I could use transplantation to evaluate the fitness of a range of aneuploid cells *in vivo* in the context of an otherwise euploid organism. I found that aneuploidy is also deleterious in the hematopoietic cell lineage, although some aneuploidies were surprisingly well tolerated. These findings warrant further investigation to find the underlying causes of the phenotypes observed. Furthermore, this system has great potential to assess how aneuploidy affects the kinetics of tumorigenesis in leukemia and lymphoma.

Fitness defects in HSCs scale with degree of aneuploidy

By performing *in vivo* competition assays, I was able to evaluate the relative fitness of three different types of aneuploid HSCs: trisomy 16, trisomy 19 and BubR1$^{H/H}$. Fetal liver cells from aneuploid embryos or their wild type littermates were competed against fetal liver cells from a common euploid donor embryo. Comparison of each aneuploid donor to a wild type littermate when competed against a common euploid donor revealed that the relative fitness of the aneuploid HSCs scaled with the degree of aneuploidy. Trisomy 16 is the largest genomic amplification, trisomy 19 is an intermediate amplification and BubR1$^{H/H}$ HSCs have the smallest amplification as assessed by single cell sequencing. Correspondingly, trisomy 16 HSCs exhibited severe fitness defects, trisomy 19 HSCs had minor fitness defects and BubR1$^{H/H}$ HSCs showed no difference in fitness when compared to HSCs from wild type littermates.

This result is consistent with other studies in aneuploid yeast strains (Torres et al., 2007) and *in vitro* studies of mouse embryonic fibroblasts (Williams et al., 2008). Together, these findings argue for the role of a general cellular response to aneuploidy that is scaled depending on the size of the genomic imbalance, suggesting that aneuploidy confers similar cellular

phenotypes in many contexts. This general, graded response to aneuploidy occurs not only *in vitro*, but also *in vivo* and in this cell particular type—which is generally thought to be highly regulated by its niche (Morrison and Scadden, 2014)— in the context of an otherwise euploid organism.

In this vein, it would be interesting to further analyze the dynamics of the bone marrow niche during transplantation of aneuploid HSCs. We found that the aneuploid fetal liver HSCs can home to the bone marrow as effectively as wild type cells upon transplantation. We thus hypothesize that the differences in fitness observed in these HSCs can be attributed to decreased proliferative capacity after reaching the niche. This difference in proliferation could be due to the cell's response to aneuploidy. However, the nature of the hematopoietic stem cell makes this proliferation defect worthy of further investigation. HSCs rely on signals from other cells to be stimulated to divide (Wang and Wagers, 2011). Perhaps aneuploid HSCs are less responsive to the signals being produced by other niche cells, especially if their transcriptional profile is altered to enact the aneuploidy stress response (Sheltzer et al., 2012; Torres et al., 2007). Alternatively, HSCs divide very infrequently and instead rely largely on progenitor cells to produce the peripheral blood (Busch et al., 2015; Sun et al., 2015). Perhaps the defects observed in this study can be attributed to reduced proliferation in the progenitor cell compartment. This is particularly relevant in the case of trisomy 16, where a lineage-specific alteration in proliferation was observed. Whether trisomy 16 has a greater impact on proliferation and differentiation in the B cell lineage than in other lineages should be evaluated.

Cell culture studies have shown that the proteomic imbalances caused by aneuploidy lead to proteotoxic stress, metabolic alterations, increased ROS production and cell cycle delays

(Santaguida and Amon, 2015). These general characteristics of aneuploid cells likely contribute to the decreased proliferative potential and eventual exhaustion of aneuploid HSCs observed.

Trisomy 16 HSCs show severe proliferation defects

We found that trisomy 16 led to greatly reduced HSC fitness in both *in vivo* competition assays and in hematopoietic reconstitution. In particular, we found that mice reconstituted with trisomy 16 had several phenotypes, including macrocytic anemia – a reduction in red blood cell number accompanied by an increase in red blood cell volume that is usually indicative of a proliferative defect – and leukopenia, a reduction in white blood cell counts, that in this casae seems to be due largely to reduced B cell numbers. Similar phenotypes have been observed previously: macrocytosis was detected in individuals with DS (David et al., 1996), and an alteration in B cell progenitors was observed in fetal livers from trisomy 21 fetuses (Roy et al., 2012). As discussed above, further work into characterizing the observed B cell defects in this model is needed to determine which part of the B cell differentiation pathway is affected by trisomy 16. Quantification of the cells that give rise to B cells – pro-B and pre-B cells – in the bone marrow of trisomy 16 recipient mice will be performed as done previously (Hardy et al., 1991) to determine if there is a perturbation in a specific progenitor pool in the B cell differentiation pathway.

Additionally, critical to understanding how aneuploidy affects cellular fitness *in vivo* is to assess the effects of multiple different aneuploidies. Therefore, more trisomic HSCs should be evaluated in reconstitution to determine whether the defects observed for trisomy 16 are more chromosome-specific or more due to the size of the genomic alteration. We now have the means to evaluate trisomy 13 HSCs. Trisomy 13 mice, which harbor a 120 Mbp amplification, can be isolated at E14.5. This genomic amplification is larger than that observed in trisomy 16 (98

Mbp). Thus, evaluation of trisomy 13 HSC fitness will clarify whether the observed fitness decrease and the specific phenotypes observed from trisomy 16 are dominated by alterations in the dosage of chromosome-specific genes, or by the overall decrease in fitness associated with the aneuploid state, or a combination of both factors. As the initial study found that mice reconstituted with trisomy 13 HSCs showed few specific defects but had the lowest rate of survival (Herbst and Winking, 1991), I anticipate that the general fitness defects of trisomy 16 may be due to aneuploidy-associated phenotypes while the B cell lineage-specific phenotypes may be accounted for by amplification of specific genes.

Trisomy 19 is well-tolerated in the blood

While trisomy 19 showed a minor decrease in HSC fitness in *in vivo* competition assays, it led to very few phenotypes in hematopoietic reconstitution, even upon serial transplantation. This result indicates that aneuploidy can be well tolerated in the hematopoietic lineage in some contexts. However, chromosome 19 is the smallest mouse autosome and thus not as large of a genomic insult as trisomy 16. Furthermore, it has been demonstrated that a single HSC can lead to long term engraftment of a recipient (Krause et al., 2001), and estimates of HSC proliferative potential based on serial transplantation assays predict that HSCs would be able to sustain hematopoiesis for 15-50 lifespans in mice (Harrison and Astle, 1982). While aging associated phenotypes have been observed in the blood (Geiger et al., 2013), these studies suggest that HSCs have a great amount of regenerative potential. Perhaps in our experimental system, we did not challenge the trisomy 19 HSCs enough to be able to detect differential fitness. Further serial transplantation experiments will help evaluate the potential of trisomy 19 HSCs. Additionally, a serial dilution experiment – in which mice are transferred with decreasing numbers of HSCs – may prove a more sensitive way to detect a fitness decrease and determine a dose of cells where

trisomy 19-specific phenotypes can be detected. However, it is possible that having a slight decrease in HSC fitness may not result in any observable phenotypes *in vivo* in this experimental system.

BubR1$^{H/H}$ HSCs

Some of the most interesting results from this study were obtained from analysis of the BubR1$^{H/H}$ HSCs. BubR1$^{H/H}$ fetal liver HSCs showed no evidence of decreased fitness by *in vivo* competition assay, despite the presence of aneuploid cells in the peripheral blood (Figure 1a). The same was true of bone marrow HSCs in hematopoietic reconstitution experiments. However, upon secondary transfer of BubR1$^{H/H}$ bone marrow cells from primary recipients, a decrease in fitness was observed, as CD45.1 recipient-derived cells that escaped irradiation began to dominate the peripheral blood of the secondary recipients. Intriguingly, the peripheral blood cells derived from the BubR1$^{H/H}$ donor in secondary recipients were euploid (Figure 1b). These data generate a number of hypotheses about how aneuploidy is tolerated and regulated in these experimental set ups.

