Clonal selection of stable aneuploidies in progenitor cells drives high-prevalence tumorigenesis

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Chromosome gains and losses are a frequent feature of human cancers. However, how these aberrations can outweigh the detrimental effects of aneuploidy remains unclear. An initial comparison of existing chromosomal instability (CIN) mouse models suggests that aneuploidy accumulates to low levels in these animals. We therefore developed a novel mouse model that enables unprecedented levels of chromosome missegregation in the adult animal. At the earliest stages of T-cell development, cells with random chromosome gains and/or losses are selected against, but CIN eventually results in the expansion of progenitors with clonal chromosomal imbalances. Clonal selection leads to the development of T-cell lymphomas with stereotypic karyotypes in which chromosome 15, containing the Myc oncogene, is gained with high prevalence. Expressing human MYC from chromosome 6 (MYC Chr6) is sufficient to change the karyotype of these lymphomas to include universal chromosome 6 gains. Interestingly, while chromosome 15 is still gained in MYC Chr6 tumors after genetic ablation of the endogenous Myc locus, this chromosome is not efficiently gained after deletion of one copy of Rad21, suggesting a synergistic effect of both MYC and RAD21 in driving chromosome 15 gains. Our results show that the initial detrimental effects of random missegregation are outbalanced by clonal selection, which is dictated by the chromosomal location and nature of certain genes and is sufficient to drive cancer with high prevalence.

Keywords: aneuploidy; cancer; chromosomal instability; MYC; oncogene; RAD21

Supplemental material is available for this article.

Received February 2, 2021; revised version accepted June 4, 2021.

Aneuploidy, or the presence of a number of chromosomes that is not an exact multiple of the haploid number, is a common finding in human cancer. The degree of aneuploidy correlates with poor prognosis and resistance to therapy, although how aberrant chromosome numbers contribute to cancer development is not clear (Weaver and Cleveland 2006; Duijf and Benezra 2013). A number of mouse models of aneuploidy induced by chromosomal instability (CIN) have been generated to study the direct effects of abnormal chromosome numbers at the molecular, cellular, and physiological level. These studies have suggested that aneuploidy can function both as a tumor suppressor or an oncogenic alteration, and whether this outcome is related to aneuploidy levels is unclear due to the different alterations to induce aneuploidy and technical differences in how the degree of aneuploidy was studied (Weaver and Cleveland 2006; Holland and Cleveland 2009; Schwartzman et al. 2010). Mutations in a surveillance pathway known as the spindle assembly checkpoint (SAC) cause CIN. In wild-type cells, the SAC ensures that anaphase only occurs after chromosomes have properly attached to the metaphase spindle (Musacchio and Salmon 2007). SAC components assemble on unattached kinetochores, where they

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facilitate the inhibition of a ubiquitin ligase known as the anaphase-promoting complex/cyclosome (APC/C) by sequestering its activator CDC20 throughout the cell. Mutations in CDC20 (CDC20AAA) that prevent binding of CDC20 to the SAC component MAD2 render the ubiquitin ligase refractory to SAC inhibition (Li et al. 2009). As a result, cells enter anaphase before chromosomes are properly bioriented on the mitotic spindle, leading to high levels of chromosome missegregation. When expressed in mice as the sole source of CDC20, the Cdc20AAA allele causes lethality at embryonic day (E) 12.5, indicating that the allele causes levels of chromosome missegregation that are incompatible with embryonic development (Li et al. 2009). Animals heterozygous for the Cdc20AAA allele are viable and develop hepatomas and lymphomas only late in life (Li et al. 2009).

In this work, we performed a systematic analysis of the prevalence of aneuploid cells in previously reported CIN models by using single-cell DNA sequencing technology in freshly isolated cells. To increase the levels of aneuploidy in vivo, we generated an inducible mouse model in which the wild-type Cdc20 allele can be excised at will, generating cells that express CDC20AAA as the sole source of CDC20 (Fig. 1A). Analysis of the dynamics of chromosomal gain and losses in the thymus suggests that random aneuploidies form at initial stages and are eventually substituted by stereotypical karyotypes, including gains in chromosome 15, harboring the Myc gene, leading to high T-cell lymphomas with short latency. Expressing human MYC from chromosome 6 changes tumor karyotypes, leading to universal gains of this chromosome, suggesting that clonal selection of random aneuploidies determines tumor karyotypes and is sufficient to cause malignant cell transformation and cancer. Unexpectedly, the gene encoding the cohesin subunit RAD21 is a major driver of chromosome 15 gains in CIN- and MYC-driven lymphomas.

Results

A novel model with high levels of chromosomal instability and high prevalence of T-cell lymphomas

We initially assessed the extent of aneuploidy in young [3-mo-old] mice from representative models of CIN with specific genetic modifications in genes encoding mitotic kinases (Plk1, Plk4, and Aurkb) or SAC components [Mad2 and Bub1b, encoding BUBR1] (Supplemental Table S1) using single-cell DNA sequencing (Knouse et al. 2014). A comparative analysis in all these models identified 2%–
20% of aneuploid cells in peripheral blood or the intestine (Fig. 1B), in agreement with previous data obtained using a variety of different techniques [see Supplemental Table S1 for details].

