Dottorando: Francesca FAGGIOLI

Single-cell analysis of ploidy and centrosomes underscores the peculiarity of normal hepatocytes

Direttore della Scuola: Ch.mo Prof. Mario CLERICI

Tutore: Prof. Cristina BATAGLIA

Correlatore: Prof. Paolo Maria VEZZONI

Albert Einstein College of Medicine, New York
Istituto clinico Humanitas, Rozzano, Milano
Alla mia famiglia

“Only the ideas that we really live have any value”

Hermann Hesse
ABSTRACT

STATE OF ART: The literature reports that, contrary to most other cell types, adult hepatocytes are polyploid cells with a DNA content of 4, 8 or even 16 haploid genomes. In fetal and early neonatal life, hepatocytes are mononucleated diploid cells that, quite abruptly, become binucleated and polyploid soon after weaning. The generation of tetraploid intermediates is not an uncommon event in the liver. These cells have the potential to generate aneuploid progeny in the subsequent cell division, because of the presence of four centrosomes. Normally in diploid cells, at the beginning of mitosis, a single centrosome duplicates and the mother and daughter organelles migrate to opposite cell poles, directing the formation of the spindle, to guarantee a balanced chromosomal segregation. Four centrosomes can cluster together, mimicking a bipolar spindle, or, as reported for tumoral cells, act as single entities that generate multipolar spindle. The result of a multipolar division is a progeny with an unbalanced DNA content, differing in 1 or a few chromosomes. The formation of aneuploid progeny in hepatocytes has never been supported by experimental evidence for two reasons: 1) the classical approaches chosen to assess the hepatocyte ploidy lack the sensitivity to detect the small differences in DNA content that result from unbalanced chromosomal segregation; in addition, a quantitative and behavioural analysis of centrosomes in normal liver cells has not been thoroughly investigated; thus the presence of extranumerary centrosomes (i.e., more than four) has never been postulated 2) The detrimental effects commonly attributed to aneuploidy made difficult to only hypothesize that a highly regenerative tissue such as the liver can contain aneuploid cells. It is thus of interest to note that some tissues are naturally aneuploid, like the embryonic brain, and in these tissues chromosome instability confers advantages properties to the cells.

AIM: The main goal of my present work is to determine whether aneuploidy is a common feature of hepatocytes in physiological conditions. To address this goal I performed a quantitative analysis of the DNA content in normal hepatocytes during liver development and adulthood (at 18.5 post coitum, at 15 days, 1.5 months, 4 months) combined with a quantitative and behavioural analysis of centrosomes.

MATERIALS AND METHODS: I applied a novel approach employing a 2-color FISH on interphase cells that provides highly quantitative and reproducible polyploidy data for individual chromosomes by assessing ploidy of a cell based on a comparison between an autosome (17 or 18) and a sexual chromosome (Y). I used a double staining for centrosome associated proteins to assess the number of centrosomes at different time point and I combined this approach to the interphase FISH to determine a correspondence between the DNA content and the number of centrosomes.

RESULTS: I have demonstrated that aneuploidy and unbalanced DNA content in binucleated hepatocytes are common features of the normal adult liver. Despite the common belief that hepatocytes contain 1, 2 or no more than 4 centrosomes, our double staining for centrosome associated proteins reveals extranumerary centrosomes in a high percentage of cells as early as 15 days of age. I showed that in mice the period between 15 days and 1.5 months marks the transition from a liver with a prevalence of mononucleated cells to a liver with up to 75% of binucleated cells. My data demonstrate that this timing correlates with a switch of
specific centrosomes numbers. At 15 days, in addition to cells that show the expected number of centrosomes (1 or 2), we also found several hepatocytes with 3 centrosomes; at 1.5 months the percentage of cells with 3 centrosomes decreased concomitantly with the increase of cells with more than 4 centrosomes. My analysis shows that the number of extranumerary centrosomes develops in concomitance with the process of binucleation and polyploidization. In addition, supernumerary centrosomes maintain the ability to nucleate α-tubulin, one of the main components of the cytoskeleton and of the mitotic spindle. This observation is intriguing based on the knowledge that adult hepatocytes are commonly considered to reside in G0 phase. Finally, by integrating interphase FISH and immunofluorescent approaches, we detected an imbalance between centrosome number and DNA content in liver cells that deviates from the equilibrium expected in normal cells.

CONCLUSIONS: We demonstrated that half of the mature hepatocytes in mice are aneuploid. This discovery could have a different impact to several fields. On one hand it provides new insights on the role of aneuploidy in adult somatic tissues. Thus, the low tumorigenicity of liver suggests that this unique feature is relevant to the peculiar biological function of hepatic cells, which are continuously challenged by metabolic stress and other insults rather than a cause of tumor formation. We can speculate that the liver, with its high level of aneuploidy detected consistently overall the ages analyzed can be pictured as a store of well tolerated genetic heterogeneity. In response to toxic stresses and diseases the liver may select the more beneficial chromosomal pattern to promote cellular fitness against cell deterioration. On the other hand, not less important is the contribution of this discovery to the field of liver cell therapies with mature hepatocytes and the consequences of aneuploidy after transplantation.
SOMMARIO

SITUAZIONE INIZIALE: La letteratura riporta, che contrariamente alla maggior parte dei tipi cellulari, gli epatociti adulti sono cellule poliploidi con un contenuto di DNA pari a 4, 8 o anche 16 genomi aploidi. Durante lo sviluppo fetale e nella vita neonatale, gli epatociti si presentano come cellule mononucleate diploidi, che abbastanza bruscamente, diventano binucleate e poliploidi subito dopo il periodo di svezzamento. Una delle peculiarità del fegato è la presenza di intermedi tetraploidi. Queste cellule hanno il potenziale di generare progenie aneuploide nelle successive divisioni cellulari, a causa della presenza di quattro centrosomi. Normalmente in cellule diploidi, all'inizio della mitosi, un singolo centrosoma si duplica, a ciò segue la migrazione dei due organelli, madre e figlia, ai lati opposti della cellula, dove andranno ad organizzare la formazione del fuso mitotico e garantiranno una corretta segregazione dei cromosomi. Quattro centrosomi possono formare dei cluster, mimando una divisione bipolare, oppure, come è già stato descritto per le cellule tumorali, agire come singole entità e generare un fuso mitotico multipolare. Il risultato di questa divisione è una progenie con una quantità di DNA non bilanciata, che differisce di uno o più cromosomi dall'atteso contenuto diploide. La formazione di progenie aneuploide nel fegato non è mai stata documentata sperimentalmente per due motivi: 1) gli approcci classici utilizzati nel determinare la ploidia degli epatociti mancano della sensibilità necessaria per rilevare piccole differenze di contenuto di DNA, come i singoli cromosomi, che derivano da una segregazione non bilanciata; inoltre un'analisi quantitativa e funzionale dei centrosomi e quindi dell'eventuale presenza di extra-centrosomi non è mai stata realizzata nel fegato in condizioni fisiologiche, tanto da supporre eventi di segregazione non corretti; 2) Gli effetti negativi comunemente legati al concetto di aneuploidia hanno reso difficile anche solo ipotizzare che un tessuto altamente rigenerativo come il fegato possa contenere delle cellule aneuploidi. Ma è certamente importante sottolineare che non è strano trovare cellule aneuploidi in condizioni fisiologiche; alcuni tessuti sono naturalmente aneuploidi, come il cervello durante lo sviluppo embrionale, e in quei tessuti l'instabilità dei cromosomi addirittura conferisce dei vantaggi alle cellule, invece che creare un danno.

SCOPO: Il principale obiettivo del mio presente lavoro è quello di determinare se l'aneuploidia è una caratteristica tipica degli epatociti in condizioni fisiologiche. Per rispondere a questo quesito io ho realizzato un'analisi quantitativa del contenuto di DNA in epatociti in condizioni fisiologiche durante lo sviluppo del fegato e durante l'età adulta considerando diversi time points, quali i 18.5 giorni post coitum, 15 giorni, 1.5 mesi e 4 mesi. Gli stessi time points sono stati utilizzati per un'analisi quantitativa e funzionale dei centrosomi.

MATERIALI E METODI: Il mio approccio nel determinare la ploidia degli epatociti consiste nell'utilizzo di una FISH a due colori applicata a cellule in interfase, perché basata sul confronto tra i segnali derivanti da un autosoma (17 o 18) e un cromosoma sessuale (Y) applicati all'interno della stessa cellula. Per stabilire il numero di centrosomi nei diversi time points ho utilizzato un doppio staining, costituito dall'uso contemporaneo di due proteine diverse che co-localizzano. Inoltre ho combinato questo staining con la FISH in interfase per determinare sulle
stesse cellule una corrispondenza tra il contsenuto di DNA e il numero di centrosomi corrispondente.

**RISULTATI:** Per la prima volta abbiamo dimostrato che l'aneuploidia e un contenuto genetico non bilanciato negli epatociti binucleati sono caratteristiche comuni nel fegato adulto che non presenti uno stato patologico. Nonostante che si ritenga comunemente che gli epatociti contengano 1, 2 o non più di 4 centrosomi, il nostro duplice staining per due diverse proteine strutturali del centrosoma ha mostrato che un elevato numero di cellule contiene extra- centrosomi anche in giovane età (15 giorni). Abbiamo dimostrato che nel topo la fascia temporale tra i 15 giorni e 1.5 mesi di età rappresenta una fase di transizione da un fegato popolato prevalentemente da cellule mononucleate a un tessuto composto fino al 75% di cellule binucleate. I nostri dati dimostrano che questo passaggio è correlato con un cambiamento specifico anche nel numero dei centrosomi. A 15 giorni, assieme a cellule con 1 o 2 centrosomi ce ne sono numerose che presentano già un numero insolito, 3 centrosomi; a 1.5 mesi la percentuale di cellule con 3 centrosomi diminuisce assieme a un concomitante aumento di cellule con più di 4 centrosomi. Questo significa che l'acquisizione di centrosomi sovrannumerari è correlata con il processo di binucleazione e poliploidizzazione. In aggiunta, gli extra-centrosomi trovati nel fegato di topo adulto hanno dimostrato di mantenere la capacità di nucleare α-tubulina, uno dei maggiori componenti del citoscheletro e del fuso mitotico. Quest’ultimo dato è particolarmente interessante se si pensa che gli epatociti adulti sono da sempre noti come cellule quiescenti, che risiedono in fase G0. Infine, l'integrazione di due approcci sperimentali, quali la FISH in interfase e l'immunofluorescenza, ci ha permesso di rilevare una mancata corrispondenza tra il numero di centrosomi e il supposto contenuto di DNA, a dimostrazione che anche in cellule normali le regole di duplicazione dei centrosomi e di sintesi di DNA non vengono sempre rispettate. Questi nuovi risultati ci spingono a credere che a questa unicità di struttura corrisponda una complessa funzione biologica delle cellule epatiche, che sono continuamente sottoposte a stress, una condizione che potrebbe predisporre a instabilità genetica.