First, in the context of an *in vivo* competition assay, aneuploid cells derived from the BubR1$^{H/H}$ donor were observed in the peripheral blood. However, these cells did not show phenotypes characteristic of fitness defects. Furthermore, aneuploidy was not detected in the peripheral blood of secondary recipient mice reconstituted with BubR1$^{H/H}$ bone marrow cells. Aneuploidy may have been observed in the competition assay and not in the bone marrow reconstitution experiments for a few reasons. First, there may be a difference between BubR1$^{H/H}$ fetal liver and bone marrow HSCs. Fetal liver HSCs were isolated at E14.5, and therefore did not experience the remaining week of embryonic development or the first months of life as did the bone marrow HSCs. There is evidence that apoptosis plays a role in the embryonic development

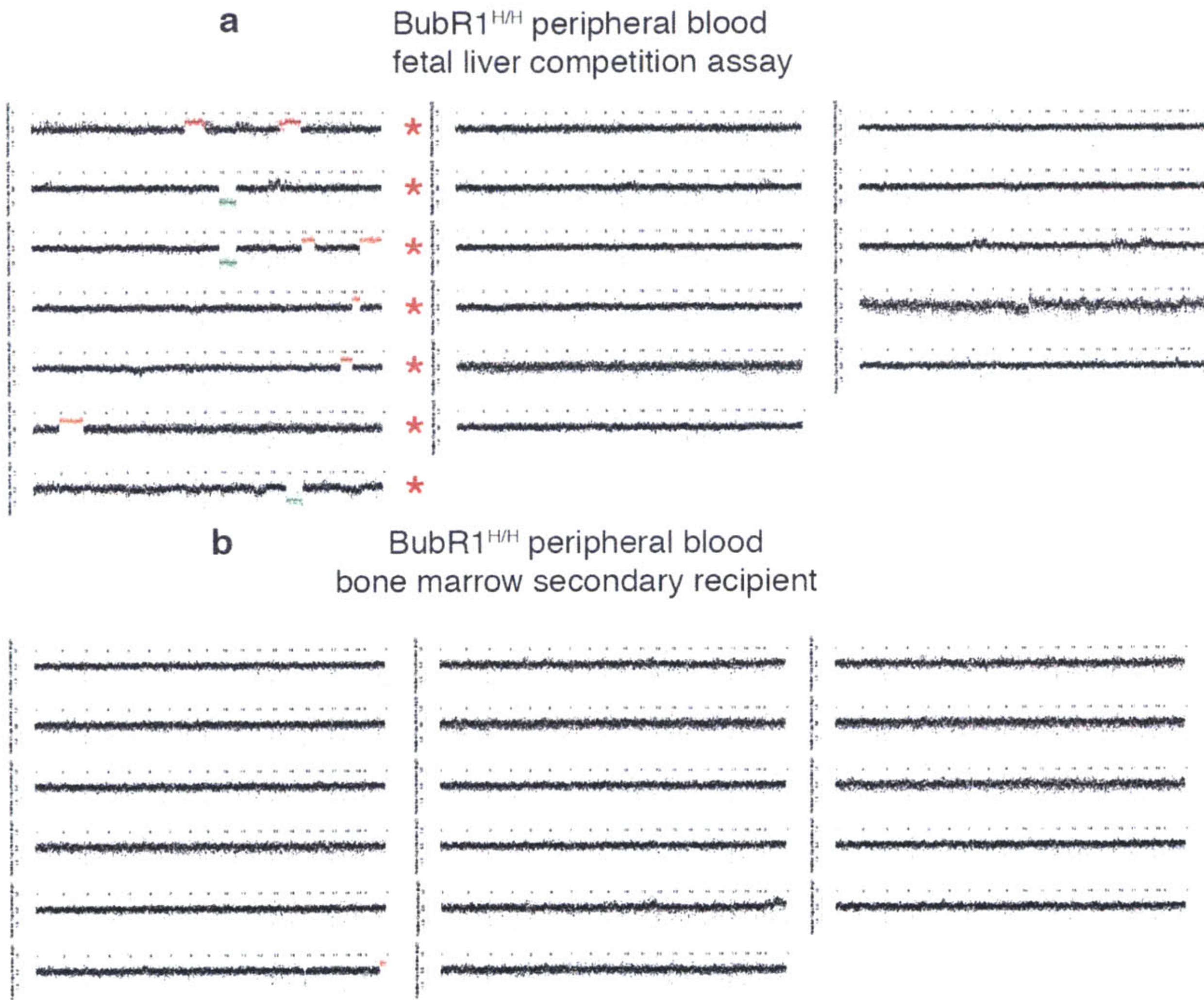

Figure 1. *Single cell sequencing analysis of a BubR1$^{H/H}$ fetal liver competition assay recipient and BubR1$^{H/H}$ bone marrow secondary recipient*

Segmentation plots of single cell karyotypes determined by single cell sequencing of CD45.2 positive peripheral blood cells derived from BubR1$^{H/H}$ fetal liver HSCs from Fig. 1d (a) and from bone marrow HSCs from secondary recipients from Fig. 4c (b). Segmentation plots show the copy number of single cells from chromosome 1 to X relative to a euploid reference on a $\log_2$ scale. Segments above the threshold for whole chromosome gain are shown in red, and segments below the threshold for whole chromosome loss are shown in green. Cells that are classified as aneuploid are indicated on the right by a red asterisk.

of HSCs at earlier stages of development (Orelio et al., 2004); thus, perhaps HSCs undergo further apoptotic elimination or selection at later embryonic stages and the isolated fetal liver cells were not subject to this selective culling. The fetal liver of later stage BubR1$^{H/H}$ embryos should be investigated for evidence of apoptosis to evaluate this possibility.

Additionally, there is evidence for selective repopulation of lymphoid lineages by recipient cells when mutant cells are subject to adoptive transfer (Rivera et al., 2003). Perhaps the aneuploid cells observed in the peripheral blood of the *in vivo* competition recipients were only tolerated because euploid competitor cells could generate enough euploid cells to compensate for the aneuploid cells present. Single cell sequencing of peripheral blood cells isolated from recipient mice of the BubR1$^{H/H}$ recipient mice could provide insight into whether euploid cells buffer aneuploid cells produced by BubR1$^{H/H}$ bone marrow HSCs. Whether these mice harbor aneuploid cells would also shed light on whether there are fundamental differences between fetal liver and bone marrow HSCs in this model as discussed above. In the *in vivo* competition assays, differentiation of each donor's HSCs into the different blood lineages was not assessed. However, it would also be interesting to evaluate whether BubR1$^{H/H}$ fetal liver HSCs contribute aneuploid cells equally to all lineages in these experiments and whether different chromosome-specific aneuploidies are tolerated or notably absent in different lineages. Such data would provide evidence for or against the hypothesis that certain aneuploidies are better tolerated in different hematopoietic lineages. For example, based on our work with trisomy 16, I would expect that BubR1$^{H/H}$-derived peripheral blood cells harboring trisomy 16 would be more likely to be myeloid than lymphoid. While such an experimental system would be rather low-throughput because it would rely on random mis-segregation events, it would provide incredible insight into how aneuploidy is tolerated in the hematopoietic system. Further such

analysis could provide hints as to which genes are especially dosage-sensitive in the proper regulation of specific hematopoietic lineages.

