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The energy and proteotoxic stress-inducing compounds AICAR and 17-AAG antagonize proliferation in aneuploid cells

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Summary

Aneuploidy, an incorrect chromosome number, is a hallmark of cancer. Compounds that cause lethality in aneuploid but not euploid cells could therefore provide new cancer therapies. We have identified the energy stress-inducing agent AICAR, the protein folding inhibitor 17-AAG and the autophagy inhibitor chloroquine as exhibiting this property. AICAR induces p53-mediated apoptosis in primary mouse embryonic fibroblasts (MEFs) trisomic for either Chromosome 1, 13, 16 or 19. AICAR and 17-AAG, especially when combined, also show efficacy against aneuploid human cancer cell lines. Our results suggest that compounds that interfere with pathways essential for the survival of aneuploid cells could serve as a new treatment strategy against a broad spectrum of human tumors.

Introduction

Aneuploidy, a condition where the chromosome number is not a multiple of the haploid complement, is associated with death and disease in all organisms where this has been studied. In budding and fission yeast aneuploidy inhibits proliferation (Niwa et al., 2006; Torres et al., 2007). In flies and worms most or all whole-chromosome trisomies and monosomies are lethal, respectively (Hodgkin, 2005; Lindsley et al., 1972). In the mouse all monosomies and all trisomies, but trisomy 19, result in embryonic lethality. In humans, all whole-chromosome aneuploidies except trisomy 13, 18, or 21 lead to death during embryogenesis. The viable trisomies display severe abnormalities (Lin et al., 2006; Moerman et al., 1988) (Antonarakis et al., 2004).

Aneuploidy is also detrimental at the cellular level. Budding and fission yeast cells carrying an additional chromosome display cell proliferation defects (Niwa et al., 2006; Pavelka et al., 2010; Torres et al., 2007). Primary aneuploid mouse embryonic fibroblasts (MEFs) trisomic for any of four chromosomes, Chromosome 1, 13, 16 or 19; primary foreskin fibroblast cells derived from Down's syndrome individuals (trisomy 21); and human cell lines with decreased chromosome segregation fidelity exhibit cell proliferation defects (Segal and McCoy, 1974; Thompson and Compton, 2008; Williams et al., 2008). Two systematic studies in disomic budding yeasts and trisomic MEFs furthermore showed that the presence of an additional chromosome elicits a set of phenotypes that is shared between

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All Experimental Procedures are described in detail in the Supplemental Information.

different aneuploidies in both yeast and mouse. Yeast cells carrying an additional chromosome display metabolic alterations, and increased sensitivity to compounds that interfere with protein folding and turnover (Torres et al., 2007). These shared traits are due to the additional proteins produced from the additional chromosomes (Torres et al., 2007). Similar phenotypes are seen in trisomic MEFs. Trisomic cells show increased sensitivity to proteotoxic compounds, higher basal levels of autophagy and elevated amounts of the active form of the molecular chaperone Hsp72 (see below) and increased uptake of glutamine, a major carbon source for the TCA cycle (DeBerardinis et al., 2007; Williams et al., 2008). Based on these findings it was proposed that aneuploidy leads to a cellular response (Torres et al., 2010; Torres et al., 2007). Cells engage protein degradation and folding pathways in an attempt to correct protein stoichiometry imbalances caused by aneuploidy. This increases the load on the cell's protein quality control pathways and results in heightened sensitivity to proteotoxic compounds and an increase need for energy. Whether the cell proliferation defects observed in aneuploid cells are also a part of the response to the aneuploid state, as is seen in many other stress responses, or caused by the mis-regulation of individual cell cycle proteins is not yet known.

Although aneuploidy adversely affects cell proliferation, the condition is associated with a disease characterized by unabated growth, cancer (reviewed in (Luo et al., 2009)). More than 90 percent of all solid human tumors carry numerical karyotype abnormalities (Albertson et al., 2003). Studies in mouse models of chromosome instability indicate that aneuploidy is not simply a byproduct of the disease but directly responsible for tumor formation. Impairing spindle assembly checkpoint activity or halving the gene dosage of the motor protein CENP-E causes chromosome mis-segregation. Remarkably, it also causes increased tumor formation in mice (Li et al., 2010; Sotillo et al., 2007; Weaver et al., 2007). How aneuploidy promotes tumorigenesis despite its anti-proliferative effects is an important question that remains to be answered.

Irrespective of how aneuploidy promotes tumorigenesis, the stresses caused by the aneuploid state could still exist in aneuploid cancer cells, a condition termed "non-oncogene addiction" (Luo et al., 2009). Compounds that exhibit lethality with the aneuploid state either by exaggerating the adverse effects of aneuploidy and/or by interfering with pathways essential for the survival of aneuploid cells could represent new tumor treatments. We have identified the energy and proteotoxic stress-inducing compounds AICAR, 17-AAG and chloroquine as exhibiting this selectivity. They induce p53-mediated apoptosis in primary mouse embryonic fibroblasts trisomic for either Chromosome 1, 13, 16 or 19. AICAR and 17-AAG also show efficacy against aneuploid human cancer cell lines. When combined, the two compounds are more effective in inhibiting the proliferation of human colorectal cancer cells that exhibit high grade aneuploidy (chromosome instability lines, CIN) compared to lines that show low grade aneuploidy (microsatellite instability lines, MIN). Our results raise the interesting possibility that the aneuploid state of a cancer cell can be exploited in cancer therapy.

Results

Identification of compounds that preferentially antagonize the proliferation of aneuploid cells

To identify compounds that exhibit adverse synthetic interactions with the aneuploid state, we employed MEFs trisomic for either Chromosome 1, 13, 16, or 19. We generated these cells using mice that carry Robertsonian fusion chromosomes (Williams et al., 2008), and compared their drug response to that of littermate control cells that carry a Robertsonian chromosome but are euploid (note that these controls were included in all experiments described here). Chromosomes 1, 13, 16, and 19 were chosen because they cover a large

portion of the size and coding spectrum of mouse chromosomes [Chr1, 197 Mbp and 1228 genes; Chr13, 120 Mbp and 843 genes; Chr16, 98 Mbp and 678 genes; Chr19, 61 Mbp and 734 genes](Williams et al., 2008).

Since aneuploidy leads to cell proliferation defects as well as proteotoxic and energy stress (Torres et al., 2007; Williams et al., 2008; reviewed in Luo et al., 2009;), we selected compounds with similar effects, with the rationale that further interference with pathways that are already impaired in aneuploids or essential for their viability may lead to lethality. We tested compounds that cause genotoxic stress (aphidocolin, camptothecin, cisplatin, doxorubicin, hydroxyurea; see Supplemental Information for effects of these compounds), proteotoxic stress (17-allylamino-17-demethoxy-geldanamycin (17-AAG), cycloheximide, chloroquine, lactacystin, MG132, puromycin, tunicamycin) and energy stress (5-aminoimidazole-4-carboxamide riboside (AICAR), compound C, 2-deoxyglucose, metformin, rapamycin and torin1). Approximately 2×10^5 MEFs were plated into 6-well plates and after 24 hours exposed to compound or vehicle alone. The effects on cell number were determined for three days. Because cell accumulation is impaired in trisomic cells even in the absence of treatment (Williams et al., 2008) (Figure 1A), cell number is presented as a percentage of cells observed in the absence of treatment. For a few compounds (e.g. aphidicolin) this percentage is greater in some trisomic cells than euploid controls (Table S1). While this indicates that trisomic cells tolerate the compound better than euploid cells, it is important to note that trisomic cells still grow significantly worse than euploid cells.

The majority of compounds did not exhibit selectivity towards trisomic MEFs or did so for only a subset of the trisomies tested (Table S1). However three compounds - the energy stress inducer AICAR, the Hsp90 inhibitor 17-AAG, and the autophagy inhibitor chloroquine impaired the accumulation of all four trisomic MEFs to a higher degree than that of euploid control cells (Table S1).