**CONCLUSIONI:** Con questo lavoro abbiamo dimostrato che la metà degli epatociti maturi sono aneuploidi. L'elevata percentuale di cellule aneuploidi nel tessuto epatico adulto in condizioni fisiologiche conferisce un nuovo significato al ruolo giocato dall’aneuploidia nei tessuti somatici adulti. Infatti la bassa tumorigenicità del fegato suggerisce che questi alti livelli di aneuploidia siano più da interpretare come una caratteristica unica e peculiare della biologia delle cellule epatiche, che sono continuamente sottoposte a stress metabolico e ad altri tipi di insulti, piuttosto che come causa di trasformazione cancerosa. A questo proposito possiamo avanzare l’ipotesi che il fegato sia un magazzino di cellule altamente eterogenee dal punto di vista genetico, il che comprenderebbe alti livelli di aneuploidia ben tollerata. A seguito di stress ed eventi patologici il fegato potrebbe selezionare il corredo cromosomico più vantaggioso in grado di promuovere in quelle circostanze la salute della cellula o del tessuto contrastando il danno tissutale o cellulare inferto. D’altro canto non meno importante è il contributo di questa scoperta al settore delle terapie cellulari del fegato eseguite con epatociti maturi e alle conseguenze dell’aneuploidia dopo trapianto.
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ABBREVIATIONS

FISH= Fluorescence in situ hybridization;
RES= Reticuloendothelial system;
TGF= Trasforming growth factor alpha;
EGF= Epidermal groth factor;
HGF= Hepatocyte growth factor;
FAH= Fumarylacetoacetate hydrolase;
FACS= Fluorescence-activated cell sorting;
PH= Partial hepatectomy;
LEC= Long Evans Cinnamon;
WD= Wilson’s disease;
SAC= Spindle assembly checkpoint;
APC= Adenomatous Polyposis Coli;
CIN= Chromosomal instability;
MEFs= Mouse embryonic fibroblasts;
ESR= Environmental stress response;
SILAC= Stable isotope labeling by amino acid;
MudPIT= multidimensional protein identification technology mass spectroscopy;
MVA= Mosaic variegated aneuploidy;
NTBC= 2-(2-nitro-4-trifluoro- methylbenzoyl)-1,3-cyclohexanedione;
HGD= homogentisic acid dioxygenase.
1. LIVER

1.1 Structure

The liver, as highly specialized tissue, regulates a wide variety of high-volume biochemical reactions with a major role in metabolism. Its functions include glycogen storage, decomposition of red blood cells, plasma protein synthesis, hormone production, detoxification and the synthesis and breakdown of small and complex molecules, many of which are necessary for normal vital functions. Hepatocytes are the chief functional parenchymal cells of the liver, contributing to roughly 80% of its mass. In three dimensions, hepatocytes are arranged in plates separated by vascular channels (sinusoids). The cells are polygonal in shape and very heterogeneous in size, with diameters ranging from 8 to 40 µm [1]. The remaining liver volume represented by non-parenchymal cells includes Kupffer cells, specialized macrophages that are part of the reticuloendothelial system (RES), hepatic stellate cells, producer of extracellular matrix and collagen and located among endothelial cells, and the sinusoidal endothelial cells. Liver is exceptionally active in synthesis of protein and lipids for export; among these, the bile secretion occurs through bile duct epithelial cells, that constitute the well-known biliary tree, developed within and outside the liver (Figure1).
Figure 1. Liver anatomy. A) The liver consists of four lobes. The Right lobe is by far the larger and the Left lobe, made up by multisided units called lobules. B) Each lobule consists of a central vein surrounded by tiny liver cells grouped in sheets or bundles. C) The vascular channels of the liver, called sinusoid, are lined by liver sinusoid endothelial cells (LSEC), which separate the sinusoid lumen from hepatocytes. They receive blood from the terminal branches of the hepatic artery and portal vein at the periphery of lobules and deliver it into central veins where the exchange of oxygen and nutrients occurs. Kupffer cells are specialized macrophages that patrol the sinusoids and bind to LSEC and occasionally hepatocytes through the gaps of two adjacent LSEC.

In common with few other tissues, including heart, muscle cells and platelet-progenitors megakaryocytes, the liver parenchyma (hepatocytes) develops a certain degree of polyploidy during its lifespan. Diploid cells contain two copies of the genome (2n), whereas polyploid cells show an increase in genome size caused by the inheritance of one or more additional sets of chromosomes. Indeed, by contrast to aneuploidy, the genomic state is balanced in polyploid cells.

Under physiological conditions, the rat liver develops from the ventral foregut endoderm in the form of epatoblasts, undifferentiated bipotential cells that retains the ability to divide and proliferate throughout life to provide progenitor cells that can differentiate into hepatocytes and cholangiocytes [2-7]. Starting from E14, during the remaining period of gestation and the first four post-natal weeks, hepatoblasts acquire functions of differentiated hepatocytes, correlated with the onset of polyploidy [8-9].

This process generates the successive appearance of mono and binucleated hepatocytes, with a DNA content that spans from a range of 2n, 4n, 8n to even greater DNA amounts [10-11]. Earlier reports have also identified in C3H mice the existence of nuclei in the liver with ploidies of 3n, 6n and 12n, avoiding the geometric $2^n$ progression proposed by Epstein and coworkers in 1967 [12]. Albeit the percentage of cells with intermediate ploidy was pretty high (in the first 2 weeks of life it amounts to 25 to 50%) [13], this phenomenon has been considered as a consequence of systematic errors possibly produced by the methods of preparation and measurement of the material, since these old reports were performed on sections of fixed tissues with micro-spectrophotometric methods that were less than optimal). In rodents, the hepatocyte ploidy level reaches a plateau three months after birth [14].
1.2 The reasons of mammalian liver polyploidy

Although most eukaryotic organisms are diploid, cells that have more than two chromosome sets are not exceptional. Polyploidy is a surprisingly common phenomenon in nature, suggesting that changes in DNA content may have an effect on the phenotype and the fitness of the cells. In particular, liver polyploidization is generally considered to indicate advancement toward differentiation and cellular senescence [15] and to lead to both progressive loss of cell pluripotency and markedly decreased replication capacity [16]. A study based on the analysis of fetal, suckling and adult rats, associated the decrease of DNA synthesis with the progression of polyploidy and binucleation. The incorporation of radiolabeled thymidine into DNA revealed that hepatocytes in newborns are exclusively diploid mononucleated cells with a considerable DNA synthesis (up to 18%) [17]. However this percentage decreases rapidly at weaning, with fewer of 5% nuclei being in the S phase. The predominance of binucleated hepatocytes (53% of the cells are at least tetraploid, with a 2n content in each nucleus) [18] in the adult young rat, with a mitotic index ranging from 0.10-0.40%, indicates that parenchymal cells lose their replicative potential upon the onset of terminal differentiation [15, 18]. Two alternative hypotheses may explain the causes of mammalian liver polyploidization: the theory of “economy in mitosis” and the theory of “metabolic stress defense”. According to the first hypothesis, the omission of mitosis is beneficial in rapidly growing and differentiating tissues that should early perform their specialized functions [19, 20]. As a matter of fact, in physiological conditions, the onset of polyploidy in the liver is clearly associated with weaning and assumption of independent feeding [21-23]. Hepatocyte polyploidy could be viewed as a cheap short term adaptation of selected animals allowing them to funnel additional resources in rapid development and reproduction, typical of short lived species. Indeed, the degree of polyploidization varies between mammals [24]. In the adult rat
the level of hepatocyte ploidy is as high as 80 to 90% [14], whereas in humans, the mean percentage of polyploid cells is 30 to 40% [25-26]. On the other side, the liver of mammals is known to be the place where many exo- and endogenous toxic chemical are processed and detoxicated. Therefore the additional gene copies provided through polyploidization could help the cells to express detoxifying genes to higher levels and survive under these conditions [27]. This hypothesis is grounded on the observation that short-lived species, that have been shown to have less effective DNA repair system [28-29], develop higher hepatocytes ploidy.

1.3 Induction of polyploidy in pathological states and in the regenerating liver

Unlike blood, skin and intestine, tissue maintenance in the liver is not driven by stem cells, but rather by division of the mature cells, hepatocytes and bile duct epithelial cells. Parenchymal cells lose their replicative potential upon the onset of terminal differentiation [18, 30]. Indeed, hepatocytes in the normal liver are quiescent (G0 phase) and exhibit only a minimal response to potent in vitro mitogens, such as transforming growth factor alpha (TGF), epidermal growth factor (EGF), and hepatocyte growth factor (HGF). However, the regenerative capacity of hepatocytes has been assessed in animal models of liver repopulation, in which transplanted cells have a selective advantage over the host [31]. Mature hepatocytes were transplanted into livers of FAH knockout mice, which lack fumarylacetoacetate hydrolase, an enzyme that disrupts tyrosine catabolism. The FAH mouse is an adequate model for hereditary tyrosinemia type I, a severe recessive autosomal metabolic disease. The disorder is characterized by severe liver and kidney defects [32]. After transplantation large, binucleated hepatocytes that represented 70% of the hepatocytes population were found to mediate most of the liver repopulation, to restore its biochemical function and to rescue the mouse
A second report still related to the FAH mouse model showed that fusion-derived hepatocytes, which by definition are at least tetraploid, after serial transplantation were capable of at least 30 cell divisions without loss of function [34]. To examine the process of liver repopulation by transplanted hepatocytes in a different mouse model, Weglarz and colleagues developed a mouse with an urokinase plasminogen activator transgene which induced diffuse hepatocellular damage beginning at 3 weeks of age. The transplantation of healthy donor hepatocytes into the liver of these mice was responsible for the parenchymal repopulation [35]. By use of such models, it has been shown that mature transplanted hepatocytes, that were sorted for a polyploid content by FACS (Fluorescence-activated cell sorting), have a stem cell–like regenerative capacity rivaling that of hematopoietic stem cells and are able to divide more than 100 times without loss of function [33]. It is important to note that the nature of the human cells that are capable of liver repopulation [36] has not been definitively determined. However, in human patients with hereditary tyrosinemia, the large size of clonal revertant nodules (i.e., healthy liver tissue derived from spontaneous hepatocyte mutations that correct the underlying genetic defect) indicates that mature human hepatocytes also have extensive regenerative potential [37].

The adult liver also involves the mitosis of mature cells in response to specific injuries. During liver regeneration, induced by two-thirds of hepatectomy (PH) in the rat, quiescent hepatocytes undergo one or two rounds of replication to restore the liver mass by a process of compensatory hyperplasia. The excised parts do not grow back, rather, the remaining liver expands in mass to compensate for the lost tissue. Many studies have shown that during this process hepatic polyploidy is modified [15, 38-41]. Indeed, regenerative liver growth differs markedly from developmental liver growth, the most striking difference being the rapid disappearance of binucleated hepatocytes. In rodents, after PH, the
proportion of binucleated cells decreases to less than 5%, while there is an overall increase in ploidy characterized by a decrease in 2n hepatocytes and an increase in 4n and 8n mononucleated hepatocytes. Interestingly, the diploid hepatocytes seem to have a higher tendency than the polyploid ones to undergo several rounds of division [40]. Moreover, after partial hepatectomy, polyploid hepatocytes exhibit senescence-type changes with increased lipofuscin accumulation, β-galactosidase activity [42] and accumulation of p21 [40]. Only when liver injury is combined with an inability of hepatocytes to divide in response to the damage, liver mass is replenished by the replication of the (intrahepatic) facultative progenitor cells, also known as oval cells [43-46]. Oval cells are not liver stem cells, rather bi-potential offsprings of stem cells that can differentiate into both bile duct and hepatocytes [47-49]. They also provide information about the lineage decision making in the liver [50-51]. Oval cells have high levels of phase II detoxifying enzymes, which may ensure their survival in the presence of toxic agents [52-53]; in the regeneration of the liver that follows loss of parenchymal cells and the delay of hepatocyte DNA synthesis induced by toxins, such as galactosamine, the oval cells proliferate and differentiate in mature hepatocytes.