To increase levels of aneuploidy in vivo, we generated a new inducible model by combining the Cdc20AAA allele [Li et al. 2009] with a conditional knockout model (Cdc20lox/lox) in which the Cdc20 allele can be excised by a tamoxifen-inducible CRE recombinase ubiquitously expressed from the RNA polymerase II [Polr2a] promoter (ERT2 allele) [Fig. 1A; Manchado et al. 2010]. Dietary tamoxifen administration [Supplemental Fig. S1A] resulted in the excision of the functional Cdc20lox allele, thereby generating cells that express CDC20AAA as the sole source of CDC20 (ERT2-Cdc20AAA mice, referred to here as Cdc20AAA). Cdc20AAA spleocytes displayed normal levels of DNA replication but were insensitive to mitotic inactivation in the excision of the functional Cdc20lox allele, thereby generating cells that express CDC20AAA as the sole source of CDC20 (ERT2-Cdc20AAA mice, referred to here as Cdc20AAA). The presence of aberrant mitoses (Supplemental Fig. S1B) was due to increased susceptibility to thymic lymphomas within the first 10 mo of tamoxifen treatment [Fig. 1F; Supplemental Fig. S1E]. These tumors were mostly comprised of proliferating CD8+ or immature CD4+ CD8+ double-positive (DP) T cells [Fig. 1G; Supplemental Fig. S1F]. We also detected hyperplasias in other Cdc20AAA tissues such as squamous epithelia of the forestomach, ovaries, endometrium, and the pancreas [Supplemental Fig. S1G]. Carcinomas were rare, likely because animals succumbed to T-cell lymphomas before more aggressive disease could develop. We conclude that high levels of CIN correlate with tumorigenesis and can induce hyperplastic lesions in a variety of tissues, leading to frequent lethal malignant transformation in the T-cell compartment.

T-cell lymphomas harbor characteristic chromosome gains

Bulk DNA sequencing of Cdc20AAA T-cell lymphomas revealed that these tumors were aneuploid in all cases [Fig. 2A,B]. When applying a cutoff in the CNV score that deviates three standard deviations from the euploid value (see the Materials and Methods), all chromosomes with the exception of chromosomes 7 and 19 were found to show numerical alterations in at least some lymphomas [Fig. 2B]. Whole-chromosome losses or focal deletions were not a frequent occurrence in Cdc20AAA T-cell lymphomas. However, we did note the loss of the distal region of chromosome 12 in some tumors [Fig. 2A]. This region contains the tumor suppressor Bcl11b, as well as several tumor suppressor microRNAs, and has been previously reported to be lost in T-cell lymphomas in different models [Bueno et al. 2008; De Keersmaecker et al. 2010]. However, two chromosome gains stood out. Chromosome 15 was gained in all Cdc20AAA tumors, whereas chromosome 14 was gained in 89% of tumors [Fig. 2B,C]. These chromosome number alterations were also observed using complementary techniques such as Giemsa staining (Fig. 2D) or spectral karyotyping (Fig. 2E) of Cdc20AAA lymphoma cells. We also confirmed the presence of these alterations using single-cell DNA sequencing from several cells isolated from individual tumors [Fig. 2F]. We conclude that T-cell lymphomas harboring recurrent chromosome gains develop with high penetrance in mice missegregating chromosomes at a high frequency.

We next compared the karyotype of Cdc20AAA tumors with those induced by lack of P53, a well-studied model of T-cell lymphomas. Whereas chromosome 15 was gained in two out of five tumors, Trp53−/− lymphomas displayed a higher karyotypic heterogeneity with frequent gains in chromosome 5 [Supplemental Fig. S2A,B]. To analyze whether chromosome 15 gain was dispensable in the presence of a compromised P53 pathway, we crossed the Trp53 conditional knockout mice with RERT2.Cdc20AAA mice. Unfortunately, both the Trp53 locus and the Polr2a locus, where the Cre-ERT2 cassette is integrated, are located in close proximity on chromosome 11; hence, we could...
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not generate and analyze Trp53\textsuperscript{\textDelta}; Cdc20\textsuperscript{\Delta/AAA}. However, ablation of one Trp53 allele significantly accelerated tumorigenesis in Trp53\textsuperscript{+/\Delta}; Cdc20\textsuperscript{\Delta/AAA} mice, reducing the median tumor free survival to 6.13 mo [Supplemental Fig. S2C]. These tumors, similarly to Cdc20\textsuperscript{\Delta/AAA}, showed universal gain of chromosome 14 and 15 but additionally displayed frequent gains of chromosome 4 and 11 [Fig. 2, cf. G, H and A, B, Supplemental Fig. S2D]. Interestingly, the presence of functional Trp53 alleles might prevent the gain of chromosome 11, never observed in Cdc20\textsuperscript{\Delta/AAA} mice [Fig. 2B] but present in more than half of Trp53\textsuperscript{+/\Delta}; Cdc20\textsuperscript{\Delta/AAA} tumors [Fig. 2H]. As described previously

Figure 2. Karyotype distribution in Cdc20\textsuperscript{\Delta/AAA} T-cell lymphomas. (A) Representative segmentation plots of three different T-cell lymphomas from Cdc20\textsuperscript{\Delta/AAA} mice, depicting relative copy number per chromosome to a euploid reference on a log\textsubscript{2} scale as scored by bulk DNA sequencing. Chromosomal gains are in red and losses in green. (B) Quantification of the percentage of Cdc20\textsuperscript{\Delta/AAA} tumors in which each of the individual chromosome gain or loss is present (n = 35 tumors). A chromosome was considered gained when \(\log_{2}\) relative copy number > 0.23 and lost when this value is < −0.32 [see the Materials and Methods]. Statistics refer to the comparison with lack of gains and losses in 18 Cdc20\textsuperscript{\Delta/AAA} thymic cells analyzed. (C) Relative copy number [\(\log_{2}\)] of chromosomes 14 and 15 in Cdc20\textsuperscript{\Delta/AAA} lymphomas. Each dot indicates an individual tumor. The dashed region indicates samples in which the copy number is above the threshold selected to define chromosomal gains (mean value + 3 SD in euploid cells). (D) Giemsa staining of a representative metaphase spread from a Cdc20\textsuperscript{\Delta/AAA} lymphoma. (E) Spectral karyotype of a representative metaphase spread from a Cdc20\textsuperscript{\Delta/AAA} lymphoma. (F) Schematic representation of chromosomal gains (red) and losses (green) as detected by single-cell DNA sequencing (scDNA-seq) in primary tumors or bone marrow infiltrations from a tumor. Each row represents a single cell. (G) Representative examples of chromosomal gains [red] or losses [green] in three different T-cell lymphomas from Trp53\textsuperscript{+/\Delta}; Cdc20\textsuperscript{\Delta/AAA} mice as scored by bulk DNA sequencing. (H) Quantification of the percentage of Trp53\textsuperscript{+/\Delta}; Cdc20\textsuperscript{\Delta/AAA} tumors with the indicated specific chromosome gains or losses (n = 20 tumors). Statistics refer to the comparison with gains and losses in Cdc20\textsuperscript{\Delta/AAA} lymphomas in B. In B and H, (*) \(P < 0.05\), (***) \(P < 0.001\) [Fisher exact test]; nonsignificant (\(P > 0.05\)] differences not shown.
(Baker et al. 2009), we predicted that Trp53+/Δ, Cdc20+/AAA tumors might lose the wild-type Trp53 allele. Indeed, we observed an almost complete absence of P53 at the protein level in Trp53+/Δ, Cdc20+/AAA tumors [Supplemental Fig. S2E]. In addition, P53 was not detected in these double-mutant T-cell lymphomas after a sublethal dose of γ-irradiation, suggesting loss of the wild-type allele. Interestingly, mutant T-cell lymphomas after a sublethal dose of S2E. In addition, P53 was not detected in these double-mutant T-cell lymphomas are different from P53-deficient tumors, maintaining at least partially P53 functionality.