Perhaps the difference between BubR1$^{H/H}$ fetal liver and bone marrow HSCs is due to differences in experimental timing. The competition assay mice were sequenced 16 weeks after transfer, while secondary recipient mice about one year after transfer. Perhaps the BubR1$^{H/H}$ HSCs giving rise to aneuploid peripheral blood cells in the secondary recipient have already undergone stem cell exhaustion by this time, and there are no longer any aneuploid HSCs contributing to the peripheral blood. Thus, the peripheral blood of secondary recipient mice should be assessed for the presence of both aneuploid HSCs and peripheral blood cells at an earlier time point. However, we think this hypothesis is less likely, as single cell sequencing of HSCs and peripheral blood cells from a BubR1$^{H/H}$ adult mouse reveals very little aneuploidy as well (data not shown). Additionally, it is possible that these mice restored BubR1 function through mutation. While this is unlikely as the alteration of the gene that causes this loss of function phenotype is the insertion of an exon with a STOP codon that contains both splice donor and acceptor sites (Baker et al., 2004), it should still be evaluated to rule out this possibility.

Most intriguingly, however, this discrepancy between fetal liver and bone marrow HSCs could be due to selective elimination of aneuploid cells in the hematopoietic lineage (in the absence of buffering by additional euploid donor cells) or loss of engraftment potential due to increased levels of HSC aneuploidy. BubR1$^{H/H}$ bone marrow HSCs may lose fitness with successive transfers without giving rise to aneuploid cells due to increased selection against aneuploid cells in the hematopoietic lineage. Perhaps the BubR1$^{H/H}$ HSCs are becoming more aneuploid as they are continually challenged to proliferate, and these aneuploid HSCs are being selectively eliminated or are giving rise to aneuploid cells that are eliminated by the immune

system. However, in the absence of an active culling mechanism for aneuploid cells, aneuploid HSCs that arise from the BubR1$^{H/H}$ mutation may not be able to efficiently produce differentiated cells to be detectable in the peripheral blood by a small sample. Further, aneuploid HSCs may not be fit enough to reconstitute further recipients and so when challenged to home and proliferate in a serial reconstitution experiment, they fail to engraft and therefore do not end up contributing to the recipient. Thus, BubR1$^{H/H}$ HSCs will be diluted out with each serial transfer.

To evaluate these possibilities, BubR1$^{H/H}$ HSCs and peripheral blood cells from each donor and recipient should be quantified and sequenced to determine if HSC numbers are progressively decreasing and to assess the prevalence of aneuploidy in each of these cell types. *In vivo* competition assays could also be performed with cells from primary and secondary recipient mice to evaluate their relative fitness after serial transplantation. Additionally, to determine whether aneuploid cells are being actively eliminated in the hematopoietic lineage, we could inhibit apoptosis in HSCs by overexpressing an anti-apoptotic protein such as Bcl-2, transfer HSCs into a recipient mouse and then karyotype cells derived from BubR1$^{H/H}$ HSCs by single cell sequencing to determine whether aneuploid cells now become evident. Finally, evaluation of an additional CIN model in serial reconstitution– for example, mice harboring the Cdc20^{AAA} mutation (Li et al., 2009), a gain of function allele that is resistant to spindle assembly checkpoint inhibition – could be used to distinguish between the possibility that BubR1 itself has a role in reconstitution or whether chromosomal instability and the aneuploid cells that result from mis-segregation are responsible for the observed experimental outcomes.

Regardless of the reason for this discrepancy, this model will prove a useful and versatile tool for evaluating the effect of a wide range of aneuploidies on HSCs fitness. If we find

evidence for an active culling mechanism for aneuploid cells, this could also provide insight into mechanisms for organisms to detect aneuploid cells.

Testing the role of aneuploidy in leukemia and lymphoma

The reason why cancer cells are aneuploid remains a mystery. However, adaptation of this cellular system could provide a unique way to assess the effects of a range of aneuploid cells on tumorigenesis *in vivo*. HSCs can be transduced *in vitro* and then transplanted into irradiated recipients. If transduced with oncogenes, this system can be used to induce leukemias and lymphomas (Hemann et al., 2005; Schmitt et al., 2000; 2002).

As a proof of principle experiment, I transduced trisomy 16 fetal livers and fetal livers from wild type littermates with either GFP or a vector containing a mutant allele of human myc that is found in Burkitt's lymphoma followed by and internal ribosome entry site and GFP (Figure 2a) as in (Hemann et al., 2005). As this system is dependent on cell proliferation *in vitro* and trisomic cells have proliferation defects, it was important to determine whether this cell type would be amenable to retroviral infection. I transferred infected cells into lethally irradiated recipients and monitored them for several months. Indeed, I found I was able to induce a lymphoma-like disease in one recipient each of either wild type cells or trisomy 16 cells transduced with myc-T58A (Figure 2b, c). These mice succumbed at 84 days and 90 days after transfer, respectively. Both exhibited splenomegaly, with the mouse transferred with wild type cells displaying macroscopic infiltration into the liver, and the mouse transferred with trisomy 16 cells experiencing paralysis of the back legs. Thus, I was able to generate a hyperproliferation in these fetal liver-derived cells which exhibited some features of lymphoma cells (Adams et al., 1985; Hemann et al., 2005), demonstrating the feasibility of this model system. Although the initial throughput is low, leukemias and lymphomas are often transplantable and amenable to