AICAR is a cell-permeable precursor of ZMP (an AMP analog), which allosterically activates AMP-activated protein kinase (AMPK) thereby mimicking energy stress (Corton et al., 1995). AMPK is sensitive to the intracellular AMP:ATP ratio and up-regulates catabolic pathways to produce more ATP and down-regulates anabolic pathways to conserve energy charge (Hardie, 2007). AICAR significantly inhibited the accumulation of cells trisomic for the large Chromosomes 1 and 13. Accumulation of cells carrying the gene-poorer Chromosome 16 was less affected (Figure 1). Proliferation of cells trisomic for the smallest chromosome, Chromosome 19, was only subtly inhibited by AICAR (Figure 1). Importantly, whereas euploid cells continued to proliferate in the presence of high concentration of AICAR (0.5 mM), cell numbers declined in all trisomic cultures (Figure 1A), indicating that AICAR in fact kills trisomic MEFs. Treatment of cells with metformin, a type 2 diabetes drug that also induces energy stress and activates AMPK (Canto et al., 2009), also impaired the accumulation of trisomy 13 and 16 cells in culture, although the effects were not as dramatic (Table S1, Figure S1A). Why AICAR and metformin show different efficacy in trisomic MEFs, despite both causing AMPK activation, is at present unclear (see Discussion).

17-AAG inhibits the chaperone Hsp90. This chaperone together with others is needed for the folding, activation and assembly of a specific set of client proteins (Young et al., 2001). 17-AAG inhibited proliferation of all aneuploid cells at a concentration of 100 nM (Figure 2A; Table S1). Furthermore, cells trisomic for the largest chromosome, Chr1, exhibited higher sensitivity to the compound than cells harboring an additional copy of the smaller chromosomes, Chr16 or 19. This finding suggests that aneuploid cells rely on protein quality control pathways for their survival, which is consistent with the finding that levels of the chaperone Hsp72 are increased in trisomic MEFs (Figure 5D).

Chloroquine also induces proteotoxic stress because it inhibits late stages of autophagy, a homeostatic mechanism critical for the elimination of damaged proteins and organelles (Levine and Kroemer, 2008; Mizushima et al., 2008). Chloroquine preferentially inhibited the proliferation of trisomic MEFs, although the anti-proliferative effects were not as dramatic as those caused by AICAR or 17-AAG. Similar results were obtained when autophagy was impaired by the knock down of the autophagy factor Beclin 1 in trisomy 13 cells (Figure S1B). As observed for AICAR and 17-AAG, the increased sensitivity of trisomic cells correlated with the size of the additional chromosome (Figure 2B; Table S1). We conclude that interference with autophagy is detrimental in aneuploid MEFs, perhaps because aneuploid cells rely on autophagy to produce energy and/or reduce proteotoxic stress. Indeed autophagy is increased in trisomic MEFs (Figure 5A–C).

Interestingly, the combined treatment of trisomic cells with AICAR and 17-AAG significantly impaired the proliferative abilities of trisomic MEFs but had little effect on euploid control cultures (Figure 2C). Similar results were obtained when cells were treated with a combination of AICAR and chloroquine (Figure 2D). We conclude that compounds exist that selectively inhibit the proliferation of trisomic MEFs. Their combined application, especially, leads to significant differential effects in euploid and aneuploid cells.

AICAR, 17-AAG and chloroquine induce apoptosis in trisomic MEFs

To examine how AICAR, 17-AAG and chloroquine preferentially antagonize the proliferation of trisomic MEFs, we asked whether the compounds induce apoptosis in trisomic but not euploid cells. At high dose, AICAR inhibits the proliferation of wild-type MEFs by inducing cell cycle arrest and apoptosis ((Jones et al., 2005), Figure 3). At a concentration of 0.2mM AICAR did not induce apoptosis in wild-type cells, but 0.5mM AICAR led to a 66% increase in early apoptotic cells (Figure 3A, B). The effects of AICAR on trisomic cells were more dramatic. Apoptosis was not increased in untreated trisomic MEFs, but addition of 0.2mM or 0.5mM AICAR led to a 2-fold increase in early apoptotic cells (Figure 3A, B). 17-AAG and chloroquine also induced apoptosis in trisomy 13 MEFs (Figure 3C).

Is apoptosis the only anti-proliferative effect of the identified compounds? We addressed this question for AICAR. We did not detect substantial cell cycle delays in AICAR-treated trisomic MEFs (Figure S2A), although subtle cell cycle alterations cannot be excluded when examining unsynchronized cells. AICAR did not appear to induce premature senescence in trisomic MEFs either, as judged by the production of senescence-associated β-galactosidase (Figure S2B). Treatment of cells with necrostatin-1 (Nec-1), an inhibitor for necroptosis (Degterev et al., 2005), did not suppress the anti-proliferative effects of AICAR either (Figure S2C). AICAR is known to inhibit the mTOR pathway (Sarbassov et al., 2005). Inhibition of the mTOR pathway either through treatment of cells with the mTOR kinase inhibitors rapamycin or torin1 or knock-down of mTOR did not inhibit the proliferation of trisomic MEFs nor enhanced the anti-proliferative effects of AICAR (Figure S3). We conclude that AICAR treatment inhibits proliferation by increasing apoptosis in trisomic MEFs. 17-AAG and chloroquine have a similar effect at least in trisomy 13 cells.

The anti-proliferative effects of AICAR are mediated by AMPK and p53

How do AICAR, 17-AAG and chloroquine induce apoptosis in trisomic MEFs? We addressed this question for AICAR. First we tested whether AICAR antagonizes the proliferation of trisomic MEFs by affecting AMPK. Knock-down of AMPK using short hairpins not only effectively lowered AMPK protein levels (Figure 4A), it also ameliorated the cell accumulation defect brought about by AICAR treatment (Figure 4B, note that the effects of AICAR treatment were assessed after only 24 hours in this experiment. Thus, its

effects on control trisomic cells were not as dramatic as after 3 days, as is shown in Figure 1B). Inhibition of AMPK by other means had similar effects.

Compound C is a pyrazolopyrimidine compound that functions as an ATP-competitive inhibitor of AMPK and other protein kinases (Bain et al., 2007). Treatment with compound C increased the proliferative abilities of trisomic cells (Figure 4C) and suppressed the adverse effects of AICAR (Figure 4D). AICAR thus inhibits the accumulation of trisomic MEFs at least in part by activating AMPK.

The sensitivity of trisomic cells to AICAR could be due to hyperactivation of AMPK in trisomic but not euploid cells. To test this possibility we measured AMPK activity in euploid and aneuploid MEFs in the presence or absence of AICAR. The basal activity of AMPK was not increased in untreated trisomic MEFs as judged by *in vitro* AMPK kinase assays and phosphorylation of Threonine 172 on AMPK, a modification indicative of active AMPK (Lamia et al., 2009) (Figure 4E, F). AMPK activation occurred faster in aneuploid MEFs upon AICAR treatment (Figure 4G) but the degree of activation was similar in euploid and aneuploid MEFs 24 hours after AICAR addition (Figure 4E, F). We conclude that hyperactivation of AMPK is not responsible for the adverse effects of AICAR on trisomic MEFs. However, our results suggest that AMPK is activated more readily by AICAR in trisomic cells.

Having established that the effects of AICAR on trisomic cells are at least in part mediated by AMPK activation, we next determined how this could lead to apoptosis. AMPK activates p53 through phosphorylation of Serine15 (Jones et al., 2005). We find that AICAR treatment subtly induced S15 phosphorylation and p53 stabilization in both wild-type and trisomy 13 MEFs (Figure 3D), but both events occurred significantly faster in trisomy 13 cells (Figure 3D). We also examined two p53 targets, the CDK inhibitor p21 and the proapoptotic protein Bax. p21 protein levels were not increased in response to AICAR treatment. In contrast, Bax activity was induced by AICAR (Figure 3D, E). Bax integrates into the outer membrane of mitochondria causing the activation of the apoptotic program (Vander Heiden and Thompson, 1999). AICAR treatment led to an increase in mitochondrially associated Bax in both wild-type and trisomy 13 cells, but the amount of Bax associated with this organelle fraction was higher in trisomy 13 cells (Figure 3E). These results suggest that p53 induces apoptosis in trisomic MEFs.

Consistent with this idea we find that, p53 knockdown suppressed the anti-proliferative effects of AICAR in trisomy 13 and 16 MEFs (Figure 3F, G). We conclude that the anti-proliferative effects of AICAR in trisomic cells are, at least in part, mediated by p53-mediated apoptosis.