Clonal evolution of karyotypes in premalignant tissues
To determine whether and how CIN interferes with T-cell maturation and leads to lymphomagenesis, we assessed T-cell development prior to tumor development. Beginning at 3 mo post initiation of tamoxifen treatment, Cdc20+/AAA mice presented with a pronounced reduction in total thymocyte number as judged by significant atrophy of the thymus [Fig. 3A], akin to that observed in other T-cell lymphoma models (De Keersmaecker et al. 2010). In these atrophic thymuses, we noted a significant increase in the relative percentage of double-negative [DN] 1 population (CD25low, CD44highb) [Supplemental Fig. S3A] that was accompanied by a reduction in DN3, DN4, and CD4+ CD8+ double-positive [DP] cells [Fig. 3B], suggesting an inefficient transition from DN1 to later stages.

We next studied the karyotypic landscape of T-cell precursors in Cdc20+/AAA animals prior to disease onset. We analyzed karyotypes at single-cell resolution in sorted hematopoietic stem cells [HSCs] and common lymphoid progenitors [CLPs] in the bone marrow, as well as DN1–4 and DP cells from tumor-free thymuses at 4 mo after the induction of tamoxifen treatment. This analysis revealed that 32% of HSCs were already aneuploid and this number further increased in the CLP population [Fig. 3C, Supplemental Fig. S3B,C]. Interestingly, the degree of aneuploidy significantly dropped in the DN1 stage [14% in DN1 vs. 55% in HSCs and 41% in CLPs] [Fig. 3C], suggesting the existence of a bottleneck for aneuploid CLPs during their transit to the thymus and the subsequent development of DN1 progenitors. However, the ratio of aneuploid cells steadily increased in later stages, reaching peak levels of up to 80% in the DP population [Fig. 3C, Supplemental Fig. S3C], in agreement with the
known proliferative expansion of T-cell progenitors in the thymus [Supplemental Fig. S1F].

Given that chromosome missegregation during mitosis in SAC-perturbed models happens randomly, we analyzed whether specific chromosome gains or losses were selected. Early progenitors in the bone marrow displayed random gains or losses, affecting most chromosomes in a similar manner without any specific chromosome being gained or lost in >5% of the cells analyzed [200 HSCs and 163 CLPs] (Fig. 3D). Similarly, DN1 cells displayed no enrichment in specific gains or losses, although this analysis was limited due to the low number of aneuploid DN1 cells. Interestingly, the overrepresentation of specific imbalances increased toward the later stages of T-cell maturation (DN2 to DP) (Fig. 3D; Supplemental Fig. S3B,C). Enrichment in specific losses affecting chromosome 11 or gains in chromosomes 14 and 15 was especially obvious and significant, suggesting that cells with these karyotypes are endowed with an advantage. We defined the cell clonality index as the ratio of aneuploid cells carrying identical chromosomal gains or losses [see the Materials and Methods]. Interestingly, whereas ~40% bone marrow cells were aneuploid, most of these alterations were random and <13% of these aneuploid cells were clonal (Fig. 3E). However, the clonality index increased from 28% up to 60% during the DN2–DP transitions. The increase in cell clonality was mostly a consequence of the increase in aneuploid cells with gains of chromosomes 14 and 15 among others [Supplemental Fig. S3D]. These results suggest that engraftment into the thymus, which occurs during the transition from the CLP to DN1 stage, favors euploid cells, and proliferative expansion during T-cell maturation is accompanied by clonal selection of cells carrying specific aneuploidies such as chromosomes 14 and 15 gains in Cdc20Δ+/AAA animals.

**Aneuploid cells require proliferation to undergo clonal selection**

To test the specific effect of CIN in later developmental stages during T-cell development, we induced chromosome missegregation in the DN3 state by driving CRE expression from the LCK promoter, or during the DN4–DP transition by driving CRE expression from the CD4 promoter (Supplemental Fig. S3A). We found that animals in which chromosome missegregation was induced during these later stages of T-cell development did not develop T-cell lymphomas during the first 15 mo. Analysis of the degree of aneuploidy in tissues provided a potential explanation for why mice in which aneuploidy was driven by LCK-CRE did not develop tumors. This CRE driver yielded low levels of aneuploidy (Fig. 3F), which is consistent with the known incomplete penetrance of LCK-CRE expression [Lee et al. 2001; Pai et al. 2003]. In contrast, CRE expression driven by CD4 led to relatively high levels of aneuploidy in DP cells [50%] (Fig. 3F,G; Supplemental Fig. S3E), similar to levels found in Cdc20Δ+/AAA cell populations in which clonal selection has not been taken place (e.g., HSC and CLP in Fig. 3C). Intriguingly, CD4-CRE, Cdc20Δ+/AAA DP cells did not accumulate specific aneuploidies [Supplemental Fig. S3D], and gains and losses affected most chromosomes similarly [cell clonality ~ 0%]. The fact the vast majority (~90%) of DP cells do not proliferate [Seitan et al. 2011; Mingueneau et al. 2013] is in agreement with the observation of random losses of chromosomes, which are typically selected against in proliferating cells. It is therefore tempting to speculate that lack of proliferation could preclude clonal selection in this model.