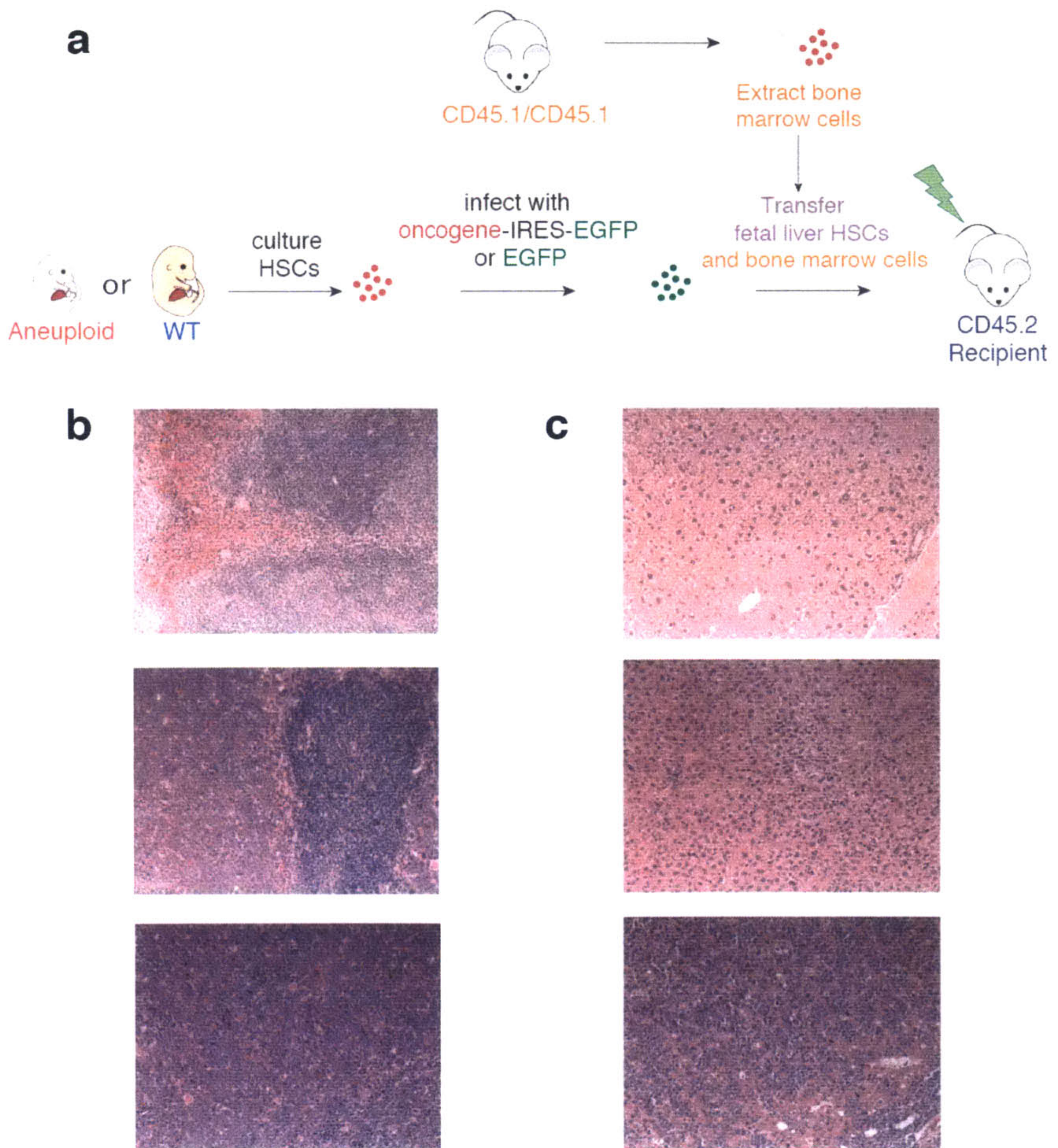

Figure 2. *Pilot experiment transducing trisomy 16 fetal livers with myc-T58A*

(a) Trisomy 16 or wild type litter mate fetal livers were cultured to permit retroviral infection of HSCs with an expression vector containing either EGFP or human myc-T58A-IRES-EGFP. After infection, cells were co-transferred into recipient mice with a small dose of wild type bone marrow cells to reduce lethality due to irradiation. Recipient mice were monitored over time for evidence of lymphoma. (b) Histology showing hematoxylin and eosin staining of spleens from a wild type mouse, the mouse receiving trisomy 16 myc-T58A, and the mouse receiving wild type myc-T58A. Infiltration of the spleen by lymphoma cells – particularly in the wild type sample – is evident by the expansion of highly pigmented blue cells. (c) Infiltration of lymphoma cells is evident in the wild type myc-T58A mouse (bottom), but is not detected in the wild type (top) or trisomy 16 myc-T58A (middle).

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manipulation in culture, making this a potentially powerful system to assess the role of cellular aneuploidy in tumorigenesis in the future. This system will greatly expand the range of our *in vivo* analysis model, allowing us to assess the combinations of many aneuploidies and oncogenes on the kinetics of transformation in leukemia and lymphoma in mice and enabling us to test the effect of aneuploidy-targeting drugs (Tang et al., 2011) in aneuploid blood cancers.

Concluding Remarks

Aneuploidy is a cellular state associated with human conditions characterized by both developmental defects and hyperproliferation. In this thesis, I have described my efforts to establish a system to assess the consequences of cellular aneuploidy *in vivo* using hematopoietic stem cell transplantation. Evaluation of three different types of aneuploid HSCs has demonstrated that relative fitness of aneuploid HSCs correlates with the size of the genome that is present in excess, a trend observed previously in several *in vitro* systems. However, the reasons why this fitness trend is observed *in vivo* are complex: some aneuploid cells are greatly impaired in rather specific ways, some are largely unaffected and some require repeated challenge to demonstrate fitness defects. Uncovering the reasons for why this range of

phenotypes is observed may provide molecular insights into the pathogenesis of Down syndrome hematopoietic perturbations and excitingly has the potential to reveal aneuploidy surveillance mechanisms in a highly selective organ system. I hope that future work using this system as both a means to characterize new aneuploid models and to assess the role of aneuploidy in cancer will provide us with a deeper knowledge of the impact of aneuploidy on cellular growth and physiology *in vivo* and enable systematic dissection the impact of aneuploidy on tumorigenesis in hematologic cancers to better clarify the precise role of aneuploidy in cancer.

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Appendix: Effects of backcrossing on trisomic embryonic lethality

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Gropp, A. (1982). Value of an animal model for trisomy. Virchows Arch a Pathol Anat Histol 395, 117–131.

Pfau, SJ and A. Amon. "A system to study aneuploidy *in vivo*." (2015) Cold Spring Harb Symp Quant Biol. 80. *In press.*

Introduction

To generate trisomic embryos, we employ a genetic system that relies on Robertsonian translocations (described in Chapter 1). This breeding scheme was originally developed by Alfred Gropp and colleagues (Gropp et al., 1972). Gropp and colleagues discovered that feral house mice caught in Switzerland had 26 chromosomes instead of the 40 chromosomes typically associated with the species (Gropp et al., 1969). This mouse, called the "tobacco mouse" because of its dark brown coat color, was initially thought to be a different species due to its different number of chromosomes and its reduced fertility when mated with laboratory strains. However, further analysis of the chromosomes by cytogenetics and matings with other wild caught mouse populations revealed that these mice had the same complement of chromosomes as other mice but that it was comprised in part by 7 Robertsonian translocations (Gropp et al., 1972; Hsu and Benirschke, 1970). A number of other Robertsonian translocations have since been isolated from wild caught feral mouse populations in a number of regions in Europe, Africa and Asia (Capanna and Castiglia, 2004), enabling the isolation of most combinations of Robertsonian translocations for laboratory use.

Initial characterization of all possible trisomic mice generated using the Robertsonian translocations revealed that this condition is generally embryonic lethal, with different trisomies experiencing lethality at different developmental stages ((Gropp, 1982), Figure 1). However, this group did observe live birth of some animals trisomic for chromosomes 16, 18 and 19.

All of these studies were performed on a wild hybrid background: they were outcrossed to laboratory strains to isolate specific chromosomes. Such mating procedures typically result in "hybrid vigor," or heterosis, a condition of increased fitness due to heterozygosity of alleles at many loci (Birchler et al., 2006). While this system makes study of trisomic embryos easier

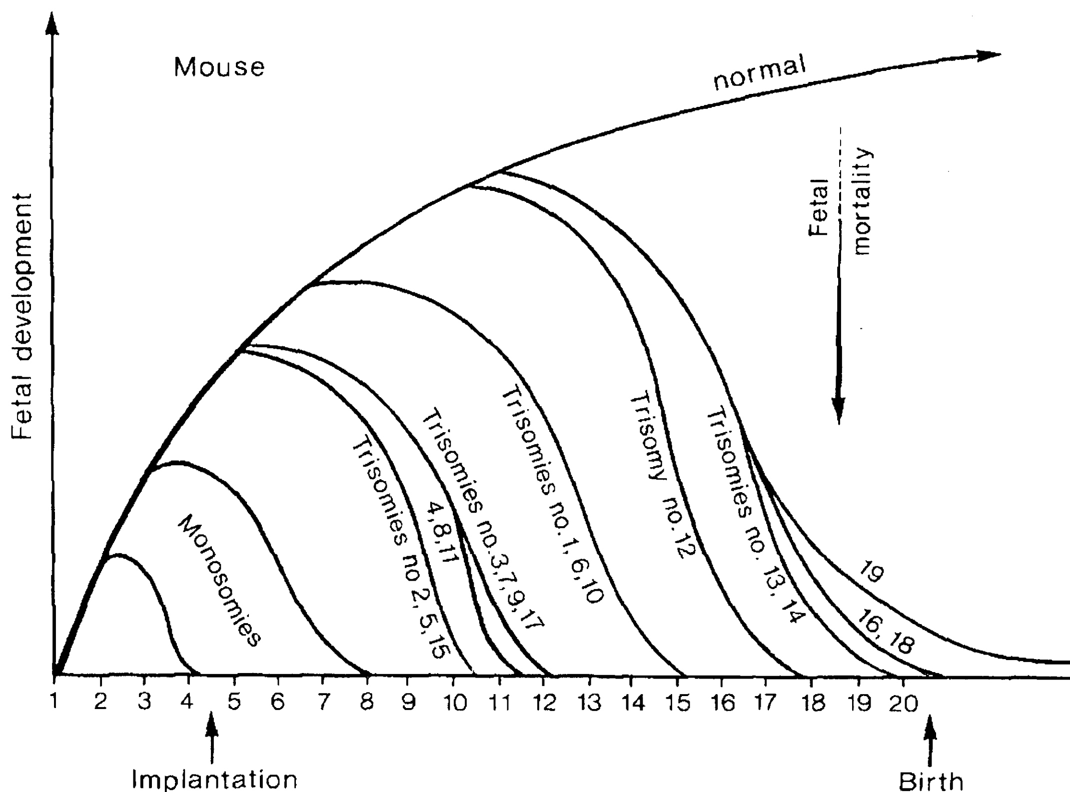

Figure 1. *Embryonic lethality of original trisomic strains* (reproduced from Gropp, 1982)

Characterization of trisomic mouse strains isolated using specific combinations of Robertsonian chromosomes revealed that trisomy is generally embryonic lethal in mice. Different trisomies have different embryonic survival times depending on the chromosome, and initial studies observed live birth of three trisomic strains.