Hepatic polyploidy can be also modified by metabolic overload that induces liver lesions. The Long Evans Cinnamon (LEC) rat bears a spontaneous mutation in the \textit{atp7b} gene, which is known to regulate the biliary copper excretion. The absence of the normal function of this gene leads to abnormal copper metabolism and, as a consequence, LEC rats develop hepatitis and liver cancer. LEC rats develop excessive copper in the liver, reduced excretion of copper into bile, a reduced level of serum copper and a remarkable decrease in serum ceruloplasmin activity [54-55], similar to patients with Wilson's disease (WD). In this animal model, hepatocytes present large polyploid nuclei and a delay in mitotic progression has been also observed [56-57]. Interestingly, in normal mice, the injection of iron-
dextran induces liver polyploidization; this effect is inhibited by the oral intake of iron chelator [58]. Oxidative damage to the liver is also associated with a pronounced increase in the population of polyploid hepatocytes. Gorla and coworkers have demonstrated that subsequent to radiation, hepatocytes exhibit evidence for oxidative injuries with elimination of intracellular anti-oxidants (as glutathione and catalase) and for increase of polyploidy [16, 59]. In the same line, another study has demonstrated that the over-expression of antioxidant enzymes (glutathione peroxidase, Cu, Zn-superoxide dismutase) in transgenic mice decreases hepatocyte ploidy during liver regeneration [60]. All these results agree with the fact that an extensive correlation exists between the generation of polyploid hepatocytes and a variety of cellular stress in the adult liver.

1.4 Several routes to polyploidy

One fascinating question is how diploid organisms develop polyploid cells. In a physiological or pathological state, polyploid cells can arise by any one of the following mechanisms: endoreplication, mitotic slippage, cytokinesis failure and cell fusion (Figure 2).

The term “endoreplication” indicates different mechanisms that arise from variation of the canonical G1-S-G2-M cell cycle [61]. One mechanism is addressed as “re-replication”, which results from the perturbation of the molecular mechanism that controls “ones and only ones” firing of replication. DNA synthesis is initiated multiple times at individual origins of replication within a single S phase, with an indistinct accumulation of DNA. In contrast, in the process of endomitosis, cells fail to complete the mitosis. After entering in mitosis, they condense the chromosomes but instead of proceeding in the segregation step, they enter into a G1-like state and re-enter into the S phase. The best-studied example of endomitosis occurs in polyploid megakaryocytes [62]. Endomitotic megakaryocytes reach metaphase or anaphase A, but never fully separate sister chromatids or
undergo cytokinesis, resulting in globulated polyploid nuclei. Endomitosis is a variant of mitosis without nuclear (karyokinesis) and cytoplasmic division (cytokinesis).

Somatic polyploidization can also occur by cell fusion, a process in which two or more cells become one by merging their plasma membranes [63]. This process inevitably leads to the formation of a cell with at least a double amount of DNA compared to the cells of origin. In cancer, cell fusion has been shown to promote the formation of tetraploid hybrids, which are believed to play a role in generating cell diversity and increase tumor malignancy. Faster growth rate, drug-resistance and increased metastatic potential are some of the new features developed by hybrids that arise by cell fusion. It is noteworthy that polyploidy hybrids in the following cell division can generate aneuploid progeny as a consequence of abnormal chromosome segregation. In contrast, in physiological conditions, cell fusion is a tightly controlled process that is restricted to only few cell types in humans, i.e. osteoclasts, trophoblasts and skeletal muscle cells [64-65]. In physiological conditions, with the exception of fusion of gametes and stem cells, in which cell fusion contributes to tissues proliferation and cell mass growth, cell fusion results in terminally differentiated multinuclear cells incapable of proliferation [66-67].

Polyploid cells can be also formed by a mechanism of mitotic slippage. During this pathological process, cells present an altered spindle assembly checkpoint (SAC). The SAC prolongs mitosis until all kinetochores are stably attached to spindle microtubules; when the SAC cannot be satisfied, cells exit mitosis without undergoing anaphase or cytokinesis (genesis of mononucleated tetraploid cells). Mitotic slippage has been observed for example in cells after prolonged mitotic arrest in response to spindle toxins [68] or in APC-deficient cells (Adenomatous Polyposis Coli is a gene frequently mutated in colon cancers) [69]. Finally, cytokinesis failure is observed in certain pathological contexts and leads to
the genesis of binucleated tetraploid cells [69, 70]. Indeed, these cells can appear following dysfunction of any of a large number of different proteins controlling cytokinesis process [71]. In addition, bulk chromatin or even a single lagging chromosome trapped in the cleavage furrow can induce cytokinesis failure and tetraploidization [72-73].

The most widely accepted paradigm is that polyploidy in the liver originates from endoreplication followed by aborted cytokinesis [10]. Further data corroborating this mechanism have been reported by Desdouets’ [74-75] and Grompe’s groups [76]. However, after several studies on bone marrow transplantation of liver in deficient FAH<sup>-/-</sup> mice, it has been demonstrated that binucleated cells can be generated through cell fusion between exogenous cells and mature hepatocytes [34, 77]. The observation spewed by these studies, that liver cells are prone to cell fusion, led us to investigate whether cell fusion could also contribute to the polyploidization in liver under physiological condition, avoiding ablation regimens, mouse model of damaged livers and artificial injections of adult cells into host recipients [78]. By using several approaches in chimeric mice, we showed that cellular patterns suggestive of cell fusion are present in adult mouse liver. These data include single cell analysis performed with chromosome probes which demonstrated that some binuclear hepatocytes from male/female chimeric mice contain one male genome (XY) in one nucleus and one female genome (XX) in the other ([78], Figs 6 and 7). In addition we showed by an immunohistochemical approach that both transgenes in LACZ/GFP chimaeric mice coexist in 20% of analyzed hepatocytes. This recent study attributes a new role to cell fusion: a programmed step in normal development of liver.
Figure 2. Several routes to polyploidy. A) Endoreplication: DNA replication in the absence of a complete mitosis. Cells that undergo endoreplication can skip different aspects of mitosis. B) Cell fusion: following fusion, the cell enters the cell cycle with two centrosomes, which are subsequently duplicated. The nuclear envelope disassembles and cells go through mitosis, giving rise to a binucleated cell. The same final product arises from defect in cytokinesis C). Finally when the mitotic arrest persists, the cell bypasses anaphase, telophase and cytokinesis and progress into the next G1 phase without correcting the mitotic error that triggered the arrest (D). As a consequence, cell derived from a mitotic slippage contain a single tetraploid (4N) nucleus with two centrosomes. Whenever a cell contains extra-centrosomes, it can then undergo either a bipolar or a multipolar mitosis, giving rise to aneuploidy.
2. ANEUPLOIDY

In the context of this discussion, it is especially important to distinguish between ‘aneuploidy’ and ‘polyploidy’. These terms describe two 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 shows alterations in chromosome number that are not a multiple of the haploid complement. Therefore, aneuploidy refers to an unbalanced genomic state. By contrast as already mentioned, 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. In contrast to polyploidy, that is well tolerated in specific cases at both the cellular and organismal level, aneuploidy has paradoxical effects on cellular fitness and depending on the context it can be associated with severe abnormalities, cell death or even growth-advantageous properties. Aneuploidy can arise in gametes/embryonic cell (costitutional) or in differentiated somatic cells (somatic) and can involve autosomes (chromosome 1-22 in humans) and gonosomes (chromosomes X or Y).

2.1 Costitutional aneuploidy

Studying the development of sea urchin eggs undergoing abnormal mitotic division, the German zoologist Theodor Boveri showed that aneuploidy is detrimental on cell and organism physiology when it occurs at embryonic level. The embryos that resulted from dispermic fertilizations exhibited developmental defects and died [79]. Boveri concluded that chromosome gain or loss leads to abnormal development and lethality. Thus, among
Boveri’s many seminal contributions to biology is the discovery that an abnormal number of chromosomes disrupts development. This deleterious effect has been well established in many species, including *Drosophila Melanogaster*, *Caenorhabditis elegans*, mice, plants and humans. In flies, with the exception of chromosome 4, all whole-chromosome trisomies and monosomies are lethal [80]. Similar results are observed in worms, where all trisomies and monosomies are inviable [81]. In the mouse, all monosomies and trisomies, except for trisomy 19, are embryonic lethal. In humans, all whole-chromosome aneuploidies, except for trisomy 13, 18, or 21, result in embryonic lethality. Even these viable trisomies display severe abnormalities. Trisomy 13 or 18 individuals die within the first few months of life and exhibit developmental abnormalities such as cardiovascular and craniofacial defects, developmental abnormalities of the nervous system, as well as growth retardation [82-83]. These phenotypes are also seen in the only viable human trisomy, trisomy 21 [84].

### 2.2 Acquired autosomal aneuploidy

In addition to constitutional aneuploidy, chromosomal missegregation can occur in differentiated somatic cells, resulting in acquired aneuploidy.

#### 2.2.1 When aneuploidy is detrimental

The most prominent process where aneuploidy is observed in somatic cells is cancer, a disease of hyper-proliferation. Here aneuploidy is not restricted to one chromosome but the disease is characterized by a high degree of numeric as well as structural karyotypic abnormalities. The 90% of solid tumors are aneuploid [85], suggesting that some patterns of chromosome gain and loss enable cells to escape normal growth restraints and develop into malignant tumors, for example by acquiring extra-copies of an oncogene or losing a tumor suppressing gene [86-87]. Even the role of aneuploidy in cancer has been the center of debate for almost a century,
whether it is a cause or a consequence of the malignant transformation is still under debate.

The role of aneuploidy in tumorigenesis has been extensively studied in mouse models of mitotic checkpoint dysfunction. So far, conventional gene knockouts have been constructed for almost all known mitotic checkpoint genes, including those encoding MAD1, MAD2, BUB1, BUB3, BUBR1 and centromere protein E (CENP-E) [88-94]. In addition, hypomorphic alleles that express dramatically reduced levels of BUB1 and BUBR1 have also been generated [95-96]. Whereas complete loss of these gene products results in early embryonic lethality, heterozygous and hypomorphic mice are viable and fertile. In all cases, mice with genetically reduced levels of mitotic checkpoint components have an increased level of aneuploidy and chromosomal instability (=CIN) [97] in mouse embryonic fibroblasts (MEFs) and tissues [88-94]. It is important to note that aneuploidy and CIN are not synonymous: whereas aneuploidy describes the state of the karyotype, CIN refers to the ‘rate’ of karyotypic change. Although CIN leads to aneuploidy, not all aneuploid cells exhibit CIN; some cells are aneuploid with a uniform, stable karyotype — a phenomenon exemplified by Down’s syndrome, a condition that is associated with widespread aneuploidy but not CIN. In general, the degree of aneuploidy, including the proportion of aneuploid cells and the range of chromosome losses and gains, varies depending on the gene product and to what level it has been reduced. However, as these animal models induce aneuploidy through continued CIN, the effect of aneuploidy in tumor development independently of CIN cannot be assessed.