AICAR exaggerates the cellular stresses caused by aneuploidy

AICAR treatment leads to increased p53-dependent apoptosis in trisomic but not euploid MEFs. However, other compounds that induce p53-mediated apoptosis, *i.e.* genotoxic compounds, do not show this selectivity. This indicates that in addition to inducing p53, AICAR must have other adverse effects on trisomic MEFs. The increased sensitivity of aneuploid cells to AICAR could be due to aneuploidy and AICAR affecting parallel pathways and/or due to AICAR exaggerating defects already present in trisomic MEFs. To test the latter possibility we analyzed proteotoxic stress indicators in trisomic cells in the presence and absence of AICAR.

In both aneuploid budding yeasts and MEFs, the majority of genes located on an additional chromosome are expressed (Pavelka et al., 2010; Torres et al., 2010; Torres et al., 2007; Williams et al., 2008) This observation, together with the finding that aneuploid yeast cells

are sensitive to conditions that interfere with protein folding and turnover led to the proposal that in yeast excess proteins produced by the additional chromosomes place stress on the cell's protein quality control systems (Torres et al., 2010; Torres et al., 2007). To determine whether trisomic MEFs are under proteotoxic stress we examined basal levels of autophagy and the Hsp72 chaperone in trisomic MEFs and their behavior in response to AICAR treatment. During autophagy, the autophagosomal membrane component LC3 is lipidated and incorporated into autophagosomal structures (Mizushima et al., 2008). In the absence of AICAR, trisomy 13 and 16 cells contained increased levels of LC3 mRNA and lipidated LC3 that was incorporated into autophagosomes (Figure 5A, C). Expression of Bnip3, a component of the autophagy machinery that is induced by many different stresses (Mizushima and Klionsky, 2007), was also increased in trisomy 13 and 16 MEFs (Figure 5B). AICAR treatment further induced Bnip3 expression as well as LC3 expression and LC3 incorporation into autophagosomes (Figure 5A–C).

Trisomic MEFs also harbor elevated levels of the inducible form of the chaperone Hsp72 (Figure 5D). AICAR treatment led to a further increase in Hsp72 levels in all but trisomy 16 cells in which Hsp72 levels were already very high (Figure 5D). Our results indicate that the activities of protein quality control pathways are elevated in aneuploid MEFs. They further show that AICAR enhances the proteotoxic stress present in aneuploid cells. We propose that this enhancement of the proteotoxic stress in trisomic cells contributes to the aneuploidy-selective anti-proliferative effects of AICAR.

AICAR and 17-AAG inhibit the proliferation of primary MEFs with decreased chromosome segregation fidelity

Having characterized the effects of AICAR, 17-AAG and chloroquine on defined aneuploidies, the trisomic MEFs, we next wanted to determine whether the compounds also inhibit proliferation of MEFs in which aneuploidies are spontaneously generated due to increased chromosome mis-segregation. To this end, we tested the effects of AICAR and 17-AAG on primary MEFs with a compromised spindle assembly checkpoint (SAC). Partial inactivation of the SAC by impairing BUBR1 function using the hypomorphic $Bub1b^{H/H}$ allele or by expressing a checkpoint resistant CDC20 allele ($Cdc20^{AAA}$) causes chromosome mis-segregation and the accumulation of aneuploid cells in culture over time (Baker et al., 2004; Li et al., 2009). Primary MEFs carrying these mutations were sensitive to 17-AAG and AICAR (Figure 6A, B). The effects were not as dramatic as in the trisomic MEFs, presumably because only 36% and 52% of the $Bub1b^{H/H}$ and $Cdc20^{AAA}$ MEFs are aneuploid after several passages, respectively (Baker et al., 2004; Li et al., 2009). Our results indicate that AICAR and 17-AAG also antagonize the proliferation of MEFs with decreased chromosome segregation fidelity.

AICAR and 17-AAG inhibit proliferation of aneuploid human cancer cells

A key question that arises from our findings is whether AICAR, 17-AAG and chloroquine also show efficacy against aneuploid cancer cell lines. To address this question, we analyzed the effects of these compounds on the proliferative abilities of colorectal cancer cell lines with high-grade aneuploid karyotypes (CIN lines) and of colorectal cell lines with near euploid karyotypes (MIN lines) (Cunningham et al., 2010). MIN (microsatellite instability) colorectal cancer lines (HCT-116, HCT-15, DLD-1, SW48 and LoVo) maintain a near-euploid karyotype ((Bhattacharyya et al., 1994); Figure 6C); CIN (chromosome instability) colorectal cell lines (Caco2, HT-29, SW403, SW480 and SW620) harbor between 50 and 100 chromosomes ((Rajagopalan et al., 2003), Figure 6C). Chloroquine did not affect CIN or MIN tumor cell line growth (Figure S4A), which is perhaps not surprising given the compound's modest anti-proliferative effects in trisomic MEFs. AICAR and 17-AAG showed greater growth inhibitory effects in CIN cell lines than in MIN cell lines or in

euploid cell lines (CCD112 CoN and CCD841 CoN; Figure 6C). Treating cells with both AICAR and 17-AAG had an even more significant differential effect (Figure 6C).

We also examined the effects of AICAR, 17-AAG and chloroquine on aneuploid lung cancer cell lines. As in colorectal cancer cell lines, chloroquine did not show a differential effect in lung cancer cell lines (Figure S4B). The effects of AICAR on lung cancer cell lines were modest. Of the eight aneuploid lung cancer lines (A549, NCI-H520, NCI-H838, NCI-H1563, NCI-H1792, NCI-H2122, NCI-H2170 and NCI-H2347) examined, only a subset of cell lines exhibited sensitivity to AICAR (Figure 6D). However all eight cell lines showed significant sensitivity towards 17-AAG. Furthermore, a slight additive effect between AICAR and 17-AAG at high concentrations of compound (0.2 mM AICAR+200 nM 17-AAG) was observed (Figure 6D; P=0.03). Interestingly, all aneuploid cancer cell lines exhibited increased sensitivity to AICAR and/or 17-AAG irrespective of whether p53 was functional or not (Figure 6C, D; see Discussion).

AICAR and 17-AAG also inhibited tumor cell growth in xenograft models. Two MIN (HCT15 and LoVo) and two CIN (HT29 and SW620) cell lines were injected into the flanks of immuno-compromised mice and were then treated with either AICAR, or 17-AAG, or both compounds. Consistent with the cell culture analyses, the combination treatment was more effective in inhibiting CIN tumor growth than in preventing MIN tumor growth (Figure 7A, B). The reduced ability of CIN lines to form tumors could in part be due to increased apoptosis. The two CIN lines but not the MIN lines exhibited high levels of apoptosis when treated with AICAR or AICAR+17-AAG in culture (Figure 7C). Furthermore as in trisomic MEFs, AICAR treatment induced the transcription of a number of autophagy genes in the two CIN (HT29 and SW620) cell lines but not the two MIN (HCT15 and LoVo) cell lines and increased the levels of the lipidated form of LC3 (Figure S5). Hsp72 levels were also higher in CIN lines but AICAR did not cause a further increase in Hsp72 levels (Figure S5B). AICAR and 17-AAG most likely inhibit tumor cell growth in multiple ways. Our results raise the interesting possibility that one reason for their growth inhibitory effect is the aneuploid state of these cancer cells.

Discussion

A response to the aneuploid state

In yeast, aneuploidy causes cell proliferation defects and increased sensitivity to proteotoxic stress (Torres et al., 2007). The data presented here together with our previous analyses of trisomic MEFs (Williams et al., 2008) indicate that the consequences of aneuploidy in mouse cells are remarkably similar to those in yeast. Cell proliferation is impaired (Williams et al., 2008) and cells show signs of energy and proteotoxic stress ((Williams et al., 2008); this study). Cells take up more glutamine and are sensitive to the energy stress-causing compound AICAR. Autophagy and active Hsp72 are elevated in trisomic MEFs and cells are sensitive to compounds that induce proteotoxic stress. It thus appears that the effects of aneuploidy on cell physiology are conserved across species. The findings described here also lend further support to our previous proposal (Torres et al., 2007; Williams et al., 2008) that cells respond to the aneuploid state by engaging protein quality control pathways in an attempt to correct protein stoichiometry imbalances caused by aneuploidy. Two recent studies showed that p53 is also part of this response (Li et al., 2010; Thompson and Compton, 2010). We did not detect elevated levels of active p53 in trisomic MEFs. We speculate that aneuploidy of a single chromosome is not sufficient to induce a p53 response.