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because of increased fitness, it can make further genetic study of the progeny of these strains difficult to interpret due to a wide range of genetic variability (Simpson et al., 1997). Mating strategies used to reduce this variability include the use of inbred syngeneic strains, or mouse lines that have been maintained for hundreds of generations by breeding siblings (Silver, 1995). This results in homozygosity of all alleles in the genome – except when *de novo* mutations arise – but also results in decreased fitness, called an inbreeding depression (Charlesworth and Charlesworth, 1987). This decreased fitness is thought to be due to, among other things, fixation

of deleterious alleles in the strain background and a reduced ability to respond to environmental challenges. Thus, backcrossing, or crossing mice carrying a genetic marker of interest with mice of an established inbred strain for many filial generations, reduces genetic variability of strains – making phenotypes easier to interpret – but also results in decreased fitness.

Our lab undertook the effort of backcrossing a number of mouse strains harboring Robertsonian translocations into the inbred mouse strain C57BL/6J. While it would reduce the genetic variability in all of our experiments, it was important to perform backcrossing for my research project in particular because of graft-versus-host disease. Graft-versus-host disease occurs during bone marrow transplantation when donor-derived T cells recognize the recipient as "non-self" and begin attacking recipient cells (Shlomchik, 2007), often resulting in death or a chronic autoimmune-like condition following transplantation. While this is a major problem in human bone marrow transplants, the use of inbred strains eliminates this issue in mice by using syngeneic donors. Therefore, backcrossing the Robertsonian strains into an inbred line was especially important for the reconstitution experiments: it both reduces lethality and ensures the phenotypes observed are due to differences in the donor HSCs and are not a consequence of chronic graft-versus-host disease. However, backcrossing also led to generally reduced fitness in the trisomic embryos derived from the Robertsonian strains. To evaluate the impact of backcrossing on trisomic embryo fitness, I documented the results of my efforts to isolate embryos by timed matings for trisomies 13, 16 and 19.

Results

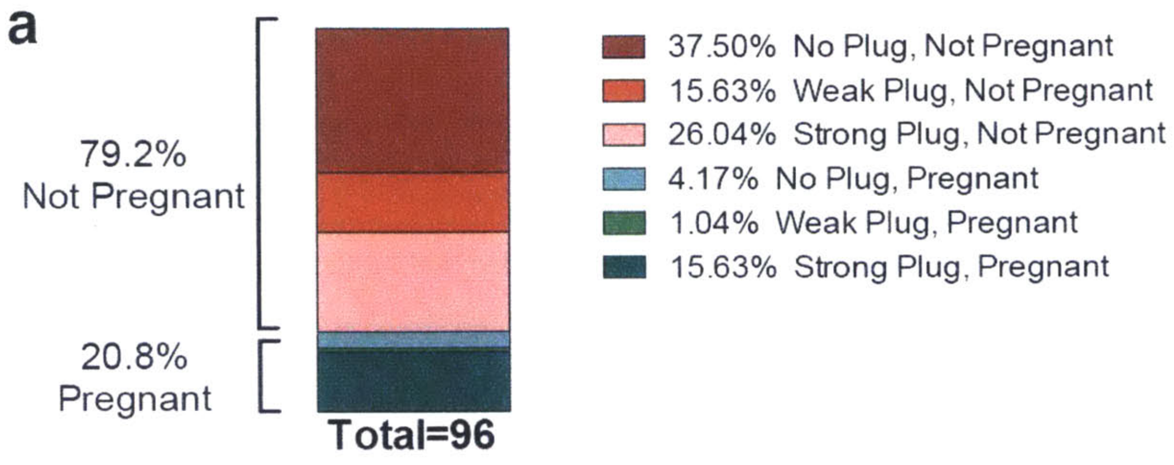
Timed matings were employed to harvest trisomic embryos at a developmental stage where hematopoietic stem cells could be isolated from fetal livers at E13.5 or E14.5. Matings are

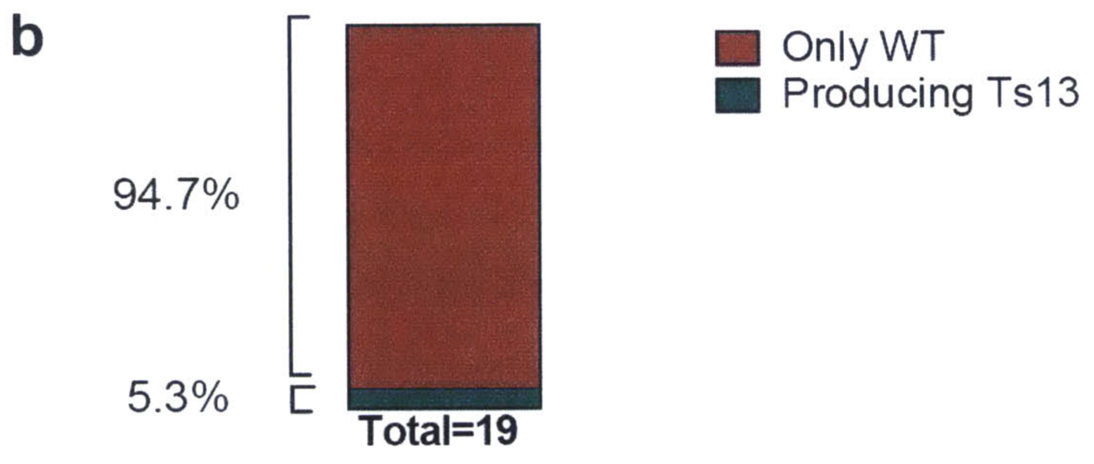
timed by observation of a vaginal mating plug in the morning, and embryos were dissected from females that appeared pregnant 13 to 14 days later.

Trisomic embryos can be distinguished by some major morphological characteristics: trisomic embryos are hypomorphic and generally paler than their wild type littermates and exhibit nuchal edema, a build-up of fluid on the spine (Gropp et al., 1983; Williams et al., 2008). Thus, trisomic embryos can be immediately distinguished from their wild type littermates by morphological observation (although karyotype of embryos deemed trisomic is always later confirmed by chromosome spreads and qPCR as described in Chapter 2). Embryos that die mid-gestation are resorbed into circulation over the course of a few days and can be detected in the uterine horn during this time (Flores et al., 2014). A previous study detected on average about 1.5 resorbed embryos between E13.5 and E15.5 in 60% of 3-7 month old C57BL/6J female mice mated with wild type C57BL/6J mice (Holinka et al., 1979), and C57BL/6J average litter size at birth is 6.2 pups (Nagasawa et al., 1973). Because trisomy is embryonic lethal over a range of a few days (Figure 1), resorptions are frequently observed when isolating trisomic embryos. To determine the range of embryonic lethality after backcrossing, I tabulated the number of wild type, trisomic and resorbed embryos from each mating.