**Chromosomal location of MYC determines clonal selection of specific aneuploidies**

How does CIN drive lymphomagenesis? To address this question, we compared the gene expression profiles of pretumoral T-cell populations and T-cell lymphomas in RERT2, Cdc20Δ+/AAA mice. Dimensornality reduction analysis of pretumoral and tumoral samples separated the different T-cell populations and indicated that T-cell lymphomas expressed intermediate levels of CD8 and CD4 (Fig. 4A; Supplemental Fig. S4A). We cannot exclude the possibility that tumor cells clustered between DN4 and DP population as a consequence of increased proliferation, a characteristic feature of DN4 cells opposed to the mainly nonproliferative DP population. The gene expression pattern of pretumoral T-cell precursor populations DN1–4 did not significantly differ between Cdc20Δ+/AAA and Cdc20Δ+/Δ animals (Fig. 4A, Supplemental Table S2), but we observed differences in the DP population. Pretumoral DP Cdc20Δ+/AAA cells displayed enrichment in pathways related to DNA replication, MTOR, and glycolysis, compared with Cdc20Δ+/Δ DP cells (Fig. 4B, Supplemental Fig. S4B; Supplemental Table S3). Interestingly, the genes significantly deregulated in Cdc20Δ+/AAA DP cells were enriched for targets of MYC:MAX [Zeller et al. 2003], a transcription factor of major relevance in many of the biological processes deregulated in these cells [Fig. 4B,C; Supplemental Table S4]. Consistent with this observation, Myc RNA levels were significantly up-regulated in pretumoral Cdc20Δ+/AAA DP cells when compared with control Cdc20Δ+/Δ DP cells (Fig. 4D), and this tendency was also observed at the protein level [Fig. 4E]. Analysis of the gene expression pattern of T-cell lymphomas in Cdc20Δ+/AAA animals suggested that Myc expression was further increased in these tumors when compared with pretumoral DP cells from mice of the same genotype [Fig. 4D,E], and up-regulation of MYC target genes was more pronounced [Fig. 4B,C; Supplemental Fig. S4C; Supplemental Tables S5, S6]. These results suggested that MYC, a well-known driver of T-cell lymphoma and other hematopoietic malignancies [Gaudet et al. 2003; Meyer and Penn 2008], was also driving tumorigenesis in the Cdc20Δ+/AAA CIN model.

The observation that chromosome 15, where Myc resides, is gained in all Cdc20Δ+/AAA-driven T-cell lymphomas [Fig. 2B,C] further raised the interesting possibility that copy number increase of Myc drives this chromosome gain. We directly tested this hypothesis by examining the consequences of expressing MYC from chromosome 6 during karyotype evolution of Cdc20Δ+/AAA-driven T-cell lymphomas. To this end, we made use of a MYC transgenic allele [Calado et al. 2012] in which the human MYC
Myc, using the Lck- and CD4-Cre recombinase alleles (Supplemental Fig. S3A). The total number of thymic cells or distribution of populations was not altered in these models (Supplemental Fig. S5C,D). Despite this early lethality due to tumorigenesis in other tissues precluded an in-depth analysis of T-cell lymphomas. To specifically examine T-cell lymphoma development, we analyzed the consequences of expressing Myc on tumorigenesis using the Lck- and CD4-Cre recombinase alleles [Supplemental Fig. S3A]. The total number of thymic cells or distribution of populations was not altered in these models (Supplemental Fig. S6A,B). Whereas the CRE-inducible Myc Chr6 transgene led to a 40% incidence of T-cell lymphomas in a Cdc20-Δ/Δ background, Cdc20Δ/Δ; Myc Chr6 developed thymic lymphomas with a median survival of 3.2 and 3 mo, respectively, in mice in which CIN was driven by Lck-CRE and CD4-CRE (Fig. 5D). Transcriptional profiling and differential expression analysis of the different models indicated that Cdc20Δ/Δ; Myc Chr6 tumors were also mostly driven by Myc-dependent transcription (Fig. 5E,F).

Interestingly, karyotype analysis of Lck-CRE; Cdc20Δ/Δ; Myc Chr6 and CD4-CRE; Cdc20Δ/Δ; Myc Chr6-driven T-cell lymphomas revealed that, while chromosome 6 was rarely gained in Cdc20Δ/Δ; Myc Chr6 tumors [Fig. 2B], >90% of Lck-CRE; Cdc20Δ/Δ; Myc Chr6 and CD4-CRE; Cdc20Δ/Δ; Myc Chr6 tumors displayed chromosome 6 gains [Fig. 6A–C]. Similarly, chromosome 6 was gained in two out of three tumors in Rert2; Cdc20Δ/Δ; Myc Chr6 mice [Fig. 6D]. Whereas chromosome 14 gains were not frequent in the presence of the Myc Chr6 allele, gains in chromosome 6 did not prevent concomitant gains of chromosome 15 in >80% of
these tumors (Fig. 6A,B,D). Partial or complete genetic ablation of Trp53 in CIN- and MYC<sup>Chr6</sup>-driven lymphomas (Supplemental Fig. S6C) similarly resulted in universal gains of chromosome 6. In these tumors, chromosome 6 gains were accompanied of gains of chromosome 15, as well as imbalances observed in P53-null thymic lymphomas such as gains of chromosome 5 (Supplemental Fig. S6D). All together, these data indicate that expressing MYC from a different chromosome is sufficient to lead to almost universal gains of the chromosome where MYC is expressed from, thus changing the stereotypical tumor karyotype.