Current evidence shows that the degree of aneuploidy is not an accurate predictor of tumor susceptibility in mice. Here, the first incongruence of this phenomenon that contributes to develop the “aneuploidy paradox” theory. Despite the association of aneuploidy with tumors, the accumulation of an abnormal chromosome content does not
always imply cancer progression. It is becoming increasingly clear that the consequences of aneuploidy are context-dependent and in certain circumstances aneuploidy can act as tumor suppressor [98]. This is clearly illustrated in individuals with Down syndrome, who have a significant increase in hematological cancers, but a reduced incidence of solid tumors. Although aneuploidy has long been implicated in driving cancer, aneuploidy can suppress tumorigenesis in certain cases. Cenp-E haploinsufficiency reduces the incidence of carcinogen-induced tumors and greatly extends the survival of mice that lack the p19Arf tumor suppressor by an average of 93 days [99]. Moreover, mice that are heterozygous for BubR1 develop ~50% fewer tumors in the sensitized ApcMin/+ background [100], whereas deletion of the securin gene reduces the incidence of pituitary tumors by ~50% in Rb heterozygous animals [101] (although, in the case of Rb, it remains unclear if tumour suppression results from increased levels of aneuploidy). Tumour repression has also been observed in stably aneuploid mice that are trisomic for ~50% of the orthologue genes on human chromosome 21 [102]. One explanation for these observations is that exposure to carcinogens or loss of tumor suppressor function results in low levels of genetic damage and/or chromosome missegregation that, when combined with aneuploidy, drive rates of genetic instability above a threshold compatible with cell viability [99]. Consistently, aneuploidy and apoptosis are also increased in the intestines of BubR1+/−ApcMin/+ mice, thereby providing evidence that too much aneuploidy might promote cell death and inhibit tumour growth [100].

In conclusion, under normal circumstances, aneuploidy might act as a barrier to suppress tumorigenesis by reducing the growth of pre-neoplastic cells. However aneuploidy promotes tumorigenesis in some contexts when it provides a selective pressure for the accumulation of additional mutations that allow cells to tolerate the adverse effects of chromosomal imbalances [103]. The unbalanced gene expression caused
by aneuploidy might increase the rate at which cells acquire the mutations that are necessary for their survival and proliferation. Once gained, these adaptations would unlock the oncogenic potential of aneuploidy, allowing cells to survive and continue to proliferate in the face of increased genomic instability. Aneuploidy can alter the course of tumour development. However, whether it does so in a positive or negative manner depends on the cell type and the genetic context.

2.2.2 When aneuploidy is normal
It is thus of interest to note that some tissues are naturally aneuploid. This observation raises the question of whether aneuploidy is always detrimental. In mice and humans, one third of the dividing cerebral neuroblasts in the embryonic brain is aneuploid [104]. Many of these aneuploid cells are eliminated during the course of the development, as there are fewer, around 10%, aneuploid cells in the adult brain [104-106]. Nevertheless the aneuploid cells that survive into adulthood are functional, as judged by their ability to form synapses and contribute to the normal neuronal and glia population of the adult brain [107]. Single chromosomal abnormalities, monosomies and trisomies, are the predominant form of aneuploidy detected in the brain. The apparent absence of the detrimental effects of aneuploidy in brain cells could be due to the specific functions of these cells types. Neurons, once differentiated, never divide again. In this post-mitotic state, the anti-proliferative effect of aneuploidy probably have a limited impact on cell function. Aneuploidy in the brain might allow cells to define and modify their functional capacities. In the case of neuroblasts, the high level of aneuploidy has been correlated with their capacity to confer genetic variability, necessary for the high-structural and functional complexity of cerebral circuitries [108]. Recently, I have been able to demonstrate that aneuploidy increases significantly with age in the mouse cortex, a part of the brain especially vulnerable to age related changes [109]. Based on a 2-color interphase FISH strategy we showed that the
age-related increase in aneuploidy is chromosome specific and predominantly affects the non-neuronal cells (glia). Contrary to neurons, the glia preserves the ability to divide in adulthood and especially during aging their acute proliferation provides a protective role for the integrity and nutrition of neurons [110]. Our recent data suggest that mosaic aneuploidy in the old brain is more complex than that previously predicted. The fraction of aneuploid neurons surviving embryonic selection into adulthood remains constant (1%), but coexists with a much higher level of aneuploid non–neuronal cells (9.8%) accumulated during aging. Because alteration of gene expression has been shown to occur as a consequence of single chromosome aneuploidies [111], we postulated that the accumulation of aneuploidy observed during aging in the cortex as well as during embryonic development may be responsible for changes in the transcriptome profile.

2.3 Cellular responses to extra copies of chromosomes

A major focus of current research is to determine how cells respond at the transcriptional or proteomic level to gene expression imbalances that are caused by aneuploidy. In particular, some of these studies address the question of whether aneuploidy causes transcriptional and protein expression changes in direct proportion to the copy number alteration of the DNA or whether the cell minimizes the effects of aneuploidy through dosage compensation. There is also the more complex possibility of gene expression effects beyond the chromosomes that are affected by aneuploidy through altering feedback loops of transcriptional regulators or through epigenetic effects [103]. The possibility that cells induce a specific transcriptional stress response to aneuploidy has also been raised [112]. Beyond the importance of this point in understanding the basic physiology of aneuploidy, this is also a crucial concept. If aneuploidy triggers a common stress response and all aneuploid cells need to develop specific adaptations in order to proliferate with their altered genomes, this
opens the possibility that aneuploidy itself may be targeted as a cancer therapy.

Two types of model are 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 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 [113]. We refer to aneuploid cells derived from CIN 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 separate the effects of aneuploidy from other CIN-associated phenotypes, such as structural chromosomal aberrations, or from potential functions of mutated genes that induce chromosome missegregation.

2.3.1 Chronic defined aneuploidies.

Analyses of chronic defined aneuploidies have provided insight into the consequences of changing the gene expression pattern of entire chromosomes in organisms that do not have prevalent compensatory mechanisms.

Transcriptome effects. Two recent studies in aneuploid yeast strains — which were generated using different methods, used gene expression microarrays to investigate the effects of aneuploidy on the transcriptome. Both studies report that gene expression, in general, is proportional to gene dosage in aneuploid yeast. Interestingly, Torres et al. [112] used a chromosome transfer strategy and selectable markers to generate
aneuploid yeast strains with a single extra chromosome (gain). All the aneuploid strains proliferated more slowly than the wild-type cells, although in some cases the differences were modest and only apparent in co-culture experiments. The aneuploid yeast cells demonstrated a delay in the G1 phase of the cell cycle, increased sensitivity to drugs targeting protein synthesis and folding, and metabolic changes with increased glucose uptake and use. These aneuploid yeast cells also exhibited modest but statistically significant increases in genomic instability with elevated rates of point mutations, mitotic recombination and loss of whole chromosomes, as well defective DNA repair [114]. The systematic nature of this work represents a major advance in the field and not only demonstrates that aneuploidy is detrimental to haploid yeast that has been grown under non-selective conditions, but it also begins to elucidate the mechanisms that lead to these growth defects [112, 115]. In addition, they also found that many yeast strains showed a common gene expression signature, that were observed only in strains carrying additional yeast genes, which indicate that they reflect the consequences of additional protein production as well as the resulting imbalances in cellular protein composition. They concluded that this common phenotype is independent of the identity of the individual extra-chromosome. This signature was originally described by Gasch et al. [116] as an environmental stress response (ESR) in yeast, that has been characterized by defects in cell growth, altered metabolic properties and proteotoxic stress. In particular, proteotoxicity manifests itself as temperature sensitivity, sensitivity to protein folding and degradation inhibitors, and protein aggregate formation. When normalized for growth rate in phosphate-limited conditions, the aneuploid strains showed increased expression of genes related to ribosomal biogenesis and nucleic acid metabolism [112]. Pavelka and coworkers [117] induced meiosis in yeast strains with an odd ploidy (3n or 5n), which produces aneuploid progenies at high frequencies, and then isolated aneuploid
strains without any drug selection. Using this technique, the authors generated 38 stable aneuploid strains (12.5% of spores analysed) with 35 distinct karyotypes. This group only identified this ESR signature using their most stringent analysis, and it was not correlated with either growth rate or number of aneuploid chromosomes. However, the different approaches used in these studies for strain construction, selection, growth and data analysis make a direct comparison difficult. One issue, discussed below, is whether the strains being studied are genetically stable. The genetic heterogeneity of unstable strains might mask gene expression patterns that are detectable in stable strains. Thus, although both studies agree that aneuploidy can induce a general transcriptional response beyond the copy number alteration of the affected chromosome (or chromosomes), it is less clear whether this response mainly reflects the impaired growth of some strains or whether it reflects a specific aneuploidy-sensing mechanism that is wired into cells. Chronic defined aneuploidies also have an adverse effect on mammalian cells. Williams and colleagues [118] cultured mouse cells that were engineered to express a specific additional chromosome (trisomy for chr. 1, 13, 16 or 19), by mating mouse strains carrying Robertsonian translocation with wild-type mice. Between 7 and 40% of the resulting progeny were trisomic for the chromosome that is common to the two Robertsonian translocations because of a meiotic non-disjunction event in the male germline. The consequent analysis of the effects of aneuploidy on cell proliferation and physiology revealed that these cell lines had decreased rates of proliferation, and increased cell size and metabolic rates, all conditions that reduce cell fitness and differential kinetics of spontaneous immortalization in culture. These findings are compatible with the theory that having an abnormal number of chromosomes is disadvantageous for mammalian cells.