Single-chromosome gains as a model for aneuploidy in cancer

We have used single chromosome gains to study the effects of aneuploidy on cell physiology. But can this type of aneuploidy also shed light on the role of aneuploidy in tumorigenesis? Single chromosomal gains rarely occur in cancer. Instead, severe karyotypic abnormalities, involving many chromosomes and often multiple copies of individual chromosomes are the norm. Despite this difference in degree of aneuploidy, we believe that single chromosome gains can speak to the role of aneuploidy in cancer for the following reasons: First, important features and traits of the aneuploid state can be deduced from the analysis of multiple single chromosomal abnormalities because phenotypes shared by cells carrying different single additional chromosomes will also exist in cells with multiple chromosomal abnormalities. In fact, the protein stoichiometry imbalances caused by aneuploidy and the proteotoxic and energy stress these imbalances elicit will, if anything, be more pronounced in cells with multiple numeric chromosomal abnormalities. Second, in some cancers, premalignant lesions or low-grade tumors show limited chromosomal gains or losses. For example, small adenomas and atypical ductal hyperplastic lesions show a low degree of loss of heterozygosity (Larson et al., 2006; Shih et al., 2001). The study of single chromosomal abnormalities could therefore provide important insights into the early stages of tumorigenesis. Finally, the compounds we discovered to inhibit the proliferation of trisomic MEFs also showed efficacy against aneuploid human cancer cell lines suggesting that the trisomy system can be employed to reveal features of aneuploid tumor cells.

We also note that the use of stable disomic and trisomic cell lines to study the effects of aneuploidy has technical advantages. Unlike genome-instability inducing mutations, the karyotypes of the disomic and trisomic cell lines do not change and all cells harbor the same karyotype. Thus, population-based assays can be employed to study these cell lines.

Compounds that synergize with the aneuploid state

Among 18 compounds we identified three, AICAR, 17-AAG and chloroquine, that exhibit synthetic interactions with four different trisomic MEF lines. This specificity indicates that the interactions observed are not simply a consequence of inflicting further harm on already severely impaired cells, but that the compounds interact with a specific aspect of aneuploidy. We observe a correlation between the degree of sensitivity to these compounds and chromosome size, which is also seen with other traits shared by trisomic MEFs (Williams et al., 2008). This correlation suggests that the compounds synergize with the more general effects of aneuploidy, rather than with the effects of gene copy number imbalances of individual genes.

The effects of AICAR on trisomic MEFs were especially significant. The observation that knock-down of AMPK or treatment of cells with the AMPK antagonist compound C suppressed the anti-proliferative effects of AICAR indicates that AICAR exerts its function on trisomic MEFs at least in part through activating AMPK. It is worth noting that other compounds that activate AMPK did not show the same degree of efficacy as AICAR. The effects of metformin on trisomic MEFs were subtle and 2-deoxyglucose although causing AMPK activation did not show selectivity for trisomic cells (Figure S6). In fact, 2-deoxyglucose was highly toxic even in euploid cells (Figure S6A). The differential effects of the different AMPK-activating compounds may be explained by the finding that AICAR, metformin and 2-deoxyglucose activate AMPK via different mechanisms. 2-deoxyglucose stimulates AMPK through its inhibitory effects on glycolysis. Metformin is thought to activate AMPK by inhibiting oxidative phosphorylation (Hawley et al., 2010). In contrast to these indirect ways of activating AMPK, AICAR is metabolized into ZMP in cells, which then binds AMPK (Hawley et al., 2010). This direct interaction with AMPK may have more dramatic effects in trisomic than euploid MEFs.

Mechanisms of proliferation inhibition

Our results indicate that AICAR, 17-AAG and chloroquine induce apoptosis in trisomic MEFs. The AICAR-induced apoptosis is mediated by p53. Apoptosis caused by 17-AAG and chloroquine also depends on this transcription factor at least in trisomy 13 cells (Figure S7). Simply activating p53 is however not sufficient to cause this aneuploidy specific apoptosis because DNA damaging agents (e.g. doxorubicin), which also activate p53 (Tomasini et al., 2008), do not show selectivity. What then are the origins of the aneuploidy-selectivity of the three compounds? Our data provide some insights into the synergism between aneuploidy and AICAR. AICAR induces energy stress. This exaggerates the already stressed state of aneuploid cells as judged by higher levels of autophagy and active Hsp72. We propose that this increases the cell's susceptibility to apoptosis. As AICAR also activates p53 through AMPK, the combination of these events induces apoptosis. The mechanisms whereby AICAR induces autophagy are well established (Buzzai et al., 2007) but how it increases the levels of the stress-induced chaperone Hsp72 is not clear. In budding yeast, the heat shock response transcription factor Hsf1, which induces the production of many chaperones, is activated by the AMPK homolog Snf1 under glucose starvation conditions (Tamai et al., 1994). A similar response of the protein folding pathways to AMPK activation could also exist in mammalian cells.

How aneuploidy-induced stresses sensitize trisomic cells to AICAR-induced apoptosis is not known. We did not detect elevated levels of p53 in untreated trisomic MEFs nor hyperactivation of p53 by AICAR. We did find that p53 is more readily activated by AICAR treatment in aneuploid cells. This could explain the compound's differential effects on aneuploid and euploid cells. Alternatively, the increased susceptibility of trisomic MEFs to apoptosis could be brought about by p53-independent mechanisms. Such independent mechanisms must, however, also result in increased levels of Bax insertion into mitochondrial membranes. We speculate that Bnip3 could be such an independent mechanism. Bnip3, which is induced by a variety of stresses in a p53-dependent and independent manner and is present at high levels in trisomic MEFs (Figure 5B), has been shown to induce apoptosis in a variety of cell types including murine fibroblasts (Burton and Gibson, 2009).

A synergism analogous to that proposed for aneuploidy and AICAR can be envisioned to exist between the aneuploid state and the proteotoxic-stress inducing compounds 17-AAG and chloroquine. The compounds could further exaggerate the proteotoxic stress of aneuploid cells, pre-disposing them to p53-mediated apoptosis.

Effects of AICAR and 17-AAG on aneuploid cancer cells

The proliferation inhibitory effects of AICAR and 17-AAG in colon cancer cell lines with multiple chromosomal abnormalities were more pronounced than in cancer cells with only few numeric karyotypic abnormalities. Their combined use especially had significant effects on CIN cancer cell lines compared to euploid control lines and MIN cancer cell lines, both in cell culture and xenograft models. AICAR and 17-AAG most likely inhibit tumor cell growth in multiple ways, but two observations argue that different degrees of aneuploidy contribute to the differential effects of the two compounds on MIN and CIN cell lines. (1) A synergism between AICAR and 17-AAG and the aneuploid state is also seen in two types of primary aneuploid cells, trisomic MEFs and MEFs with increased chromosome mis-segregation. (2) The response to AICAR and 17-AAG treatment is similar in CIN cells and trisomic MEFs. AICAR treatment induces autophagy in both cell type. Hsp72 is induced in MEFs and already elevated in the CIN cell lines even in the absence of AICAR treatment.

In contrast to trisomic MEFs, inactivation of p53 does not protect aneuploid CIN colon cancer and lung cancer cell lines from death by AICAR and/or 17-AAG. We have not yet identified the mechanisms underlying this p53-independent sensitivity but the two compounds do appear to induce apoptosis in at least two CIN cancer cell lines. We speculate that the aneuploidy-associated stresses are high in cells with high-grade aneuploidy, making conditions that further enhance these stresses a lethal event. In trisomic MEFs, AICAR and/or 17-AAG also exaggerate the adverse effects of aneuploidy, but in cell lines with low-grade aneuploidies, such as the trisomic MEFs, this only sensitizes cells to p53-mediated apoptosis.