Trisomy 13 embryos were not reliably isolated

Male mice that were compound heterozygotes for the 11.13 and 13.16 Robertsonian translocations were mated with wild type C57BL/6J females. These matings were not very productive (Figure 2). Most matings did not result in pregnancy, even though ~58% resulted in a visible mating plug (Figure 2a). Only one trisomy 13 embryo was isolated from the pregnant females that resulted (Figure 2b); thus adoptive transfer of trisomy 13 was not performed in this


a
79.2%
Not Pregnant
20.8%
Pregnant
Total=96
37.50% No Plug, Not Pregnant
15.63% Weak Plug, Not Pregnant
26.04% Strong Plug, Not Pregnant
4.17% No Plug, Pregnant
1.04% Weak Plug, Pregnant
15.63% Strong Plug, Pregnant


b
94.7%
5.3%
Total=19
Only WT
Producing Ts13

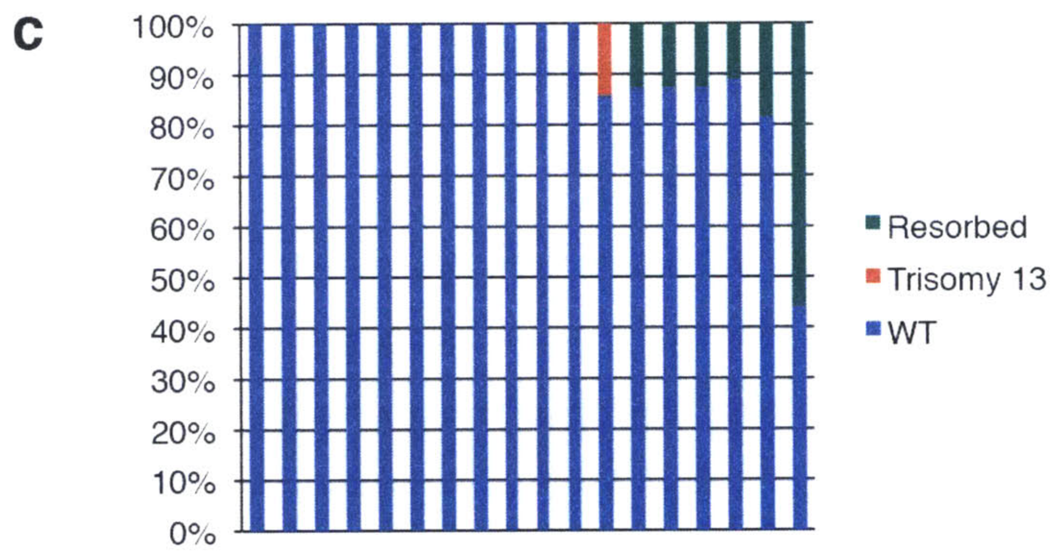

c
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Resorbed
Trisomy 13
WT

Figure 2. *Timed matings for trisomy 13 were not productive*

We aimed to isolate trisomy 13 embryos at E13.5 (a) Timed matings with 11 different 11.13/13.16 compound heterozygous males yielded very few pregnancies, despite producing a strong or weak mating plug nearly 60% of the time. (b) Of the females that became pregnant as a result of the timed mating, only 5.3% of these pregnant females (1/19) produced a trisomy 13 embryo between E12.5 and E15.5. (c) Of the 19 pregnancies observed, 12 pregnant females produced only wild type embryos with no evidence of embryo resorption, 6 pregnant females had both wild type embryos and resorbed embryos, and 1 pregnant female produced wild type embryos and one trisomy 13 embryo with no resorptions.

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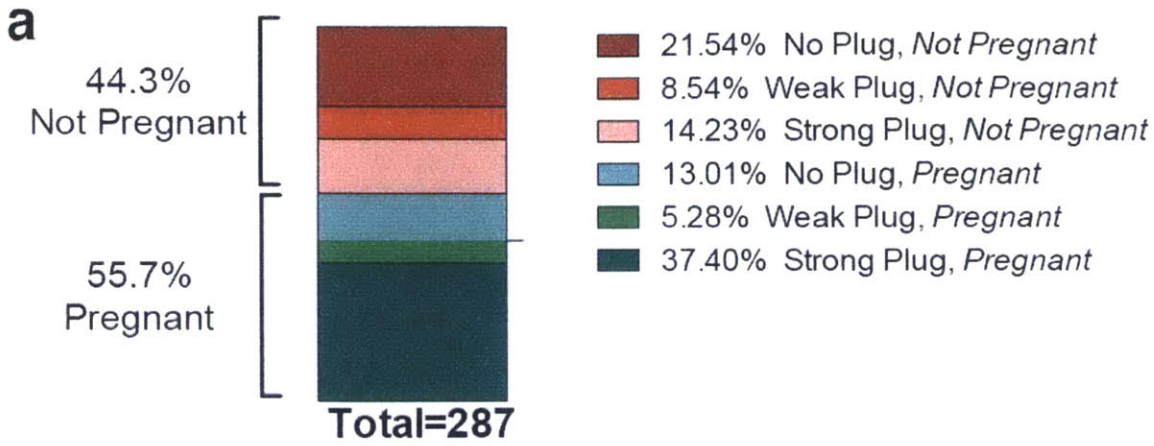
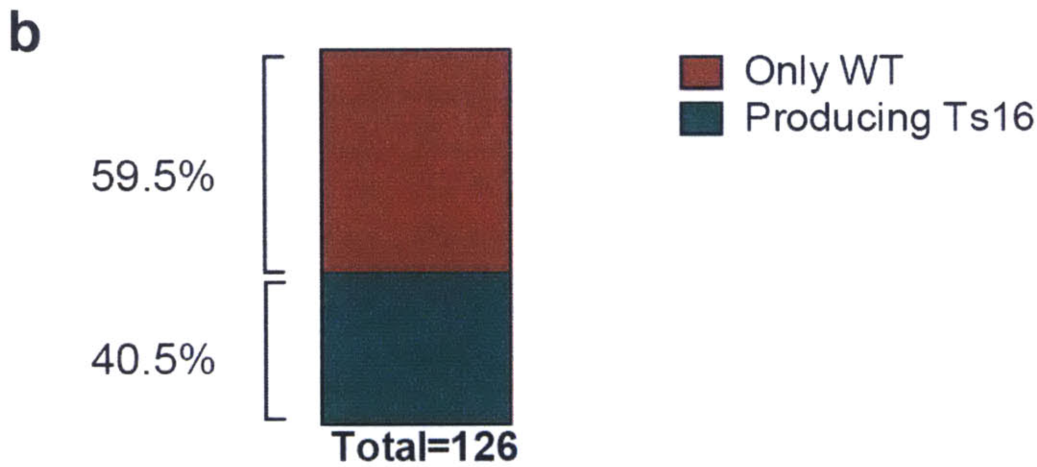
study. Analysis of the number of wild type, trisomic and resorbed embryos isolated from each pregnant female reveals no evidence of resorption in 11/19 pregnancies (Figure 2c). The average litter size including resorptions from these pregnancies was 7.3, and litters with resorptions had on average 1.83 resorptions per litter (range 1-5). Because trisomy 13 was only isolated from ~5% of pregnant females, the absence of resorptions in most pregnant females suggests that backcrossing of may have moved the mid-point of embryonic lethality for trisomy 13 to earlier than E13.5. This is a significant decrease in fitness, as initial characterization of trisomy 13 found that the mid-point of embryonic lethality for this strain was around E17.5 (Figure 1). However, this decreased production of trisomy 13 embryos could also be due to several other factors specific to these particular Robertsonian translocations (see Discussion).