Proteome effects. The effect of aneuploidy on the proteome is also controversial. The stochiometry of certain protein complexes, such as the
ribosome, is maintained by the proteolysis of subunits that fail to assemble into the complex [119-120]. In the absence of mechanisms for compensation, aneuploidy could lead to an excess of uncomplexed proteins and proteotoxic stress [112, 121]. Proteotoxic stress results from the accumulation of unfolded, misfolded and aggregated proteins in a cell and can lead to the activation of factors and pathways that are designed to mitigate the burden of these unfolded proteins. This includes the ubiquitin–proteasome and chaperone pathways and could place an energetic burden on aneuploid cells. The above yeast transcriptome studies also looked for dosage compensation at the level of the proteome. Providing evidence for dosage compensation, Torres et al. [112] found that most proteins examined (13 of 16) did not scale with gene copy number and that these proteins were members of multi-protein complexes. Consistent results were subsequently found in a more global proteome analysis [121], leading the authors to hypothesize that increasing protein degradation to compensate for gene copy number abnormalities may be a general response to aneuploidy. In further support of this hypothesis, the group found that some of their aneuploid yeast strains were more sensitive to proteasome inhibitors than the isogenic euploid control cells. By contrast, Pavelka and coworkers [117] found that the chromosome copy number generally scaled with protein abundance, that proteomic changes clustered among similar karyotypes and that there was minimal dosage compensation for core complex proteins. The reason for these differences is not completely clear; however, differences in the sensitivity of the protein detection techniques [122-123] namely, stable isotope labelling by amino acids in cell culture (SILAC) and multidimensional protein identification technology (MudPIT) mass spectrometry and/or the stability of the aneuploid yeast strains used [114, 124] are potential explanations. When characterizing cellular responses to aneuploidy with the aim of defining a cellular state or vulnerability that might form the basis of an aneuploidy-specific cancer
therapy, it is important to know whether the findings from yeast can be generalized to higher eukaryotes. Preliminary data imply that aneuploid cells that are derived from diploid HCT116 human colon cancer cells do show some evidence of protein-level dosage compensation and display an upregulation of the autophagy pathway (Z. Storchova, Max Planck Institute of Biochemistry, Martinsried, Germany, personal communication). Taken together, these results imply that aneuploid cells may share adaptive cellular responses of dosage compensation at the level of the proteome, but the details of the extent, importance and mechanism of this compensation remain to be elucidated.

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 are both independent of the specific set of genes amplified on the extra chromosome and are indicative of energy and proteotoxic stress. These general phenotypes are seen in addition to the chromosome-specific effects caused by amplification of individual genes and combinations of a small number of genes on the aneuploid chromosome.

### 2.3.2 Acute random aneuploidies.

Cells that contain acute random aneuploidies due to CIN also have proliferation defects and show 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 missegregation compromises cell proliferation [125].

The cellular response to CIN-induced aneuploidy differs depending on the degree of aneuploidy, where p53 promote cell death or cell-cycle arrest in response to massive or low level of chromosome missegregation [126]. Cells with CIN caused by chemically induced chromosome missegregation, by gain-of-function alleles of Cdc20 [126], or loss of
function alleles of Bub1B [95], or by overexpressing the checkpoint factor Mad2 [127] 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 [128], cells heterozygous for deletions in CENP-E [99] and cells that overexpress the ubiquitin-conjugating enzyme UbcH10 [129] 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 missegregation events occur [130]. 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 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 might be needed to detect proliferation defects of individual aneuploid cells. Moreover, aneuploidy could be beneficial in the presence of strong selective pressure [131-132]. For example, where yeast has two similar genes on different chromosomes, cells in which one of these paralogues is detected may compensate by the casual gain of an extra copy of the chromosome bearing the other paralogue [132].

2.4 Several routes to aneuploidy

In a human adult, millions of cell divisions occur every minute, and the maintenance of a diploid karyotype requires the proper segregation of chromosomes with every cell division. However, the chromosome segregation machinery is imperfect, and in vitro estimates suggest that normal, diploid cells missegregate a chromosome once every hundred cell divisions [133]. The basal rate of spontaneous chromosome missegregation in vivo is an unknown but important quantity that could vary
between cell types. Even if this in vivo rate is extremely low, strong selective pressure could enable the proliferation of rare aneuploid cells under certain conditions, as discussed below.

The disruption of multiple genes and pathways has been implicated in increasing the rate of chromosome gains and losses above the basal rate and generating CIN. Errors in chromosome segregation during mitosis are generally considered as one of the mechanisms leading to aneuploidy. The mitotic checkpoint, also known as the spindle assembly checkpoint, is the major cell cycle control mechanism that ensures high fidelity of chromosome segregation. The mitotic checkpoint is responsible to delay anaphase until all chromosomes are properly oriented on the microtubule spindle. Under normal conditions, the checkpoint is released only when all chromosomes are correctly attached to the kinetochore. Any perturbation of the checkpoint leads to initiation of anaphase before the spindle has established proper orientation and proper attachment to its chromosomes. This can lead to chromosome missegregation and, consequently, to aneuploidy.

There are several specific checkpoint problems that may cause gain or loss of one or more chromosome (Figure 3): a) alteration of signaling within the mitotic checkpoint where cells with unattached or misaligned chromosomes can proceed trough anaphase and lead to a daughter cell with both copies of a chromosomes. A compromised SAC (=spindle assembly checkpoint), which arrests cells with improper spindle kinetochore attachments, can lead to CIN and aneuploidy [97, 134]. The human syndrome mosaic variegated aneuploidy (MVA) is caused by inactivation of the SAC protein BUBR1 [135]; b) cohesion defects where one sister chromatid could be lost prematurely and thus be missegregated. Accurate chromosome segregation is achieved through carefully orchestrated interactions between the mitotic spindle, kinetochores and cohesion [136-137]; c) Merotelic attachment when one kinetocore is attached to both poles of the spindle
and generate lagging chromosomes that can be either excluded from both daughter cells or be missegregated. A possible explanation of the erroneous merotelic attachments could be the presence of extra-centrosomes, that can generate a multipolar cell division or more frequently a pseudo bi-polar spindle. The consequences of centrosome clustering, that enables cells to survive, is reflected in an increased frequency of merotelic attachments [138], at least in tumors.; d) **uncontrolled centrosome duplication (multipolar mitosis)**. The duplication of the centrosome normally occurs during S phase through a cdk2 dependent mechanism [139], and is under a system of constraint that ensures there is one and only one duplication event during interphase. As a result of fidelity in duplication, a non-transformed cell has two centrosomes at mitosis, which dictate the formation of two spindle poles. If more than one duplication event has occurred in interphase, a multipolar spindle will result, and the genome will be segregated in an aneuploid manner. In the future, it will be important to define precisely how centrosome amplification affects the dynamics of spindle microtubules and whether centrosome amplification increases the rate at which merotelic attachments are formed or whether it impairs the error correction mechanisms that fix them; e) **unstable tetraploid intermediate**.
Figure 3. Mechanisms that lead to aneuploidy. There are several pathways by which a cell may become aneuploid. A) A compromised SAC could allow cells to enter anaphase with unattached or misaligned chromosomes. As a result, both copies of one chromosome may end up in a single daughter cell. B) Chromosomes can be missegregated if sister chromatid cohesion is lost prematurely or if it persists during anaphase. C) A single kinetochore can attach to microtubules that arise from both poles of the spindle. If the merotelic attachments are not corrected before anaphase, then both sister chromatids can missegregate towards the same pole to generate aneuploid cells, or they can lag in the spindle midzone and be excluded from both daughter nuclei. D) Cells with centrosome amplification usually cluster extra centrosomes during mitosis to form a pseudo-bipolar spindle that can result in an increased frequency of merotelic attachments.
Tetraploidy is frequently an intermediate in tumor progression toward aneuploid status. In many human carcinomas, cells with tetraploid DNA content arise as an early step in tumorigenesis and precede the formation of aneuploid cells [70]. The first step of this process involves an aberrant mitotic exit to tetraploid status in G1, and the second, the absence of a G1 surveillance mechanism that normally would prevent cell cycle progression of cells with an abnormal chromosome complement. Tetraploid cells harbor extra-centrosomes. These supernumerary centrosomes can lead to chaotic multipolar mitoses in which chromosomes are haphazardly segregated into two or more daughter cells, a defect that directly causes whole-chromosome aneuploidy (Figure 4).
**Figure 4. The fate of tetraploid cells.** A) Most tetraploid cells will be detected by the tetraploidy checkpoint. Activation of this checkpoint results in G1 arrest, eventually followed by apoptosis. B) When the tetraploidy checkpoint is compromised (for example, in p53-defective cells), tetraploid cells proceed through cell division. This most often results in aneuploidy and cell death, but if this occurs during transformation, viable aneuploid clones can emerge. C) A reduction mitosis results by the segregation of a diploid genome to one pole during a multipolar mitosis. D) In dividing hepatocytes and many cancer cell types, the extra centrosomes in a tetraploid cell can be clustered, enabling a bipolar mitosis.
3. AIMS

Polyploidization is the most peculiar feature of the liver. The literature reports that, contrary to most other cell types, adult hepatocytes are polyploid cells with a DNA content of 4, 8 or even 16 haploid genomes and any deviation from this mathematic assumption is the result of technical errors. However, the phenomenon of polyploidization includes, as first step, the generation of tetraploid intermediates. An underestimated aspect is that these cells in a tumoral context have shown to generate aneuploid progenies in the subsequent cell division, because of the presence of four centrosomes that promote multipolar spindles. The same cell fate could be shared by the progeny of tetraploid hepatocytes that re-enter in the cell cycle or even by binucleated or mononucleated cells arisen by cell fusion, a recent well-recognized process involved in normal liver development.

Thus, we believe that the ploidy of liver is a more complex phenomenon that needs to be investigated. Because polyploidization arises through liver aging, at specific time-points, we decide to perform our analysis on aneuploidy in correspondence of those time-points, like weaning (21 days) and adulthood (4 months), and enriched of others in between, that could reveal more interesting features of liver cells (15 days, 1.5 months).

In summary, the main objectives of my present work are:

- **to check for events of chromosomal imbalance in hepatocytes at the single cell level in physiological conditions.**
- **to determine whether aneuploidy occurs in these cells at a significant degree.** These goals will be addressed performing a 2-color FISH on interphase cells for two different chromosomes (an autosome and a gonosome). The DNA content will be assessed by the comparison between the signals of the chromosomes tested. In binucleated hepatocytes, the comparison will be referred to one single nucleus as well as within the two nuclei.
to determine whether aneuploidy is a widespread phenomenon or, as is the case for polyploidization, occurs at specific time-points. My experimental analysis will be comprehensive of the developmental stages of the liver until the adulthood.

- **to check for the presence of abnormal centrosome number.** To address this aim I will perform a quantitative analysis of centrosomes at the single cell level following the same temporal outline of the DNA content analysis described above. The use of a combined immunostaining for two different proteins that co-localize at the centrosome level will guarantee a correct and trustworthy analysis of centrosomes number. Any numerical value different from the expected progression of centrosome number for a polyploid cell (i.e., 1, 2, 4 etc.) as well as dispare values will be considered.

- **to investigate whether the expected stoichiometric ratio between copies of DNA and centrosomes number within a diploid cell is conceivable also for a polyploid /aneuploid cell.** To address this aim I will perform sequential experiments of FISH/immunostaining for centrosome proteins at the single cellular level.

- **to determine the mechanism by which binucleated hepatocytes reach an unbalanced DNA content.** This goal will be address testing the hypothesis that asynchronous cell division contributes to the aneuploidy observed in binucleated cells.

- **to test whether extra centrosomes are functionally active and act as independent entity of cluster in aggregates.**

The nucleation assay performed on young and old mice will provide the answer to the behavior of extra-centrosomes.

In conclusion, a detailed analysis of hepatocytes at the single cell level is fundamental to better understand either the properties of terminally differentiated cell, or the consequences of liver cell therapies with mature hepatocytes.
Figure 5. The graph summarizes the time-points analyzed for each specific experimental approach described below.

4. MATERIAL AND METHODS
4.1 Experimental Plan
CD1 mice (18 days p.c., 15 and 21 days, 1.5, 4, 5 and 7 months old) were obtained from Charles River. Mice were maintained in accordance with the guidelines from the Italian Ministry of Health. To obtain fresh suspensions of juvenile and adult hepatocytes, liver perfusion was carried out as previously described [78].