Why the four aneuploid cell lines in which p53 is wild-type (Caco2, A549, NCI-H1563, NCI-H2347) were not more sensitive to AICAR and/or 17-AAG than cell lines in which p53 is mutated, is not yet clear either. It is possible that other components of the p53 pathway are defective in these cell lines. Alternatively, the p53 wild-type cancer cell lines may have evolved other mechanisms that help them cope with the adverse effects of aneuploidy. Clearly, it will be important to determine how AICAR and 17-AAG inhibit tumor cell proliferation and whether the selectivity for high-grade aneuploidy holds true in other tumor types. Similarly, understanding why AICAR is more effective in colon cancer cell lines than in lung cancer cell lines could shed light on how different cancer types develop mechanisms that allow them to tolerate proteotoxic and energy stress.

The observation that cancer cells lacking p53 are also sensitive to 17-AAG and/or AICAR has important implications for the potential use of the two compounds as cancer therapeutics. 17-AAG has been shown to exhibit antitumor activity in multiple myeloma and anaplastic large cell lymphoma in clinical trials (Georgakis et al., 2006; Taldone et al., 2008). AICAR is currently not approved for use in humans. Our studies predict that the combined use of AICAR and 17-AAG may be effective against a broad spectrum of human tumors. Most cancers not only lack p53 but they are also highly aneuploid and are thus likely to experience proteotoxic and energy stress. Our data raise the interesting possibility that compounds that exaggerate these stresses exhibit efficacy against many or perhaps all aneuploid tumors.

Experimental Procedures

Mouse strains and cell lines

Mouse strains were obtained from the Jackson Laboratory and are described in the Supplemental Materials; human cell lines were obtained from ATCC. Littermate-derived euploid and trisomic primary MEFs were described previously (Williams et al., 2008). All experiments were performed in at least three independent trisomic cell lines and analyzed together with euploid littermates that carried a single Robertsonian translocation. We used MEFs at early passages (≤p5) to ensure that karyotypic changes had not yet occurred. Two independent $Cdc20^{AAA}$ MEFs were kindly provided by Dr. P. Zhang; $Bub1b^{H/H}$ mice by Dr. J. M. van Deursen.

Mice xenografts

Two MIN (HCT15 and LoVo) and two CIN (HT29 and SW620) cells were inoculated s.c. into flanks of 6-week old female nude mice. Seven days after injection, animals were treated with daily i.p. injection of AICAR (500 mg/kg body weight), 17-AAG (80 mg/kg body weight), or an equal volume of vehicle. Number of animals analyzed: vehicle control: n = 4; AICAR: n = 3; 17-AAG n = 3; AICAR+17-AAG: n = 5. Mice had to be sacrificed at day 25 due to tumor size in the vehicle control group.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

Albertson DG, Collins C, McCormick F, Gray JW. Chromosome aberrations in solid tumors. Nat Genet. 2003; 34:369–376. [PubMed: 12923544]

Antonarakis SE, Lyle R, Dermitzakis ET, Reymond A, Deutsch S. Chromosome 21 and down syndrome: from genomics to pathophysiology. Nat Rev Genet. 2004; 5:725–738. [PubMed: 15510164]

Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, McLauchlan H, Klevernic I, Arthur JS, Alessi DR, Cohen P. The selectivity of protein kinase inhibitors: a further update. Biochem J. 2007; 408:297–315. [PubMed: 17850214]

Baker DJ, Jeganathan KB, Cameron JD, Thompson M, Juneja S, Kopecka A, Kumar R, Jenkins RB, de Groen PC, Roche P, et al. BubR1 insufficiency causes early onset of aging-associated phenotypes and infertility in mice. Nat Genet. 2004; 36:744–749. [PubMed: 15208629]

Bhattacharyya NP, Skandalis A, Ganesh A, Groden J, Meuth M. Mutator phenotypes in human colorectal carcinoma cell lines. Proc Natl Acad Sci U S A. 1994; 91:6319–6323. [PubMed: 8022779]

Burton TR, Gibson SB. The role of Bcl-2 family member BNIP3 in cell death and disease: NIPping at the heels of cell death. Cell Death Differ. 2009; 16:515–523. [PubMed: 19136941]

Buzzai M, Jones RG, Amaravadi RK, Lum JJ, DeBerardinis RJ, Zhao F, Viollet B, Thompson CB. Systemic treatment with the antidiabetic drug metformin selectively impairs p53-deficient tumor cell growth. Cancer Res. 2007; 67:6745–6752. [PubMed: 17638885]

Canto C, Gerhart-Hines Z, Feige JN, Lagouge M, Noriega L, Milne JC, Elliott PJ, Puigserver P, Auwerx J. AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. Nature. 2009; 458:1056–1060. [PubMed: 19262508]

Corton JM, Gillespie JG, Hawley SA, Hardie DG. 5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? Eur J Biochem. 1995; 229:558–565. [PubMed: 7744080]

Cunningham D, Atkin W, Lenz HJ, Lynch HT, Minsky B, Nordlinger B, Starling N. Colorectal cancer. Lancet. 2010; 375:1030–1047. [PubMed: 20304247]

DeBerardinis RJ, Mancuso A, Daikhin E, Nissim I, Yudkoff M, Wehrli S, Thompson CB. Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. Proc Natl Acad Sci U S A. 2007; 104:19345–19350. [PubMed: 18032601]

Degterev A, Huang Z, Boyce M, Li Y, Jagtap P, Mizushima N, Cuny GD, Mitchison TJ, Moskowitz MA, Yuan J. Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol. 2005; 1:112–119. [PubMed: 16408008]

Georgakis GV, Li Y, Rassidakis GZ, Medeiros LJ, Younes A. The HSP90 inhibitor 17-AAG synergizes with doxorubicin and U0126 in anaplastic large cell lymphoma irrespective of ALK expression. Exp Hematol. 2006; 34:1670–1679. [PubMed: 17157164]

Hardie DG. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. Nat Rev Mol Cell Biol. 2007; 8:774–785. [PubMed: 17712357]

Hawley SA, Ross FA, Chevtzoff C, Green KA, Evans A, Fogarty S, Towler MC, Brown LJ, Ogunbayo OA, Evans AM, et al. Use of cells expressing gamma subunit variants to identify diverse mechanisms of AMPK activation. Cell Metab. 2010; 11:554–565. [PubMed: 20519126]

Hodgkin J. Karyotype, ploidy, and gene dosage. WormBook. 2005:1–9.

Jones RG, Plas DR, Kubek S, Buzzai M, Mu J, Xu Y, Birnbaum MJ, Thompson CB. AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. Mol Cell. 2005; 18:283–293. [PubMed: 15866171]

Lamia KA, Sachdeva UM, DiTacchio L, Williams EC, Alvarez JG, Egan DF, Vasquez DS, Juguilon H, Panda S, Shaw RJ, et al. AMPK regulates the circadian clock by cryptochrome phosphorylation and degradation. Science. 2009; 326:437–440. [PubMed: 19833968]

Larson PS, de las Morenas A, Cerda SR, Bennett SR, Cupples LA, Rosenberg CL. Quantitative analysis of allele imbalance supports atypical ductal hyperplasia lesions as direct breast cancer precursors. J Pathol. 2006; 209:307–316. [PubMed: 16604511]

Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell. 2008; 132:27–42. [PubMed: 18191218]

Li M, Fang X, Baker DJ, Guo L, Gao X, Wei Z, Han S, van Deursen JM, Zhang P. The ATM-p53 pathway suppresses aneuploidy-induced tumorigenesis. Proc Natl Acad Sci U S A. 2010

Li M, Fang X, Wei Z, York JP, Zhang P. Loss of spindle assembly checkpoint-mediated inhibition of Cdc20 promotes tumorigenesis in mice. J Cell Biol. 2009; 185:983–994. [PubMed: 19528295]

Lin HY, Lin SP, Chen YJ, Hung HY, Kao HA, Hsu CH, Chen MR, Chang JH, Ho CS, Huang FY, et al. Clinical characteristics and survival of trisomy 18 in a medical center in Taipei, 1988–2004. Am J Med Genet A. 2006; 140:945–951. [PubMed: 16528742]