**Genes in addition to MYC drive chromosome 15 gain in CIN-induced T-cell lymphoma**

Although chromosome 15 gain was slightly less prevalent in T-cell lymphomas from Cdc20<sup>Δ/AAA</sup>; MYC<sup>Chr6</sup> samples when compared with RERT2-CRE, Cdc20<sup>Δ/AAA</sup>-driven T-cell malignancies, it was nevertheless a frequent occurrence (Fig. 6D). This result raised the possibility that, even in the presence of exogenous MYC, the endogenous Myc encoded on chromosome 15 was still contributing to T-cell lymphomagenesis. To test this possibility, we first examined the expression of transgenic human [h] and endogenous murine [m] MYC. We were able to distinguish these two forms of MYC because hMYC migrates slightly faster on SDS-PAGE than mMYC. hMYC was expressed at similar levels as mMYC in pretumoral T cells of RERT2-Cre; Cdc20<sup>Δ/Δ</sup>; MYC<sup>Chr6</sup> mice (Fig. 7A). Surprisingly, Cdc20<sup>Δ/AAA</sup>; MYC<sup>Chr6</sup> tumors, driven by both the RERT2-CRE or LCK-CRE allele, primarily expressed hMYC and not mMYC (Fig. 7B). This loss of mMYC expression was due to down-regulation of transcription. Human MYC transcripts were low in pretumoral MYC<sup>Chr6</sup> samples and significantly increased in tumoral MYC<sup>Chr6</sup> samples (Fig. 7C,D) in agreement to what observed at the protein level (Fig. 7B). Murine Myc transcripts, on the other hand, were detected in pretumoral T cells and Cdc20<sup>Δ/AAA</sup> tumors but not in T-cell lymphomas of Cdc20<sup>Δ/AAA</sup>; MYC<sup>Chr6</sup> mice (Fig. 7C,D). This down-regulation is likely a consequence of negative feedback loops described previously [Cole 2014], which limit expression of the endogenous Myc gene in presence of abundant MYC signaling. Importantly, these data indicated that increasing mMYC levels is unlikely to be the only reason for why chromosome 15 is frequently gained in Cdc20<sup>Δ/AAA</sup>; MYC<sup>Chr6</sup>-driven T-cell lymphomas. Instead, the lack of Myc expression indicates that other genes located on chromosome 15 may contribute to T-cell lymphomagenesis in Cdc20<sup>Δ/AAA</sup>; MYC<sup>Chr6</sup> mice.

To directly test the importance of endogenous Myc in MYC<sup>Chr6</sup>-driven T-cell lymphomagenesis, we deleted the endogenous Myc gene encoded by chromosome 15 using an additional Myc<sup>lox</sup> allele in a Cdc20<sup>Δ/AAA</sup>; MYC<sup>Chr6</sup> background. CRE activity in this model results in the concomitant activation of MYC on chromosome 6 and ablation of Cdc20 and Myc floxed alleles (Fig. 7E). Unexpectedly, karyotype analysis showed that chromosome 15 was invariably gained in T-cell lymphomas obtained from this model despite the complete lack of endogenous Myc in this model (Fig. 7F). This result, along with the silencing of the endogenous Myc in Cdc20<sup>Δ/AAA</sup>; MYC<sup>Chr6</sup> tumors, indicated that genes in addition to Myc may contribute to chromosome 15 gains in T-cell lymphomas in these models.
Rad21 contributes to chromosome 15 gains in CIN-induced T-cell lymphoma

MYC is commonly amplified with RAD21 in multiple human tumors (8p24 amplicon), and both genes map close together in human chromosome 8 or mouse chromosome 15. RAD21 expression, which is transcriptionally regulated by MYC, prevents excessive replicative stress caused by this oncogene, thus contributing to MYC-induced tumorigenesis (Rohban and Campaner 2015; Rohban et al. 2017). We therefore directly tested the possibility that increased copies of Rad21 may contribute to chromosome 15 gains by using a Rad21Δ/Δ conditional allele driven by CD4-CRE expression (Fig. 7G; Seitan et al. 2011). Homozygous CD4-CRE-mediated deletion of Rad21 results in lethality when thymocytes are forced to proliferate [Seitan et al. 2011], preventing further tumorigenesis studies in vivo. However, ablation of a single copy of Rad21 in a CIN- and MYCChr6-driven background (CD4-CRE, Cdc20Δ/ΔAA; MYCChr6; Rad21Δ/Δ) resulted in the development of T-cell lymphomas with slightly increased dynamics compared with Rad21+/+ models [Supplemental Fig. S7A]. This observation is in agreement with a haplinsufficient role for RAD21 in vivo and the tumorigenic effect of decreasing RAD21 expression levels in the hematopoietic system [Mullenders et al. 2015]. Interestingly, whereas gains of chromosome 6 or chromosome 14 were similar to mice with wild-type Rad21, loss of a single copy of Rad21 resulted in decreased number of tumors [four out of seven] with 15 gains compared with control models [Fig. 7H; Supplemental Fig. S7B]. Unexpectedly, MycΔ/Δ lymphomas displayed a higher CNV score for chromosome 15 gains, possibly suggesting more than three copies of this chromosome in Cdc20Δ/ΔAA- and MYCChr6-driven tumors. The CNV score for chromosome 15, on the other hand, was significantly lower in Rad21Δ/Δ T-cell lymphomas when compared with Rad21+/+ tumors [Fig. 7I]. Based on these observations, we speculate that, in the presence of exogenous MYC on chromosome 6 and a CIN background, chromosome 15 gains are driven by Rad21.