4.2 Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) was performed using a locus specific probe for the X chromosome and painting probes for the 17, 18 and Y chromosomes. The BAC clone RP23-113K2, mapping to the distal region of the X chromosome, was obtained from the Children’s Hospital, Oakland CA. This probe was labeled by nick translation using biotin-16-dUTP (Roche Diagnostic, Indianapolis, IN) and was detected by Alexa Fluor 647-conjugated streptavidin antibody (Invitrogen, Carlsbad, CA). To obtain painting chromosomes for the 17, 18 and Y chromosomes, flow sorted DNA for 17, 18 and Y chromosomes (M.A. Ferguson-Smith, University of Cambridge, Cambridge UK) were labelled by DOP-PCR with Spectrum Aqua-dUTP (Perkin Elmer, Waltham, MA) and Spectrum Orange-dUTP (Abbott Laboratories, Abbot Park, IL). Square coverslips in which hepatocytes were plated after perfusion were incubated in denaturation solution (FA/SSC) at 90°C for 1’ and 45” and then dehydrated with serial ethanol washing steps (70 ice-cold, 90, 100% for 3’ each). Probes were denaturated in the hybridization solution (50% dextran sulfate/SSC) at 85°C for 5 min, applied onto the slides and incubated overnight at 37°C in a humidified chamber. After washing with 50% formamide/2X SSC and 1X SSC for 5’ the coverslips were incubated at 37°C with blocking solution (3% BSA). Thereafter for the detection of the locus specific probe the coverslips were incubated with the previously mentioned secondary antibody. Slides
were counterstained with DAPI, dehydrated with ethanol series and mounted for imaging.

4.3 Centrosome analysis, nucleation assay and H3 staining

After perfusion, single hepatocytes were plated in chamber slides and incubated at 37°C overnight in William’s E medium (Invitrogen, Carlsbad, CA) supplemented with 15% Fetal Calf Serum (FCS) (Sigma, St Louis, MO) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). For centrosome visualization, cells were fixed in ice cold MeOH for 10’ rinsed 3 times with PBS 1x and incubated with goat serum 5% for 1h at 37°C. Hepatocytes were incubated with mouse anti γ-tubulin and rabbit anti pericentrin (Abcam, Cambridge, MA 1:500) and detected with anti-mouse Alexa-488 and anti-rabbit Alexa-647 (Invitrogen AlexaFluor 1:1000). For nucleation assay, cells were incubated with Nocodazole (10 mg/mL) for 1.5 h at 37°C followed by 15’ on ice. Hepatocytes were washed with PBS 1x at room temperature and incubated with fresh medium at 37°C for 5’ to allow for α-tubulin polymerization, thus testing centrosome nucleation activity. The cells were fixed in MeOH on ice for 10’ and store at 4°C until their use. For microtubules and centrosome detection after blocking (10% goat serum, Sigma) hepatocytes were incubated with mouse α-tubulin (Abcam 1:500), rabbit anti γ-tubulin or rabbit anti-pericentrin (Abcam 1:500) specific antibodies. Secondary detection was performed with anti-mouse 488 antibody (AlexaFluor 1:1000) and goat anti-rabbit 647 antibody (Abcam 1:1000). The cells were finally counterstained with DAPI. For phospho-histone H3 assay, hepatocytes were fixed in 1% PFA for 10 minutes, washed 3 times in PBS 1x and permeabilized with Triton X-100, 0.3% for 10 minutes at room temperature. After blocking with 5% goat serum (Sigma, St Louis MO), the cells were incubated with a mouse monoclonal
antibody against histone H3S10P (mAbcam 14955, 1:500) and detected with anti-mouse AlexaFluor 488 secondary antibody (1:1000).

4.4 Two colors combined FISH and immunofluorescence

Liver cells from a 45 day-old mouse were plated in special glass coverslips carrying a grid that allows mapping of the exact position of the cells (BELLCO, Vineland, NJ). To determine the centrosome number hepatocytes were stained with an anti γ-tubulin antibody as previously described. In the first step of our experiment images corresponding to centrosome signals were acquired with a dye specific cube together with its differential interference contrast image (DIC). The physical location of each cell was recorded with the aid of numbers and letters engraved on the grid. The second step consisted of removing the antifade and carrying out a FISH hybridization with a Y-specific painting probe to determine the ploidy (see above for the procedure). During this second step, the previously acquired DIC image was essential for recognizing the same field used for the γ-tubulin staining and used to unambiguously associate the number of centrosomes and the DNA content of each cell.

4.5 Image acquisition

For Figure 6 images were acquired using an inverted epi-fluorescent microscope (Nikon eclipse TE 200) after cytological staining. For Figure 7, Figure 8A and Figures 11 and 12 specimens were acquired using a motorized inverted fluorescence microscope, CellR (Olympus) also equipped with DIC. FISH images were acquired with fine focusing oil immersion lens (× 60, NA 1.35 and × 40) in optical sections of 0.5 µm. The microscope was equipped with a CCD Olympus Fluo View camera. For Figures 8B-C, interphase cells were imaged with an Olympus BX61 microscope with an UPlanSApo 40 X oil immersion len, an Hg arc lamp for
excitation and narrow band filters for all fluorescent emission and equipped with a Cooke SensicamQE camera with IPLab imaging software for image acquisition. Images of interphase cells for each slide were acquired for the Spectrum Orange, Cy5 and Spectrum Aqua dyes. An IP lab script was generated to acquire images; a DIC image was acquired first to ensure that bi-nucleated cells shared the same cytoplasm even though the hepatocytes were diluted enough to avoid high density cell plating. Multiple focal planes were acquired for each channel to ensure that signals on different focal planes were included: eight focal planes for chromosome painting and thirteen different focal planes for locus specific probes were acquired. For Figure 9, images were acquired with a manual inverted fluorescence microscope (Axiovert 200, Zeiss) with fine focusing oil immersion lens (× 60, NA 1.35). The microscope was equipped with a Camera Hall 100 and with the Applied Spectral Imaging software. For Figure 15, cells were acquired with fine focusing oil immersion lens (× 40) in optical sections of 0.5 μm using an FV1000 laser scanning confocal microscope (Olympus) equipped with a FV1000 software, and operating in channel mode with 405, 488 and 633 nm excitations; DIC was also used. The z stacks were acquired with resolution of 1 Airy unit to allow three-dimensional reconstructions. For Figure 8A and Figures 11 and 12, images were analyzed with tools available through ImageJ (http://rsb.info.nih.gov/ij/) and Photoshop (Adobe).
The resulting fluorescence emissions were collected using the following band-pass filters:

<table>
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<tr>
<th>DYE</th>
<th>BAND-PASS FILTER</th>
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<tr>
<td>DAPI</td>
<td>425-to-475 nm</td>
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<tr>
<td>SPECTRUM AQUA</td>
<td>430-to-450 nm</td>
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<tr>
<td>ALEXAFLUOR 488</td>
<td>500-to-550 nm</td>
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<tr>
<td>SPECTRUM ORANGE</td>
<td>565-to-615 nm</td>
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<td>ALEXAFLUOR 647 or CY5</td>
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5. RESULTS and DISCUSSION

5.1 Some binucleated hepatocytes show an unbalanced chromosome content.

Fetal and neonatal mouse hepatocytes are diploid cells. Polyploidy usually starts at the second/third week of age, in concomitance with weaning, although the appearance of binucleation and polyploidy can change according to the mouse strain examined [10-11, 18]. To confirm that this also applies to the strain we used in this study (CD1), we analyzed the frequency of binucleated and mononucleated cells with classical haematoxylin/eosin staining at different mouse ages. We performed our analysis on two CD1 mice for four different ages: fetal (18 days post coitum), before weaning (15 days), young (1.5 months) and adult (4 months) mice.

Our results show that CD1 hepatocytes are mainly mononucleated cells in fetal and perinatal life, but become binucleated at 15 days post-partum. At 1.5 months most hepatocytes are binucleated cells and their percentage does not vary appreciably later in life (Figure 6).

To investigate the ploidy of single hepatocytes we took advantage of our previous experience based on the use of chromosome specific probes for the analysis of chromosome content of liver cells [78, 140]. In general, a tool for the analysis of hepatocyte DNA content is the staining of nuclei upon digestion of liver tissue with propidium iodide followed by quantification of fluorescent intensity with a flow cytometer [10]. Another approach is based on the evaluation of fluorescence intensity of thin liver tissue sections stained with Hoechst 33342 using an epi-fluorescent microscope. The identity of mono- or binucleated hepatocytes is determined by comparing nuclear to membrane labelling [74]. These classical approaches lack the sensitivity to detect the small differences in DNA content that result from unbalanced chromosomal segregation.
Figure 6. Classical hematoxilin/eosin staining of liver during the normal development. A) Top and bottom panel show the typical staining of cytoplasm and nucleus of single hepatocytes, obtained by liver perfusion of 15 days and 3 months old mice. Magnification 40x. B) The graph summarizes the percentage of mono and binucleated liver cells at different mouse ages.

Moreover, the use of tissue sections stained with Hoechst or DAPI for the determination of DNA content incurs in several technical problems, which make difficult to determine the individual cell identity, and consequentially a correct evaluation of the staining. I therefore designed and extensively validated a two-color FISH approach that provides highly quantitative and reproducible polyploidy data for individual chromosomes in interphase nuclei by assessing ploidy of a cell based on a comparison between an autosome (17 or 18) and a sexual chromosome (Y) [78]. The chromosomes, selected for the small size, were suitable for the analysis of liver ploidy. First, I confirmed with this approach that in cells from fetal livers and in 15 day-old mice, all signals were compatible with a diploid content for both nuclei (Y and 17 probes used, Figure 7).
Figure 7. Interphase FISH on single hepatocytes before weaning. Example of FISH with 2-color labelled probes of 15 days old male liver. A) The hybridization with a Y-paint probe (spectrum orange=so) shows that each nucleus contains one single signal, as expected for male diploid cells for sexual chromosomes. The combination with DIC (=differential interference contrast image) allowed to classify the single hepatocytes, as mono or binucleated cell. B) The merged images of stained nuclei with DAPI and the 17-paint probe, detected with Cy5 dye, show that even for the autosome tested the signals corresponds to a correct diploid content.

However the ploidy analysis of the subsequent time points (1.5 and 4 month-old mouse) revealed an unbalanced DNA content between the two nuclei (Figure 8). In binucleated liver cells, it is often assumed that the chromosome content is similar in both nuclei and that binuclear cells divide synchronously into two mononuclear 4n hepatocytes [73]. However, our analysis with chromosome Y and chromosome 18 in 4 months old mouse revealed two different unbalanced conditions, as illustrated in Figure 8A. In some binucleated hepatocytes the signals of the two chromosomes tested within a nucleus didn’t match. This condition is indicative of aneuploidy and, as reported before, is generally related to a pathological condition. In addition, I also found binucleated hepatocytes that show a perfect match of signals between the two chromosomes within a nucleus, but an unbalanced DNA content between the two nuclei (i.e., one nucleus is diploid and the
other is tetraploid). Altogether, I estimated that the entity of these phenomena is in the order of 21% (18/87 cells).