Lindsley DL, Sandler L, Baker BS, Carpenter AT, Denell RE, Hall JC, Jacobs PA, Miklos GL, Davis BK, Gethmann RC, et al. Segmental aneuploidy and the genetic gross structure of the Drosophila genome. Genetics. 1972; 71:157–184. [PubMed: 4624779]

Luo J, Solimini NL, Elledge SJ. Principles of cancer therapy: oncogene and non-oncogene addiction. Cell. 2009; 136:823–837. [PubMed: 19269363]

Mizushima N, Klionsky DJ. Protein turnover via autophagy: implications for metabolism. Annu Rev Nutr. 2007; 27:19–40. [PubMed: 17311494]

Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature. 2008; 451:1069–1075. [PubMed: 18305538]

Moerman P, Fryns JP, van der Steen K, Kleczkowska A, Lauweryns J. The pathology of trisomy 13 syndrome. A study of 12 cases. Hum Genet. 1988; 80:349–356. [PubMed: 3198112]

Niwa O, Tange Y, Kurabayashi A. Growth arrest and chromosome instability in aneuploid yeast. Yeast. 2006; 23:937–950. [PubMed: 17072887]

Pavelka N, Rancati G, Zhu J, Bradford WD, Saraf A, Florens L, Sanderson BW, Hattem GL, Li R. Aneuploidy confers quantitative proteome changes and phenotypic variation in budding yeast. Nature. 2010; 468:321–325. [PubMed: 20962780]

Rajagopalan H, Nowak MA, Vogelstein B, Lengauer C. The significance of unstable chromosomes in colorectal cancer. Nat Rev Cancer. 2003; 3:695–701. [PubMed: 12951588]

Sarbassov DD, Ali SM, Sabatini DM. Growing roles for the mTOR pathway. Curr Opin Cell Biol. 2005; 17:596–603. [PubMed: 16226444]

Segal DJ, McCoy EE. Studies on Down's syndrome in tissue culture. I. Growth rates and protein contents of fibroblast cultures. J Cell Physiol. 1974; 83:85–90. [PubMed: 4273197]

Shih IM, Zhou W, Goodman SN, Lengauer C, Kinzler KW, Vogelstein B. Evidence that genetic instability occurs at an early stage of colorectal tumorigenesis. Cancer Res. 2001; 61:818–822. [PubMed: 11221861]

Sotillo R, Hernando E, Diaz-Rodriguez E, Teruya-Feldstein J, Cordon-Cardo C, Lowe SW, Benezra R. Mad2 overexpression promotes aneuploidy and tumorigenesis in mice. Cancer Cell. 2007; 11:9–23. [PubMed: 17189715]

Taldone T, Gozman A, Maharaj R, Chiosis G. Targeting Hsp90: small-molecule inhibitors and their clinical development. Curr Opin Pharmacol. 2008; 8:370–374. [PubMed: 18644253]

Tamai KT, Liu X, Silar P, Sosinowski T, Thiele DJ. Heat shock transcription factor activates yeast metallothionein gene expression in response to heat and glucose starvation via distinct signalling pathways. Mol Cell Biol. 1994; 14:8155–8165. [PubMed: 7969152]

Thompson SL, Compton DA. Examining the link between chromosomal instability and aneuploidy in human cells. J Cell Biol. 2008; 180:665–672. [PubMed: 18283116]

Thompson SL, Compton DA. Proliferation of aneuploid human cells is limited by a p53-dependent mechanism. J Cell Biol. 2010; 188:369–381. [PubMed: 20123995]

Tomasini R, Mak TW, Melino G. The impact of p53 and p73 on aneuploidy and cancer. Trends Cell Biol. 2008; 18:244–252. [PubMed: 18406616]

Torres EM, Dephoure N, Panneerselvam A, Tucker CM, Whittaker CA, Gygi SP, Dunham MJ, Amon A. Identification of aneuploidy-tolerating mutations. Cell. 2010; 143:71–83. [PubMed: 20850176]

Torres EM, Sokolsky T, Tucker CM, Chan LY, Boselli M, Dunham MJ, Amon A. Effects of aneuploidy on cellular physiology and cell division in haploid yeast. Science. 2007; 317:916–924. [PubMed: 17702937]

Vander Heiden MG, Thompson CB. Bcl-2 proteins: regulators of apoptosis or of mitochondrial homeostasis? Nat Cell Biol. 1999; 1:E209–E216. [PubMed: 10587660]

Weaver BA, Silk AD, Montagna C, Verdier-Pinard P, Cleveland DW. Aneuploidy acts both oncogenically and as a tumor suppressor. Cancer Cell. 2007; 11:25–36. [PubMed: 17189716]

Williams BR, Prabhu VR, Hunter KE, Glazier CM, Whittaker CA, Housman DE, Amon A. Aneuploidy affects proliferation and spontaneous immortalization in mammalian cells. Science. 2008; 322:703–709. [PubMed: 18974345]

Young JC, Moarefi I, Hartl FU. Hsp90: a specialized but essential protein-folding tool. J Cell Biol. 2001; 154:267–273. [PubMed: 11470816]

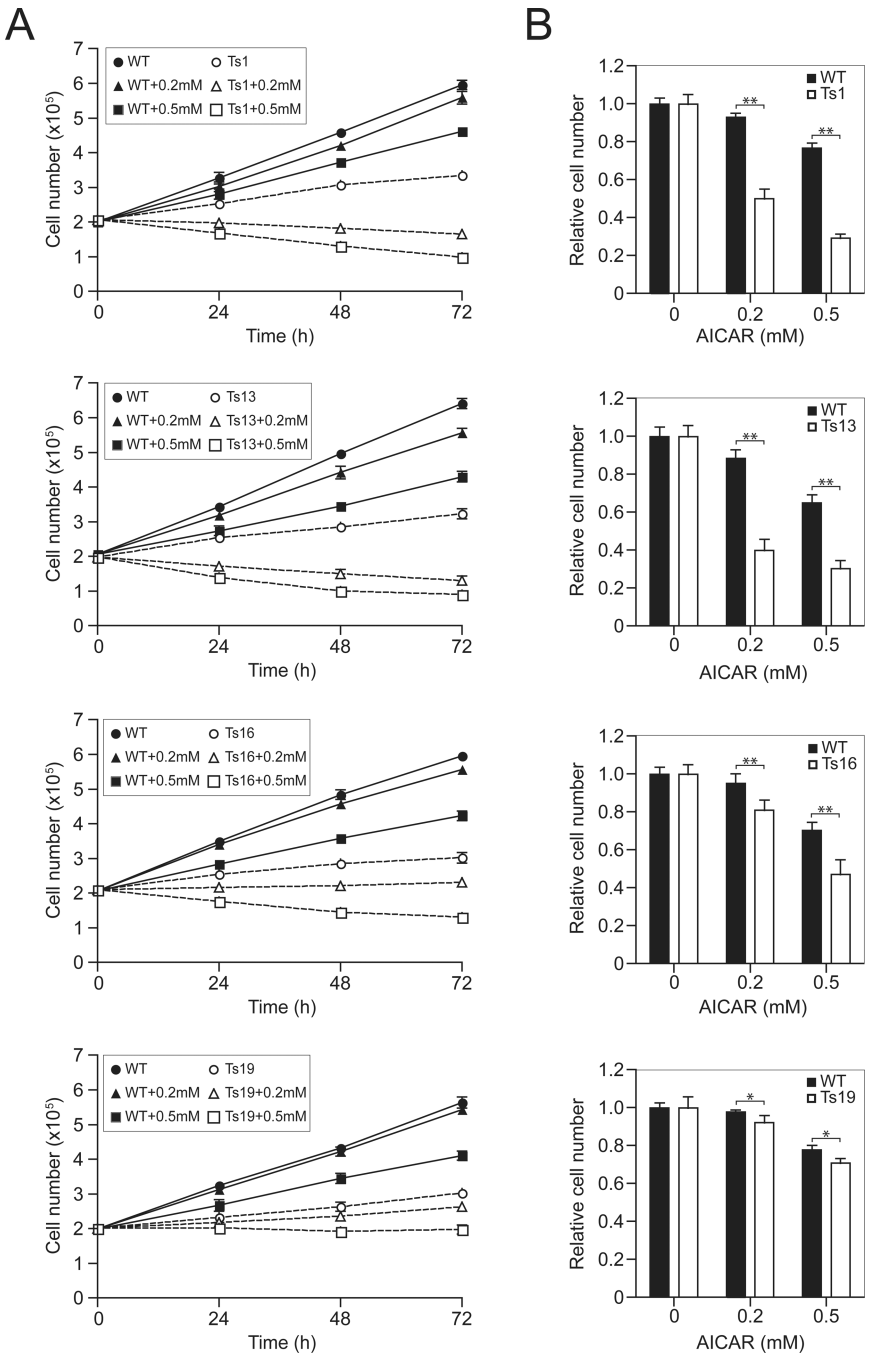

Figure 1. AICAR inhibits proliferation in trisomic MEFs

(A) Wild-type (filled symbols) and trisomic primary (open symbols) MEFs were grown for 72 hours either in the absence (circles) or presence (0.2 mM, triangles; 0.5 mM, squares) of AICAR and cell number was determined at the indicated times.