Discussion

Previous computational studies suggested that cancer aneuploidies are driven by copy number changes in oncogenes and tumor suppressor genes [Davoli et al. 2013]. However, whole-chromosome gains and losses are generally detrimental, causing multiple cellular stresses that are accompanied by a p53-dependent antiproliferative response [Thompson and Compton 2010; Santaguida and Amon 2015]. In fact, single-chromosome gains can be robust indicators of tumor growth [Sheltzer et al. 2017]. Early work investigating the role of CIN in driving LOH found that chromosome missegregation initially promotes loss of the chromosome carrying the wild-type Trp53 allele in Trp53+/- mice, thus resulting in P53-null cells. However, aneuploidy was quickly reversed as tumor cells gained a second copy of the chromosome carrying the mutant Trp53[−/−] allele [Baker et al. 2009]. The fact that Trp53[−/−] cells require two chromosome missegregation events to develop into tumors [and restore euploidy] thus indicates that losing a copy of the whole chromosome containing Trp53 antagonizes tumorigenesis, presumably due to haplinsufficiency of genes located on the monosomic chromosome. Given the significant

Figure 6. MYC drives specific chromosome gains in Cdc20Δ/ΔAA tumors. (A) Representative karyotype of three tumors from LCK-CRE, Cdc20Δ/ΔAA, MYCChr6 mice as determined by DNA sequencing. The plot to the right shows the distribution of chromosomal gains and losses in these animals [n = 23]. (B) Representative karyotype of three tumors from CD4-CRE, Cdc20Δ/ΔAA; MYCChr6 mice as determined by DNA sequencing. The plot to the right shows the distribution of chromosomal gains and losses in these animals [n = 11]. In A and B, statistics refer to the comparison versus gains and losses in Cdc20Δ/ΔAA thymic lymphomas [Fig. 2B]. [**] P < 0.05, [***] P < 0.01, [****] P < 0.001 (Fisher exact test); nonsignificant (P > 0.05) differences not shown. (C) Representative SKY analysis of a T-cell lymphoma in LCK-CRE, Cdc20Δ/ΔAA, MYCChr6 mice showing specific gains of chromosomes 6 and 15, among others. (D) Relative copy number of chromosomes 6, 14, and 15 in T-cell lymphomas from mice with the indicated genotypes. The red-shaded region indicates copy number values higher than the threshold selected for chromosome gains (the mean + 3 SD of the euploid value). Data for chromosomes 14 and 15 in RERT2, Cdc20Δ/ΔAA- are taken from Figure 2C for comparison. Each dot indicates an individual tumor.
fitness penalties associated with whole-chromosome aneuploidies, it was thus not clear whether gains or losses of specific oncogenes or tumor suppressor genes could outweigh these adverse effects to drive tumorigenesis.

Our comparative analysis of a number of CIN models, using single-cell DNA sequencing as a standardized protocol, suggests that previous murine CIN models display low or moderate levels of aneuploidy. A novel model in which high levels of CIN are induced by expressing Cdc20 AAA, the only cellular source of CDC20 in cells results in high prevalence tumorigenesis, and the aggregated data from all these models suggest that levels of aneuploidy in healthy tissues positively correlate with tumor susceptibility later in life.

A detailed analysis of the dynamics of aneuploidy during T-cell maturation suggests that CIN initially results in random accumulation of chromosomal gains and losses in early progenitors. However, specific karyotypes are selected during the proliferative phases of T-cell maturation, leading to the emergence of clones further selected during malignant transformation, giving rise to T-cell lymphomas. Gain of chromosome 15, where the Myc encoding gene resides, is an obligatory alteration that probably prevents DP cells from down-regulating pathways that stimulate proliferation. Myc is a potent oncogene involved in 70% of human cancers and is a major driver of T-cell acute lymphoblastic leukemia (T-ALL) and T-cell lymphomas, where it functions as a central node downstream from major oncogenic pathways, including NOTCH or TAL1/ARID5B (Weng et al. 2006; Palomero et al. 2007; Leong et al. 2017). In murine thymic lymphomas, Myc is typically overexpressed (McMorrow et al. 1988; Gaudet et al. 2003; Yoshida et al. 2007; Bueno et al. 2008; Timakhov et al. 2009; De Keersmaecker et al. 2010). Furthermore, gain of chromosome 15, where MYC is typically overexpressed (McMorrow et al. 1988; Gaudet et al. 2003; Yoshida et al. 2007; Bueno et al. 2008) or Rad21 mutant alleles by CRE recombinase (De Keersmaecker et al. 2010). However, specific karyotypes are selected during the proliferative phases of T-cell maturation, leading to the emergence of clones further selected during malignant transformation, giving rise to T-cell lymphomas.
with increased chromosomal instability, is also accompa-
nied by chromosome 15 gains (Venkatachalam et al. 1998; Haines et al. 2006; Bakker et al. 2016). Interestingly, p53 seems to be at least partially functional in Cdc20Δ/AAA lymphomas, suggesting that high levels of CIN may over-
come the need of eliminating p53 function. This is an in-
teresting observation that would require further research in
the future.

How does MYC drive thymic lymphomagenesis in Cdc20Δ/AAA animals? DN3 thymocytes depend on li-
gand-induced Notch signaling to maintain MYC expres-
sion [Weng et al. 2006]. Upon pre-T-cell receptor [pre-
TCR] expression, they lose their dependence on Notch and cytokine signaling and differentiate into CD4+
CD8+ DP cells, in which MYC is typically repressed. We propose that in aneuploid Cdc20Δ/AAA DP cells, this re-
pression is lost as a consequence of frequent chromosome
15 gain, which then contributes to the development of
lymphomas. Interestingly, while MYC expression is higher
in Cdc20Δ/AAA thymic lymphomas compared with pre-
tumoral DP cells, it is still lower than in normal DN2,
DN3, and DN4 cells. Thus, it appears that the amount of
MYC needed to drive T-cell lymphomagenesis is not
greater than what is observed in highly proliferative lympho-
cyte populations. In fact, Cdc20Δ/AAA MYCChr6,
MycΔ/Δ mice display higher CNV score for chromosome
15 gains than the corresponding Myc+/+ controls [Fig.
7]. One possible explanation for this observation arises
from the fact that MYC overexpression induces replica-
tive stress and a DNA damage response that acts as a bar-
er to tumor development [Halazonetis et al. 2008; Rohban and Campaner 2015]. This replication stress can
be managed in tumor cells through the expression of
RAD21, a component of the cohesin complex involved
in transcription, repair of DNA double-strand breaks, as
well as in chromatid cohesion during mitosis [Losada
2014]. MYC and cohesin cooperate in triggering tran-
scription from specific sites in the genome [Yan et al. 2013],
and RAD21 reinforces cohesion-mediated DNA synthe-