Trisomy 16 embryos have decreased fitness

Male mice that were compound heterozygotes for the 6.16 or 13.16 and 16.17 Robertsonian translocations were mated with wild type C57BL/6J females. About 56% of matings resulted in pregnancy (Figure 3a), and of those pregnancies, about 41% resulted in litters with trisomy 16 embryos (Figure 3b). Analysis of the number of wild type, trisomic and resorbed embryos isolated from each pregnant female that produced embryos between the ages of E13.5


a
44.3%
Not Pregnant
55.7%
Pregnant
Total=287
21.54% No Plug, Not Pregnant
8.54% Weak Plug, Not Pregnant
14.23% Strong Plug, Not Pregnant
13.01% No Plug, Pregnant
5.28% Weak Plug, Pregnant
37.40% Strong Plug, Pregnant


b
59.5%
40.5%
Total=126
Only WT
Producing Ts16

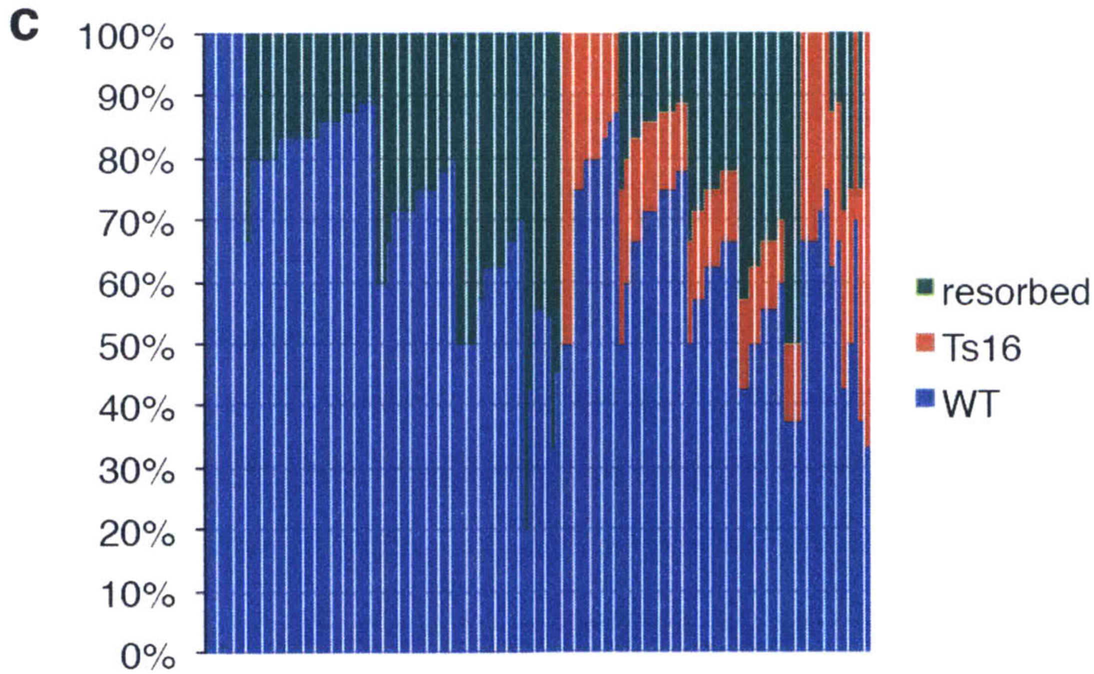

c
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
resorbed
Ts16
WT

Figure 3. *Timed matings for trisomy 16 reveal shift in embryonic lethality*

We aimed to isolate trisomy 16 embryos at E14.5 (a) Timed matings with 25 different 6.16/16.17 or 13.16/16.17 compound heterozygous males yielded resulted in pregnancy about 56% of the time. (b) Of the females that became pregnant as a result of the timed mating that produced embryos between E13.5 and E15.5, about 40% yielded trisomy 16 embryos. (c) These pregnant females produced resorbed embryos in nearly all cases.

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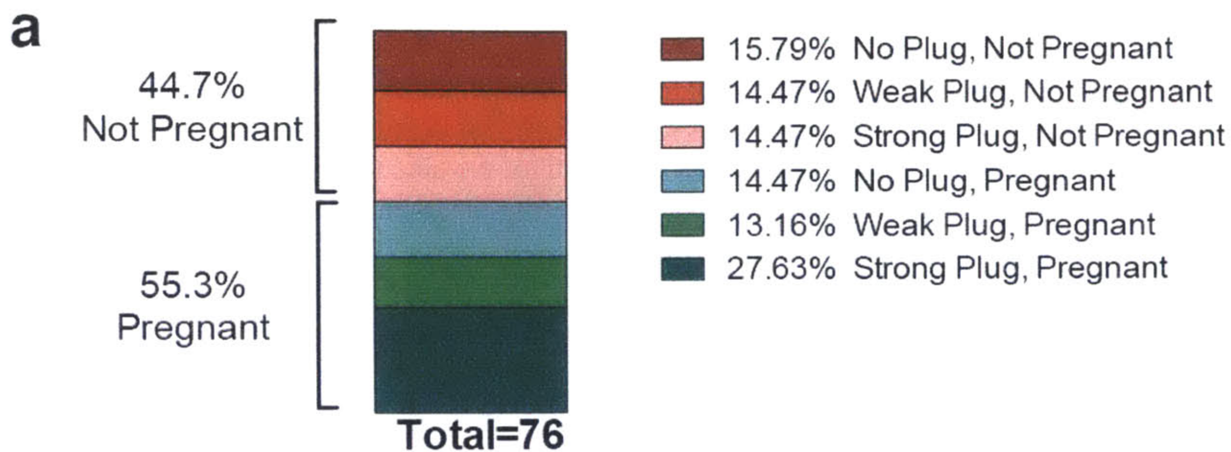
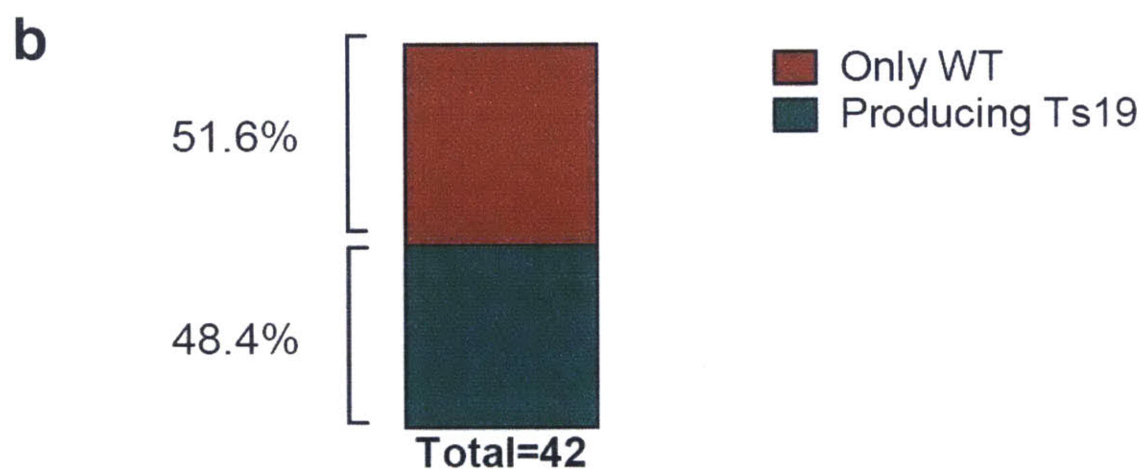
and E15.5 reveals resorption in nearly all pregnancies, with slightly less than half of litters producing trisomy 16 embryos (Figure 3c). The average litter size including resportions for these pregnancies was 6.6, and litters with resorptions had on average 2.1 resorptions per litter (range 1-6). This result argues that backcrossing may have moved the mid-point of embryonic lethality

to around E14.5. This is also a significant decrease in fitness. Although the mid-point of embryonic lethality in trisomy 16 observed previously was about E17.5 (Figure 1), the authors report observation of trisomy 16 at birth (Gropp et al., 1974).