(B) Cell number of wild-type (filled bars) and trisomic cells (open bars) was determined after 3 days and is shown as the percentage of the untreated control. The data in this and all subsequence figures are shown as the mean ± standard deviation. **P*<0.05, ***P*<0.005, *t* test.

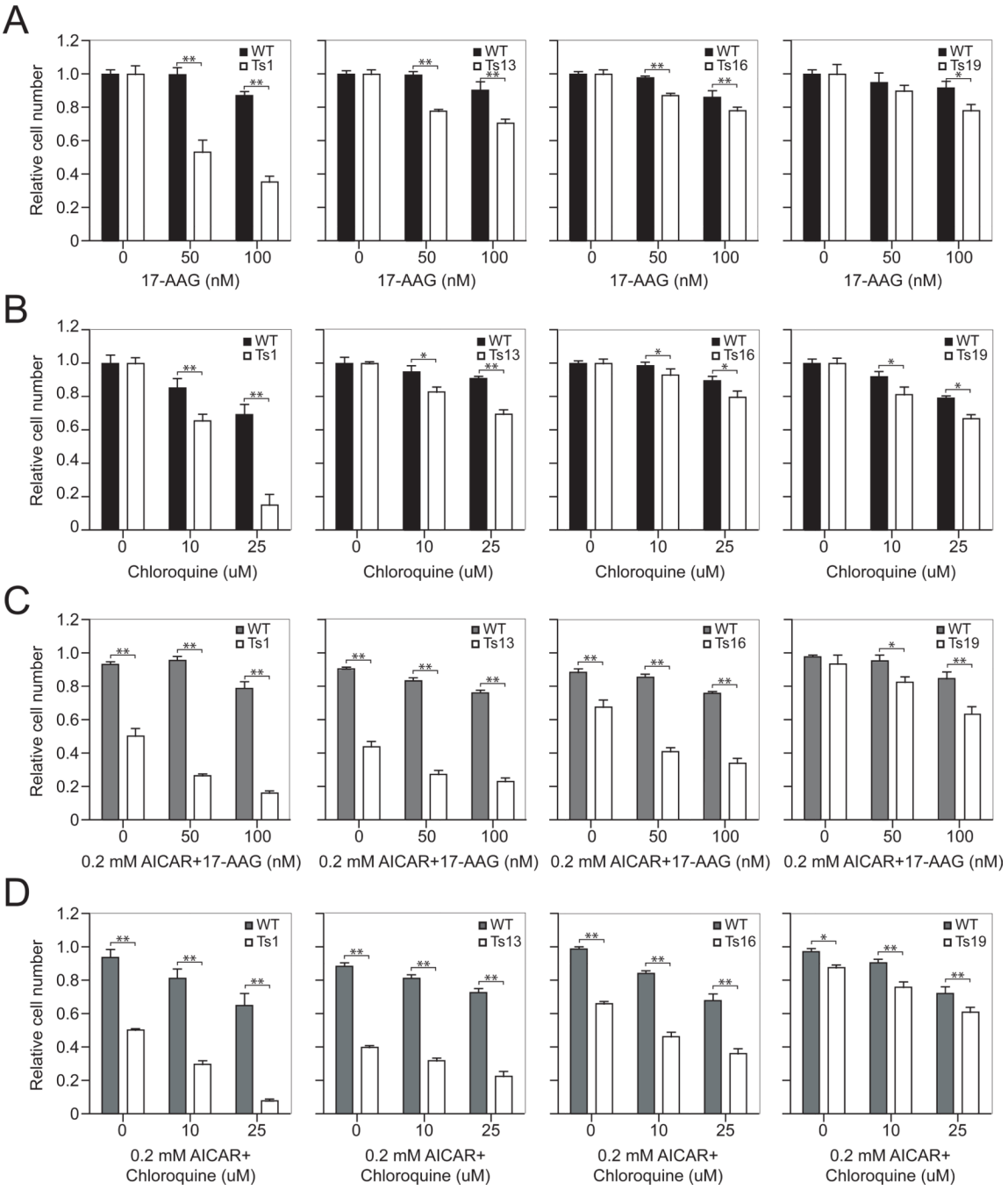

Figure 2. The proteotoxic compounds 17-AAG and chloroquine exaggerate the anti-proliferative effects of AICAR

(A, B) Wild-type (filled bars) and trisomic cells (open bars) were treated with the indicated concentrations of 17-AAG (A) or chloroquine (B) and cell number was determined after 3 days.

(C, D) Cells were treated with 0.2 mM AICAR and the indicated concentrations of 17-AAG (C) or chloroquine (D). Cell number was determined after 3 days. *P<0.05, **P<0.005, t test.