Materials and methods

Mouse models

The Cdc20AAA [Li et al. 2009], Cdc20lox/lox [Manchado et al. 2010],
Myclox/loxl (de Albornoz et al. 2001), Rad21lox/loxl (Seitan et al. 2011),
and Rosa26-LSL-CAG-Myc-hCD2 [MYCChr6]Calado et al. 2012) alleles were reported previously. For excision of loxP-flanked se-
quences, we used a tamoxifen-inducible CRE recombinase ubiq-
ityously expressed under the control of the ROSA26 R26R allele [Manchado et al. 2010]. To activate Cre during specific stages of
T-cell development, we used constitutive LCK-CRE or CD4-CRE
[Lee et al. 2001]. Tamoxifen was administered in the diet [Envigo
TD.130860], alternating tamoxifen diet with normal minimal
phytoestrogen diet (Teklad global 16% protein, Envigo). Other
mouse models used in this work are listed in Supplemental Table
S1 [Baker et al. 2004; Malureau et al. 2010; González-Loyola
et al. 2015; Levine et al. 2017; del Cárter et al. 2018]. Mice were in
a mixed C57/B16x129/1 mixed background. All animal proce-
dures were performed following the institutional guidelines and
protocols approved by the Massachusetts Institute of Technology
Committee for Animal Care and the corresponding national
legislation.

For histological analyses, dissected organs were fixed in 10% buffered formalin [Sigma] and embedded in paraffin wax. Sections of
3- or 5-μm thickness were stained with hematoxylin and eosin
[H&E]. Additional immunohistochemical examinations of the tissues and pathologies were performed using specific antibodies
against Ki67 [Abcam ab16667], phospho-histone H3 S10 [Milli-
pore 06-570], and active caspase 3 [Cell Signaling 9661].

Cell isolation from tissues and tumors and flow cytometry

Bone marrow, spleenocytes, thymocytes, and intestinal crypt
T-cells were isolated as described previously [Plau et al. 2016].
Briefly, after obtaining a single-cell suspension, cells were incu-
bated for 3 min in ACK buffer for red blood cell lysis and washed
in IMDM containing 0.5% BSA. Cells were counted with a Coul-
ter counter analyzer, and 3 million cells were stained with anti-
bodies for CD8a, CD4, CD25, and CD44 [BD Biosciences] for
thymic profile analysis. For the isolation of HSCs and CLPs, 50
million to 80 million bone marrow cells were incubated with a
mouse lineage antibody cocktail labeled with biotin obtained
from Miltenyi Biotech. Cells were incubated with antibiotin
microbeads, and subsequently, lineage-positive cells were
depleted by retention of biotin-positive cells on a MACS column [Miltenyi Biotech]. Lineage-depleted cells were stained for CD48, CD150, CD117, and Sca-1 [BD Biosciences and BioLegend] and sorted on a FACSARIA4 cell sorter [Becton Dickinson]. For splenocyte stimulation, cells were treated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) + 50 ng/mL ionomycin and treated with 10 μM EdU for 2 h before analysis by flow cytometry. In all cases, the fixable Near-IR live/dead dye [Invitrogen] was used to exclude dead cells from the analysis. FlowJo software was used for flow cytometry analysis.

Bulk and single-cell DNA sequencing

Single-cell DNA sequencing was performed as previously described [Knouse et al. 2014, 2016]. Briefly, single cells were isolated either by microaspiration or by 96-well plate sorting on a FACSARia4 cell sorter, and genomic DNA was amplified with the GenomePlex single-cell whole-genome amplification kit (Sigma 25-457-8). Amplified DNA was purified, barcoded, pooled, and sequenced on an Illumina HiSeq2000 at the Massachusetts Institute of Technology Bio-Micro Center. For bulk DNA sequencing analysis, 50 ng of purified DNA was prepared and barcoded using Nextera reagents [Illumina]. Libraries were quantified using an AATI fragment analyzer before pooling and were sequenced on an Illumina HiSeq2000. Sequencing reads were trimmed to 40 nucleotides and aligned to mouse [mm9] genome reference using the BWA [0.7.17] backtack algorithm with default options. HMMcopy [0.1.1] was used to detect copy number variations [CNVs] by estimating DNA copy number in 500-kb bins, controlling for mapability and GC content (calculated by HMMcopy gcCounter). Variability scores [VSs] were calculated as described previously [Knouse et al. 2014]. Cells with high variability in copy number across bins [VS > 0.4] were excluded from the analysis. We used the tail values of the kernel density estimation to define a chromosome as being lost when the CNV score log2 < −0.32 and gained when this value was log2 > 0.23, corresponding to the mean ± 3 standard deviations of the copy number value of chromosome scores in euploid cells.

To define the clonality of a cell population, we scored chromosomes that were gained or lost with a frequency higher or lower than the average ± 2 SD of the overall gains and losses in that cell population after single-cell DNA sequencing analysis. These chromosomes were defined as clonally gained or lost, and the percentage of cells carrying these clonal imbalances was taken as the clonality index.