To exclude that the observed phenomenon was related to the autosome selected for the experiment, I performed an interphase FISH with both sexual chromosomes (X and Y) on single hepatocytes of 7 months. I observed that, based on the ploidy of sex chromosomes, most binucleated cells from mature livers carry nuclei with the same chromosomal content (2n, 4n or even 8n). However, in two different experiments I found a numerical discrepancy between the visualized signals. The analysis with only the Y chromosome painting probe revealed that 8.7% of binucleated hepatocytes showed a discordant number of Y chromosomes between the two nuclei, (Figure 8B). In addition, a similar percentage (8%) of cells with an unbalanced DNA content in the two nuclei but with a perfect match of the signals in each nucleus (discordant nuclei for ploidy) was found when the Y chromosome painting probe was used in combination with a BAC X-chromosome specific probe (Figure 8C). The perfect match between X and Y signals found in the same nucleus ensured the quality and the reliability of my analysis, demonstrating that the observed aneuploidy is real and not a consequence of technical challenges.

Figure 8. Interphase FISH of adult binucleated hepatocytes. (A) Binucleated hepatocytes with unbalanced DNA content for chromosome Y (cyan) to which corresponds aneuploidy for the autosome 18 (in red) (top: 1 Y chr. with 3 copies of 18 chr. in the left nucleus and 2 Y with 2 copies of 18 in the right nucleus; bottom: 2 Y with 3 copies of chr. 18 in the left nucleus. In this nucleus one spot for chr. 18, the top one, could also be the result of two overlapping chromosomes 18, resulting in 4 signals. In either case the left nucleus is unbalanced with respect to the right nucleus, which contains 1Y with 2 copies of 18 (B) Representative binucleated cell with discordant DNA content between the two nuclei (8n vs. 4n, top and 2n vs. 4n, bottom) as detected by the number of copies of chromosome Y (in red) (continue to the following page).
Figure 8. (continue from previous page) (C) Ploidy of binucleated hepatocyte analyzed with a chromosome paint for Y (cyan) and a locus specific probe for X (yellow). In each nucleus the number of copies for the Y chromosome match the expected number for the X chromosome (top 3X, 3Y and 4X, 4Y; bottom 2X, 2Y and 1X, 1Y). However, the ploidy between the two nuclei is discordant). The cartoons on the right of each panel summarize the ploidy for each cell.
Although polyploid hepatocytes were historically thought to have limited mitotic capacity, several groups [141-143] have recently shown that tetraploid and octaploid mouse hepatocytes are highly regenerative. Polyploid hepatocytes could undergo reductive divisions and generate diploid daughters [140-141] both in vitro and in vivo [34], through a process termed “ploidy conveyor.” When polyploid hepatocytes divide, multiple mitotic spindles are initially established. In most cases, these multiple spindles resolve in a single bipolar spindle, but multipolar mitoses also occur. However, the asymmetry of mitotic spindle predisposes to aneuploidy, genome instability and lately neoplastic transformation [70, 144-146]. The association between tumor transformation and aneuploidy made difficult to accept that a highly regenerative tissue such as the liver contains a large percentage of aneuploid cells. However, by using probes from the sex chromosomes and two autosomes (MMU 17 and 18), we obtained unambiguous images of unbalanced chromosome content in 21% of hepatocytes analyzed for Y and 18 chromosomes and 8% for sexual chromosomes, in 4 months old male mice. The observed aneuploidy frequencies suggested that the chromosomes analyzed show a different susceptibility to be lost or gained. Because the aneuploidy for autosomes were not considered vital or highly detrimental it was quite surprisingly to find the chromosome 18 more aneuploid than the sexual chromosome analyzed (X and Y) in an adult normal tissue. To note, based on the calculated average of 4% per sexual chromosome in adult mouse liver as many as the 40% of cells may be affected. In agreement with our data, other groups recently reported that aneuploidy occurred at high frequency in the liver of young, adult and aged WT mice [141]. The aneuploidy was detected in a range of 25 to 60%, based on chromosome counting in hepatocytes metaphases. Even if both approaches have limits in determining the exact percentage of aneuploidy due to the high complexity of ploidy in adult liver, it is a fact that half of the mature hepatocytes in mice
are aneuploid and yet retain full ability to divide. Similar results were collected for humans [147]. The calculated aneuploidy for each age group analyzed (Young, Adult and Senior) was 58% (range, 31%–91%), suggesting that the degree of aneuploidy is exceptionally high even in humans but more important that it remains constant throughout postnatal life. In many cases, the calculated aneuploidy was nearly identical for each probe set (i.e., H6); in other instances, the predicted aneuploidy varied by as much as 2-fold (i.e., H18). This was also in agreement with the difference of aneuploidy rate found for the chromosomes tested in the present work. To note, the synteny of chromosome 18 in humans and mouse is really high, suggesting that the observed increase of instability of chromosome 18 during aging may play the same role in both species.

Contrary to the other reports, our data support the occurrence of aneuploidy in a completely normal setting, suggesting that the continuous duplication and/or cell fusion process facilitate the acquisition of an unbalanced genome content. Recent findings have shown that such exceptionally high levels of aneuploidy are likely to have functional consequences. First demonstrated in yeast, aneuploidy has shown to be beneficial in terms of providing a rapid cellular adaptation under pleiotropic stress, promoting the survival of the fittest phenotypes [117, 148]. Recently, a similar mechanism of adaptation has been demonstrated also for the liver [147]. To test whether aneuploidy could be beneficial in the liver, Grompe’s group utilized the Fah−/− model of hereditary tyrosinemia. Loss of Fah leads to accumulation of fumarylacetoacetate and associated toxic metabolites, resulting in liver failure and death. Liver function can be maintained by blocking the pathway upstream of FAH by treatment with the drug NTBC (= 2-(2-nitro-4-trifluoromethylbenzoyl)-1, 3-cyclohexanedione) or loss of HGD (=homogentisic acid dioxygenase). In Hgd+/−Fah−/− mice, after drug treatment interruption, livers were repopulated with reverted hepatocytes, which rapidly emerged organized in spread healthy nodules. Approximately
25% of these nodules contained spontaneous mutations in Hgd and a markedly enrichment for chromosome 16 loss (e.g., either whole chromosome loss or terminal deletion), containing functional Hgd. These mutations led to the complete loss of HGD activity, producing hepatocytes resistant to tyrosinemia and demonstrated that aneuploidy is as a mechanism for stress-induced liver adaptation to chronic injury.

Our analysis based on interphase FISH allows for a more precise quantification of DNA content of binucleated cells, compared to the chromosome counting in metaphases in which the identity of binucleated hepatocytes is lost due to the breakage of the nuclear and cytoplasmic membranes. We confirmed that the majority of hepatocytes had balanced DNA content, as reported by Guidotti et al. [74]; however a high rate of binucleated cells have a discordant chromosome number between the two nuclei. This finding suggests either that they originate by fusion of two cells with different DNA content or that the two nuclei have the ability to divide asynchronously. We previously investigated the role of cell fusion in normal liver development, demonstrating a consistent contribute of cell fusion to the liver polyploidization [78]. In this work we investigated the second hypothesis, that is that asymmetric cell division can explain the unbalanced DNA content in binucleated hepatocytes (see below).
5.2 Nuclei of binucleated hepatocytes are mostly synchronous

To test the hypothesis that the unbalanced DNA content in binucleated hepatocytes is the result of asynchronous cell division within the same cell, I visualized in a 15 day- and a 5 month-old mouse the level of chromatin modification through immunostaining with anti phosho-histone H3. The phosphorylation of H3 at serine 10 occurs during interphase and mitosis. In interphase the phosphorylation of H3 affects only a subset of genes, correlates to their transcriptional activation and appears as small H3SP10 foci. However, in late G2 phase the phosphorylation of H3 occurs also in pericentromeric heterochromatin. At the G2/M stage thus an anti-H3S10P antibody is visible as a dot-like structure and is indicative of active replication. This is consistent with a role of “mitotic marker” attributed to H3S10P. In mice from both age, mononucleated cells showed a variety of dot-like structures along with a more diffuse or weak staining suggestive of cells in different stages of the cell cycle (Figure 9 A,C,E). A similar situation was found for binucleated hepatocytes (Figure 9 B,D,F), but the pattern of staining between the two nuclei was usually identical. This observation suggests that binucleated cells nuclei are synchronous, as suggested by Guidotti et al [74]. However, it cannot be excluded the possibility of fusion of two cells in different phases, like G1 or G2 and S, in which the two nuclei are synchronized before proceeding through the next phase of the cell cycle, as shown by Wong and Stearns [149]. We can also speculate that with this approach I can detect only the final step of the phenomenon. Based on this consideration, the use of an in vitro system of cell fusion would represent an additional approach to monitor this process and establish how synchronous cells evolve.
Figure 9. Immunofluorescence with anti-histone H3S10P antibody. (A, B) Examples of binucleated and mononucleated cells from a 15 day-old mouse. In this young mouse the mononucleated cells show different status of chromatin modification as detected by an anti phospho-histone H3 antibody (green). Small H3S10P foci or a diffuse staining (white arrows) are indicative of non replicative cells, while larger H3S10P foci at pericentric chromatin (red arrows) suggest that cells are proceeding to the G2/M phase. (C-F) Cells from a 5 month-old mouse. (B,D,F) Binucleated cells from young (B) and old (D,E) mice with small H3S10P foci (B,D) and with dot-like structures (F). The staining for both nuclei is comparable.

5.3 Normal hepatocytes bear extra-numerary centrosomes

The centrosome is a complex organelle that serves as the main microtubule organizing centre as well as the regulator of cell cycle progression. Centrosomes consist of microtubule-based cylinders, defined as centrioles, with a highly conserved nine-fold radial symmetry. The centriole pair are identified as the mother centriole, the old and fully mature, and the young, the immature, daughter centriole, assembled during the
The young centriole is about 80% the length of the mother centriole. Centrosomes duplicate once and only once per cell cycle. The tight regulation of centrosome number ensures that two centrosomes are present during mitosis, leading to a correct chromosome segregation into two daughter cells. The majority of diploid cells contain either one or two centrosomes, depending on their phase within the cell cycle. In order to correlate centrosome number with the occurrence of polyploidy and aneuploidy, I tested centrosomes behaviour in mouse hepatocytes by enumerating and performing functional analysis at different ages. To provide strict controls for the entire procedure, hepatocytes obtained after liver perfusion were analyzed using two distinct antibodies against γ-tubulin and pericentrin, that both recognize the centrosome structure, and for this reason colocalize.