Trisomy 19 embryos also show decreased fitness

Male mice that were compound heterozygotes for 5.19 and 9.19 Robertsonian translocations were mated with wild type C57BL/6J females. About 55% of matings resulted in pregnancy (Figure 4a), and of those pregnancies, about 48% resulted in litters with trisomy 19 embryos (Figure 4b). Analysis of the number of wild type, trisomic and resorbed embryos isolated from each pregnant female that produced embryos between the ages of E13.5 and E15.5 reveals resorption in most pregnancies, with about half of litters producing trisomy 19 embryos (Figure 4c). The average litter size including resportions for these pregnancies was 7.3, and litters with resorptions had on average 2.25 resorptions per litter (range 1-5). This result argues that backcrossing may have moved the mid-point of embryonic lethality to slightly later than


a
44.7%
Not Pregnant
55.3%
Pregnant
Total=76
15.79% No Plug, Not Pregnant
14.47% Weak Plug, Not Pregnant
14.47% Strong Plug, Not Pregnant
14.47% No Plug, Pregnant
13.16% Weak Plug, Pregnant
27.63% Strong Plug, Pregnant


b
51.6%
48.4%
Total=42
Only WT
Producing Ts19

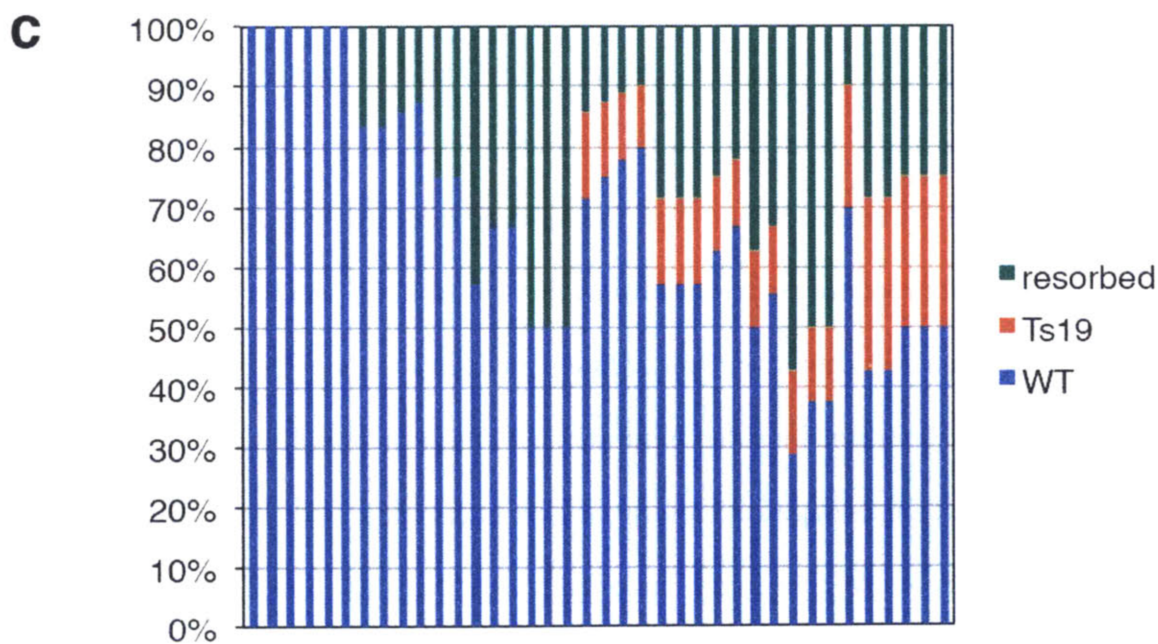

c
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
resorbed
Ts19
WT

Figure 4. *Timed matings for trisomy 19 reveal shift in lethality*

We aimed to isolate trisomy 19 embryos at E14.5 (a) Timed matings with 15 different 5.19/9.19 compound heterozygous males yielded resulted in pregnancy about 55% of the time. (b) Of the females that became pregnant as a result of the timed mating that produced embryos between E13.5 and E15.5, about 48% yielded trisomy 19 embryos. (c) These pregnant females produced resorbed embryos in most cases.

E14.5. Again, this is a significant decrease in fitness. The mid-point of embryonic lethality in trisomy 19 observed previously was about E17.5 (Figure 1); however, the authors also report numerous observations of trisomy 19 at birth (Gropp et al., 1974).

Discussion

The breeding data presented here indicates that backcrossing Robertsonian chromosomes into the C57BL/6J background has led to a shift in the mid-point of embryonic survival for trisomies 13, 16 and 19. This can make generation of trisomic fetal liver cells challenging, especially given that trisomy 16 and 19 fetal livers on average yield about 50% and 70% fewer cells than the livers isolated from their wild type littermates, respectively (data not shown).

While backcrossing likely led to a decrease in fitness, there are a number of factors that could have also influenced this shift in survival in addition to inbreeding. If trisomy segregated in a Mendelian fashion, we would expect about 1/3 of embryos surviving to the later stages of embryonic development to be trisomic (monosomies die very early in development, see Figure 1). However, variable rates of chromosome mis-segregation were observed in the original hybrid strains both for different Robertsonian translocations and in male and female mice ranging from 4 to 26% (Gropp et al., 1974). We have not evaluated the rate of chromosome mis-segregation in these strains after backcrossing; however, the presence of multiple resorbed embryos in most litters suggests that trisomic embryos are being produced. Additionally, we chose to use male

compound heterozygotes to generate trisomic embryos even though their chromosome mis-segregation rate is lower than females because they could be mated multiple times. However, recent breedings with female compound heterozygotes harboring the 11.13 and 13.16 Robertsonian chromosomes has successfully yielded several trisomic embryos at E13.5 (Pei-hsin Hsu, personal communication), suggesting that mis-segregation rate – or male sterility – in these compound heterozygotes may be an additional factor limiting embryo production. Thus, more analysis of trisomy 13 is warranted to more precisely evaluate the mid-point embryonic lethality of this aneuploidy.

Additionally, we did not attempt to isolate embryos at earlier or later stages, even though our results indicate that about half of pregnancies for trisomies 16 and 19 between E13.5 and E15.5 produce trisomic embryos while nearly all contain resorbed embryos. Thus, our data provide us with only an estimate for embryonic survival. We do not know how many trisomic embryos would be observed after E15.5 and before E13.5. Yet, it is also possible that backcrossing has led to a narrowing of the window of lethality due to reduced genetic variability. Regardless, these data demonstrate that backcrossing of the Robertsonian chromosomes has led to decreased embryonic fitness. Furthermore, additional histological analysis of these embryos could reveal more defined causes of the embryonic lethality, as backcrossing may have reduced some of the genetic variability that was present in initial characterizations.

Materials and Methods

Mouse strains

Strains used to generate trisomic embryos were B6.Cg-Rb(11.13)4Bnr/JAmonJ and B6.Cg-Rb(13.16)1Mpl/JAmonJ (Trisomy 13), B6.Cg-Rb(6.16)24Lub/JAmonJ or B6.Cg-Rb(13.16)1Mpl/JAmonJ and B6.Cg-Rb(16.17)7Bnr/JAmonJ (Trisomy 16) and B6.Cg-Rb(5.19)1Wh/JAmonJ and B6Ei.Cg-Rb(9.19)163H/J (Trisomy 19). B6Ei.Cg-Rb(9.19)163H/J was backcrossed into the C57BL/6EiJ background. All other strains were backcrossed at least 10 times into the C57BL/6J background.

Timed matings of compound heterozygous males

Compound heterozygous males were mated with young C57BL/6J females. The mice were mated for 4 consecutive nights, and females were monitored every morning for evidence of a mating plug. After the fourth night, the mice were separated. Evidence of pregnancy was evaluated between 6 and 10 days after separation, and embryos were isolated from mice that appeared pregnant. Female mice that did not appear pregnant were re-mated with a different male at least 28 days after initial mating date, and male mice were kept solitary at least one week per month.

Isolation of trisomic embryos

Embryos from pregnant females were isolated at described previously (Williams et al., 2008). MEFs were made from mice that were identified as trisomic, and these cells were karyotyped by metaphase spreads and qPCR as described in Chapter 2.

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