RNA sequencing

Total RNA was isolated from primary cells or tumors, using the RNeasy plus mini kit [Qiagen] and sequenced on an Illumina HiSeq2000. Read quality was monitored by the MIT BCM/IBG in house pipeline, including sequencing error rate estimation, sequencing reads complexity estimation, fastqc report, sample contamination estimation, intergenic reads percentile calculation, intronic reads percentile calculation, UTR reads percentile calculation, gene coding reads percentile calculation, RNA contamination estimation, and sense to antisense read ratio indicating strand specificity. Alignments were carried out using STAR/2.5.3a, and counting was performed by RSEM/1.3.0 based on /3.3.1. Sample hierarchical clustering and heat map creation were performed by TIBCO Spotfire build version 7.11.0.12 based on log2[FPKM + 1] values calculated by RSEM/1.3.0. To avoid potential noise caused by noncoding genes, as well as nonexpressed genes, clustering was computed by protein coding genes with expression (sample-wise average expression ≥1 FPKM). Normalization and differential expression were carried out by DESeq2 1.10.1 using r/3.2.3. Up-regulated genes were defined as baseMean > 10, log2 fold change > 0.5, and multtest-adjusted P-values < 0.05; down-regulated genes were baseMean > 10, log2 fold change < −0.5, and multtest-adjusted P-values < 0.05. For the analysis of human MYC expression, sequencing reads were mapped to the human genome [hg38] using bowtie2, and alignments with mismatches were filtered out. Gene counts were quantified with featureCounts and data normalized using DESeq2. Gene set enrichment analysis was performed using GSEA version 4.0.3 obtained from the Broad Institute and GSEApy [https://gsea.readthedocs.io/en/latest/index.html] using custom gene sets for MYC targets [Zeller et al. 2003] or canonical pathway gene sets available at MsigDB [https://www.gSEA-msigdb.org/gsea/index.jsp] for Enrichr [https://amp.pharm.mssm.edu/Enrichr] and DAVID [https://david.ncifcrf.gov] were used for functional annotation of gene signatures.

Cell culture, metaphase spreads, and spectral karyotyping

Single-cell suspensions were obtained from primary tumors after ACK red blood cell lysis and were cultured in the presence of the CDK1 inhibitor RO3306 [7.5 μM] for 6 h to enrich for cells in G2. The CDK1 inhibitor was then washed out, and cells were cultured for an additional hour to enter mitosis. Subsequently, samples were treated with [0.075 M KCl] hypotonic solution and were fixed in methanol:acetic acid [3:1 volume ratio]. Metaphases were prepared in a controlled humidity chamber [ThermoTech]. Spectral karyotyping was performed and analyzed as previously described [Liyang et al. 1996], using a combination of five different fluorochromes. A minimum of 20 metaphases were imaged and karyotyped using the hiSKY 7.2.7 software [ASI] on a Leica DMRXA microscope.

Protein analysis

For immunodetection in protein lysates, proteins were separated on SDS-PAGE, transferred to nitrocellulose membranes [Bio-Rad], and probed using specific primary antibodies against MYC [Cell Signaling], cyclin B1 [Cell Signaling], P53 [Cell Signaling], P21 [Santa Cruz Biotechnology], and phosphorylated H2AX [γ-H2AX; Millipore 05-636]. Actin (1:10,000; Sigma) was used as a loading control. Ki67 [Abcam ab16667], phospho-histone H3 (Cell Signaling), cyclin B1 (Cell Signaling), P53 (Cell Signaling), and probed using specific primary antibodies against MYC [Cell Signaling]. Cell culture, metaphase spreads, and spectral karyotyping was performed and analyzed as previously described [Liyang et al. 1996], using a combination of five different fluorochromes. A minimum of 20 metaphases were imaged and karyotyped using the hiSKY 7.2.7 software [ASI] on a Leica DMRXA microscope.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 8 and Python v. 3.8. All statistical tests of comparative data were performed using two-sided, unpaired Student’s t-test, Fisher exact tests, or one-way or two-way ANOVA for differential comparison between two or more groups, respectively. Data with P < 0.05 were considered statistically significant ([P < 0.05 [*]], [P < 0.01 [* *]], and [P < 0.001 [***]])). Specific statistical parameters for each assay are indicated in the corresponding figure legends.

Data availability

Sequencing data are available at the Gene Expression Omnibus [ID GSE174012] and the Sequence Read Archive [SRA] under the master accession number PRJNA728729.
Clonal selection of oncogene-driven aneuploidy


Competing interest statement
The authors declare no competing interests.

Acknowledgments
We thank past and present members of the Amon laboratory, particularly John Replogle, Hilla Weidberg, Gabriel Neurohr, Sarah Plau, Daniel Corbi, Summer Morrill, Rebecca Silberman, and Timothy Mullen, for helpful discussions, reading of the manuscript, and technical advice. We thank Shannon MacMann for assistance with mouse colony management. We thank Dr. Roderick Bronson for the histopathological analysis. We thank the Histology, Flow Cytometry, and Genomics Core Facilities at the Swanson Biotechnology Center for technical assistance. This work was supported by National Institutes of Health grant CA206157 to A.A., who is an investigator of the Howard Hughes Medical Institute, the Paul F. Glenn Center for the Biology of Aging Research at Massachusetts Institute of Technology, and the Ludwig Center at Massachusetts Institute of Technology, and by a synergy grant from the European Research Council (ERC-2019-SyG-855158).

Author contributions: M.T. and A.A. designed and directed the study. M.T. performed most assays with the help of M.A., C.S., A.C., A.H., and P.H. L.Z. contributed to the single-cell sequencing of CIN models. X.A.S. helped in the analysis of Rad21 models. D.W. performed the SKY analysis. G.P. assisted with sequencing of CIN models. D.M. conducted bioinformatic analysis. C.S. and J.Z. generated the Bub1b−/− and Mad2−/− models, respectively. T.R., A.H., and J.V.D. provided conceptual advice. M.T. wrote the manuscript with comments from all authors.

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Clonal selection of stable aneuploidies in progenitor cells drives high-prevalence tumorigenesis

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*Genes Dev.* 2021, 35: originally published online July 15, 2021

Access the most recent version at doi:10.1101/gad.348341.121

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