We found a clear relationship between ploidy and centrosome number. Diploid hepatocytes from young mice contained one or two centrosome(s) as expected, however adult hepatocytes, concomitantly with switching in ploidy, contained variable numbers of centrosomes. In tetraploid and octaploid cells, 2, 4 or an even number of centrosomes is expected. Cells with these numbers were indeed present in the adult liver and were accounted as normal. On the other hand, we also detected a large fraction of cells with an unexpected centrosome number (3 or more than 4, see Table 1). This percentage increases with the age of the mice, since no abnormal centrosome distribution was seen in fetal liver cells. At 15 and 1.5 months of age, however, 11/122 (9%) and 71/123 (58%) of hepatocytes had an abnormal centrosome count (either 3 or > 4) respectively. Therefore, the level of centrosome abnormalities correlates with the changes in ploidy occurring with mouse aging. At 4 months of age, 35/56 cells (62.5%) had an abnormal centrosome number, indicating that at around 2 months of age a plateau is reached. Interestingly, binucleated liver cells with 3 centrosomes seem to be restricted to specific liver
development times, since I identified a high percentage of 3-centrosome cells concomitantly with the appearance of binucleated cells at 15 days, and this rate declines progressively until 4 months of age, at which stage cells with more than 4 centrosomes predominate (Figure 10 A,B).

![Centrosome analysis](image)

**Figure 10.** Centrosome analysis. (A, B). The plots summarize the percentage of mononucleated (A) and binucleated cells (B) classified according to the number of centrosomes found by co-immunostaining with anti γ-tubulin and anti-pericentrin. In this plotting the percentage of cells with 4 centrosomes is kept separate from cells carrying 1 and 2 centrosomes since, depending upon the DNA content, cells with 4 centrosomes could be classified as normal or abnormal.

**Figure 11** shows examples of mono and binucleated hepatocytes with a normal number of centrosomes for the 15-day mice analyzed and an abnormal number of centrosomes for the older ages analyzed. In this regard, Wong and Stearns showed that the number of centrosomes of
fused cells is strongly related to their phase in the cell cycle. Interestingly they found that fusion of a G1-phase to an S-phase cell results in generation of cells with three centrosomes [149]. It is intriguing that we found the presence of binucleated cells with 3 centrosomes as a recurrent motive during the polyploidization process in mono- as well as in binucleated hepatocytes. However the theory of asymmetric cell division was not supported by the H3 histone assay, the possibility of fusion, previously suggested by our group [78] on the basis of chimeric binucleated cells with different patterns of sex chromosomes would explain the frequency of cells with three centrosomes.

Figure 11. Examples of normal and extranumerary centrosomes. The centrosomes detected by colocalization of γ- tubulin (green) and pericentrin (red) in mono (top) and binucleated (bottom) hepatocytes for the different ages analyzed are shown.
I next attempted to directly correlate centrosome numbers to the DNA content by simultaneously detecting ploidy by FISH and centrosomes by immunofluorescence. To this end, liver cells from a 45 day-old mouse were plated in glass coverslips with an enumerated grid that allows the identification of the localization of the cells. The cells were stained with anti-α-tubulin antibody and then hybridized for the detection of chromosome Y in a combined experiment (see Materials and methods section for detail). Examples of the obtained results are shown in Figure 12.

Figure 12. Analysis of centrosomes in relation to the DNA content. Examples of hepatocytes analyzed by combined FISH (chromosome Y red) and immunofluorescence for γ-tubulin (green). A binucleated hepatocyte with two tetraploid nuclei as detected by copies of the Y chromosome carries only three centrosomes (top). A tetraploid mononucleated cell with one centrosome (abnormal condition) is shown in the middle panel together with a binucleated tetraploid cell with 4 centrosomes. We note that the centrosomes are positioned asymmetrically. A binucleated hepatocyte with six centrosomes is shown in the bottom panel.
Among 147 binucleated cells, I found that 65 showed a diploid content for each nucleus, 50 had a tetraploid content, only 1 an octaploid content and 3 had a triploid content; in the remaining cells, the DNA content was non concordant or technically difficult to assess. I confirmed that out of 65 binucleated cells with tetraploid content (two diploid nuclei) analyzed, a large proportion (35%) had an abnormal centrosome number (Figure 13), in agreement with the results reported above. Interestingly, the majority of cells with abnormal centrosomes was represented by binucleated cells with 3 centrosomes (32%). On the other end, an octaploid content (two tetraploid nuclei) was more often associated to a highest number of centrosomes (36% of these cells had more than 4 centrosomes).

**Figure 13.** The top pie summarizes the percentage of binucleated hepatocytes with both diploid nuclei showing the distribution of centrosomes (1, 2, 3, 4 and >4); on the bottom the centrosome distribution is plotted for binucleated cells with tetraploid nuclei.
The same trend was also seen in mononucleated cells (n= 41, Figure 14), although the number of examined cells for the mononucleated hepatocytes was too small for statistical relevance. The finding of an increase in the centrosome number (>4) and ploidy occurring with age (see text and Figure 11 A,B and Figure 13), suggests that the 3-centrosome stage represents an intermediate step in the progression of the hepatocyte toward a full mature phenotype.

Figure 14. Analysis of centrosomes in relation to the DNA content of mononucleated hepatocytes. The pies summarize the distribution of centrosomes number in the group of mononucleated hepatocytes with diploid (A), tetraploid (B) and octaploid (C) DNA content determined on chromosome Y signals.
The presence of abnormalities in centrosome number is an additional conundrum in the biology of hepatocytes. How a normal division could occur in presence of these abnormalities is unclear since most centrosomes appear to be able to nucleate microtubules and direct spindle formation. The high level of aneuploidy found at different ages suggests that harbouring extra-centrosomes is probably one of the liver tools to acquire genetic heterogeneity. Alternatively, cells with a high number of centrosomes could represent terminally differentiated senescent cells that would not further divide and in this case centrosomes would be the relict of previous mitoses. The next step was to investigate whether the extra-centrosomes retain the ability to nucleate microtubules and participate actively to mitosis.

5.4 Extranumerary centrosomes maintain nucleation capacity

It is well known that most mature hepatocytes are quiescent cells. These findings regarding the presence of extra-centrosomes in adult hepatocytes prompted me to investigate their role in cell physiology. Supernumerary centrosomes are actually a specific peculiarity of certain tumour cells, and even in a high proliferative context it has been shown that not all these centrosomes maintain their ability to nucleate microtubules [150]. Therefore we investigated whether the same occurs in normal hepatocytes. We performed this analysis on 21 day- and 4 month-old mice. In the young mice, in which most cells are diploid, all centrosomes showed the ability to nucleate α-tubulin, as shown in Figure 15. Interestingly, binucleated hepatocytes with 3 centrosomes do not show functional clustering (Figure 15, row 2) since the organelles seem to be distinct entities that preserve the ability to polymerize microtubules independently. The results on adult mice (4 months) showed that most centrosomes in normal hepatocytes are still potentially active and able to nucleate microtubules, even though we expect these cells to be in a quiescent state. (Figure 15 rows 3 and 4). Therefore
centrosome “inactivation” does not appear to be at work in normal polyploid liver cells. The last possibility could suggest the hypothesis of a different, yet undiscovered role for extra-centrosomes in hepatocytes biology or function. Centrosomes are involved in cilia and flagella formation, and in some specialized cells hundreds of basal bodies are formed [151]. Centrosome proteins in liver cells could mediate cell-cell interaction and a high centrosome number could play a role in the adhesion of these large cells. Although this is highly speculative, it is noteworthy that polycystic kidney patients, who have structural abnormalities of primary cilia, have additional defects in other organs including the liver [152]. Very recently, it has been found that Joubert syndrome and related disorders, whose clinical picture includes liver fibrosis, is due to a defect in the TMEM216 gene (a protein involved in ciliogenesis and centrosomal docking) [153].
Figure 15. Nucleation assay. (A, B) Examples of one mono and two binucleated cells in a 15 day-old mouse with normal and abnormal centrosomes number. All centrosomes maintain the ability to polymerase tubulin, even the binucleated cell in B with three centrosomes. (C, D) In 5 month-old mice binucleated cells with normal (1, as shown in C) and abnormal (3, as shown in D) centrosomes the nucleation assay suggests that all centrosomes are potentially active even at 4 months of age when cells are prevalently in the Go phase.
6. CONCLUSIONS

In conclusion, for the first time, we provided a detailed single cell analysis of physiological development in the liver (at 15 days, 1.5 months, 4 months, and 7 months). In this study we have documented, at the single cell level, that:

- aneuploidy is a natural phenomenon in healthy liver and arises progressively together with polyploidization;
- the extra-centrosomes represent another peculiar feature of hepatocytes. They are still active and able to nucleate α-tubulin even in mature liver cells;
- none asynchronous cell division was observed for the binucleated hepatocytes analyzed. Therefore, the loss of stoichiometry between DNA copies and centrosomes number observed in adult hepatocytes is more likely attributed to cell fusion between two cells with a different ploidy and or to an unbalanced segregation of centrosomes.

All these unexpected features of hepatocytes provide new insights on the role of aneuploidy in adult somatic tissues. Thus, selective proliferation by subsets of aneuploid hepatocytes could yield divergent effects. On one hand, hepatic aneuploidy could have pathological consequences. Aneuploidy has been extensively described in hepatocellular carcinoma [154-155], but it is unknown whether tumors arose from preexisting aneuploid hepatocytes or simply became aneuploid during tumorigenesis. It is intriguing that whole chromosome gains/losses were reported in dozens of lesions [154]. However, the unexpected high rate of aneuploidy found in healthy liver suggests that preexisting aneuploid hepatocytes could have beneficial effects or could be better tolerated in a polyploidy context. Studies in yeast have shown that increased ploidy ‘buffers’ against the adverse effects of aneuploidy [103, 114]. Analogously, the lack of
detrimental aneuploidy-associated phenotypes in hepatocytes could be due to better tolerance of altered genomic content in the presence of higher ploidy. Consistent with this idea, hepatocytes are remarkably resistant to genomic insults such as telomere erosion and defects in the chromosome segregation machinery [156-157]. On the other hand, the selection of a chromosome-specific aneuploidy in a context of chronic liver injury that restores the hepatocytes functionality demonstrates that aneuploidy can be adaptive and beneficial to the fitness of the cells. In this regard, we can speculate that the liver, with its high level of aneuploidy detected consistently overall the ages analyzed in humans and in the mouse by our group and others, can be pictured as a store of well tolerated genetic heterogeneity. In response to toxic stresses and diseases the liver may select the more beneficial chromosomal pattern to promote cellular fitness against cell deterioration. The centrosomes amplification and the unbalanced DNA content within binucleated hepatocytes, described in this work, are both features predisposing to genomic instability and could be related to the necessity of liver cells to develop aneuploidy, as it is continuously challenged by stress events that could require high gene expression of particular loci. Taken together, these observations raise an interesting conundrum. How is it possible that the presence of extra chromosomes leads to tumorigenic transformation, but at the same time cells with severe karyotypic abnormalities, like hepatocytes, are well tolerated and beneficial? To begin to shed light on this question, Williams and coworkers [118] provide an interesting twist, by showing that harbouring an extra chromosome may or may not drive a mammalian cell into oncogenesis, depending on the chromosome itself and on the state of the cells.

Further studies, at the molecular and transcriptional level, to elucidate how the hepatocytes manage to avoid neoplastic transformation are needed to better understand in deep the biology of these peculiar cells.
However, looking for a specific cellular marker for aneuploid cells will give the chance to extrapolate aneuploid cells from their natural context and to provide an invaluable resource for the analysis of several phenomena, like aging, cancer and cellular senescence.
7. REFERENCES

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9. SCIENTIFIC PRODUCTS

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