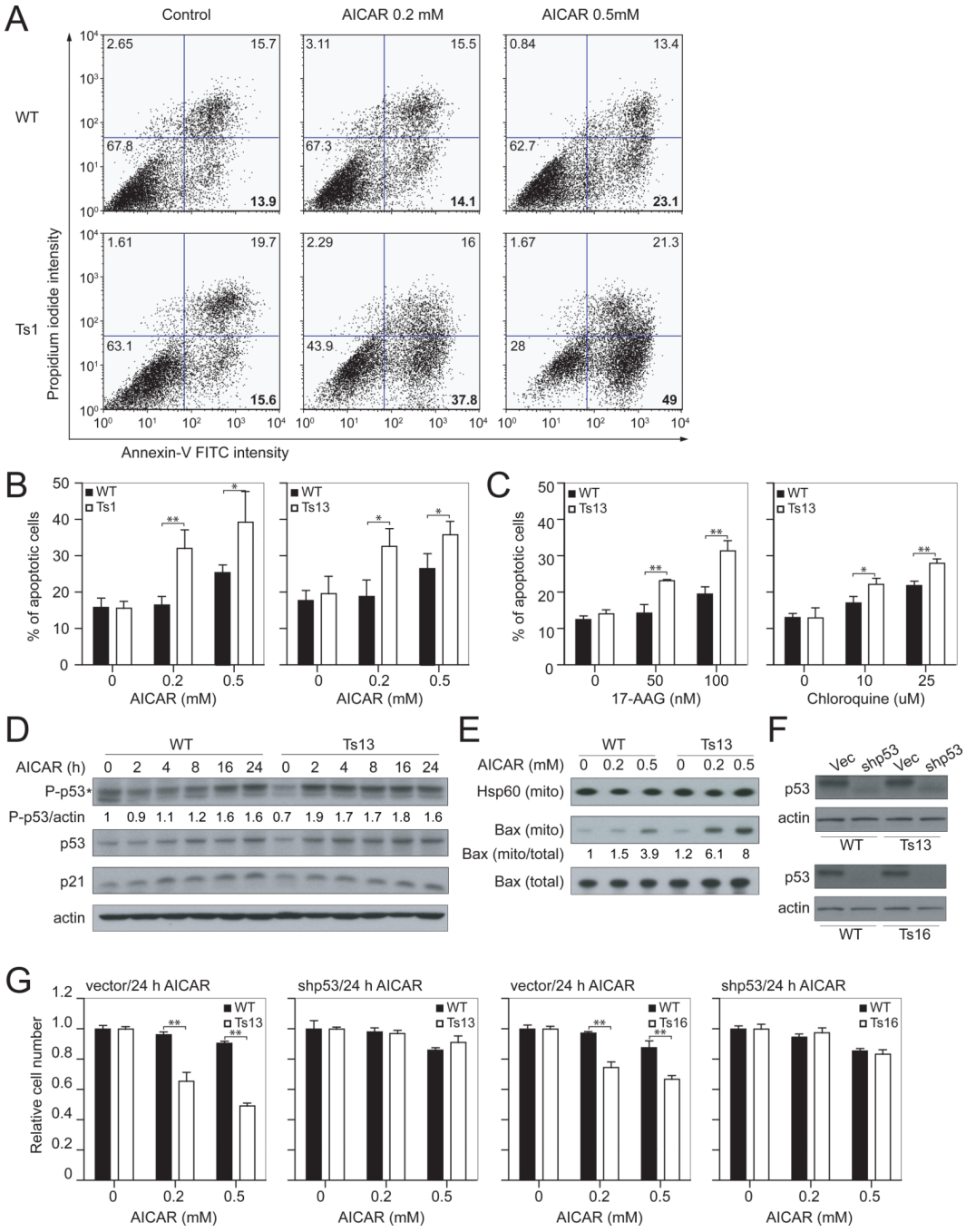

Figure 3. AICAR, 17-AAG and chloroquine induce apoptosis in trisomic MEFs

(A) Wild-type (top) and trisomy 1 cells (bottom) were treated with AICAR for 24 hours and apoptosis was measured using annexin V-FITC/ PI staining. Early apoptotic cells are found in the bottom right quadrant.

(B, C) Quantification of the percentage of annexin V-FITC positive, PI negative cells in wild-type, trisomy 1 and trisomy 13 cultures 24 hours after AICAR treatment (B) and in wild-type and trisomy 13 cultures 24 hours after 17-AAG or chloroquine treatment (C).

(D) Wild-type and trisomy 13 cells were treated with 0.2 mM AICAR and p53 Serine 15 phosphorylation, and p53 and p21 protein levels were analyzed. Quantifications of the ratio

of phosphorylated p53/actin protein are shown underneath the P-p53 blot. The ratios were normalized to untreated wild-type cells. Asterisk denotes S15 phosphorylated p53.
(E) Wild-type and trisomy 13 cells were treated with 0.2 or 0.5 mM AICAR for 24 hours. Equal amounts of cytoplasmic or mitochondrial protein extracts were probed for the presence of Bax by immunoblotting. Mitochondrial Hsp60 served as loading control in mitochondrial extracts. Quantifications of the ratio of mitochondrial Bax/total Bax protein normalized to untreated wild-type cells are shown underneath the mitochondrial Bax blot.
(F) p53 knockdown efficiency revealed by immunoblotting using an anti-p53 antibody. Actin serves as a loading control in Western blots.
(G) Cells were transfected with a p53 knockdown shRNA and treated with AICAR for 24 hours at the indicated doses. *$P<0.05$, **$P<0.005$, *t* test.

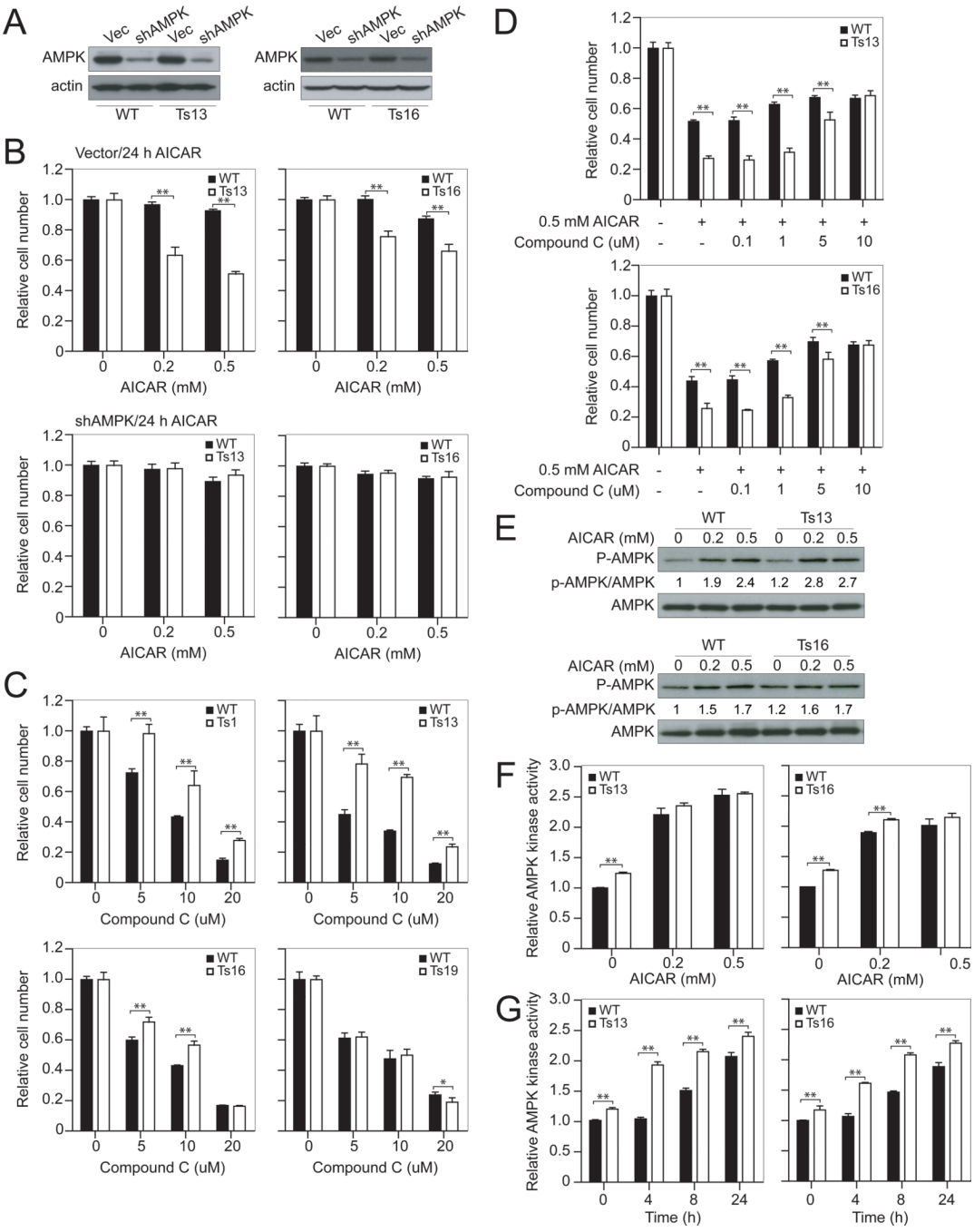

Figure 4. AICAR antgonizes proliferation of trisomic MEFs in an AMPK-dependent manner

(A) AMPKα knock down efficiency revealed by immunoblotting using an anti-AMPK antibody.

(B) Cells infected with either empty vector or an AMPKα knockdown construct were counted 24 hours after AICAR treatment.

(C) Wild-type (filled bars) and trisomic (open bars) cells were treated with the indicated concentrations of compound C for 3 days. Even though the effects of compound C were less severe in trisomic cells than euploid controls, it is important to note that the treated trisomic cells grew poorly compared to euploid control cells.

(D) Wild-type (filled bars) and trisomic cells (open bars) were treated with 0.5 mM AICAR and compound C at the indicated doses for 3 days and cell number was counted. (E, F) AMPK activity was analyzed by determining the extent of threonine172 phosphorylation on AMPK (E) or by *in vitro* kinase assays using the substrate peptide, IRS-1 S789 (F) in wild-type and trisomic cells after 24 hours of AICAR treatment. Quantifications of the ratio of phosphorylated AMPK/total AMPK protein normalized to untreated wild-type cells are shown underneath the P-AMPK blot.
(G) AMPK activity was measured by *in vitro* kinase assays at the indicated time point following addition of 0.2 mM AICAR. *$P<0.05$, **$P<0.005$, *t* test.

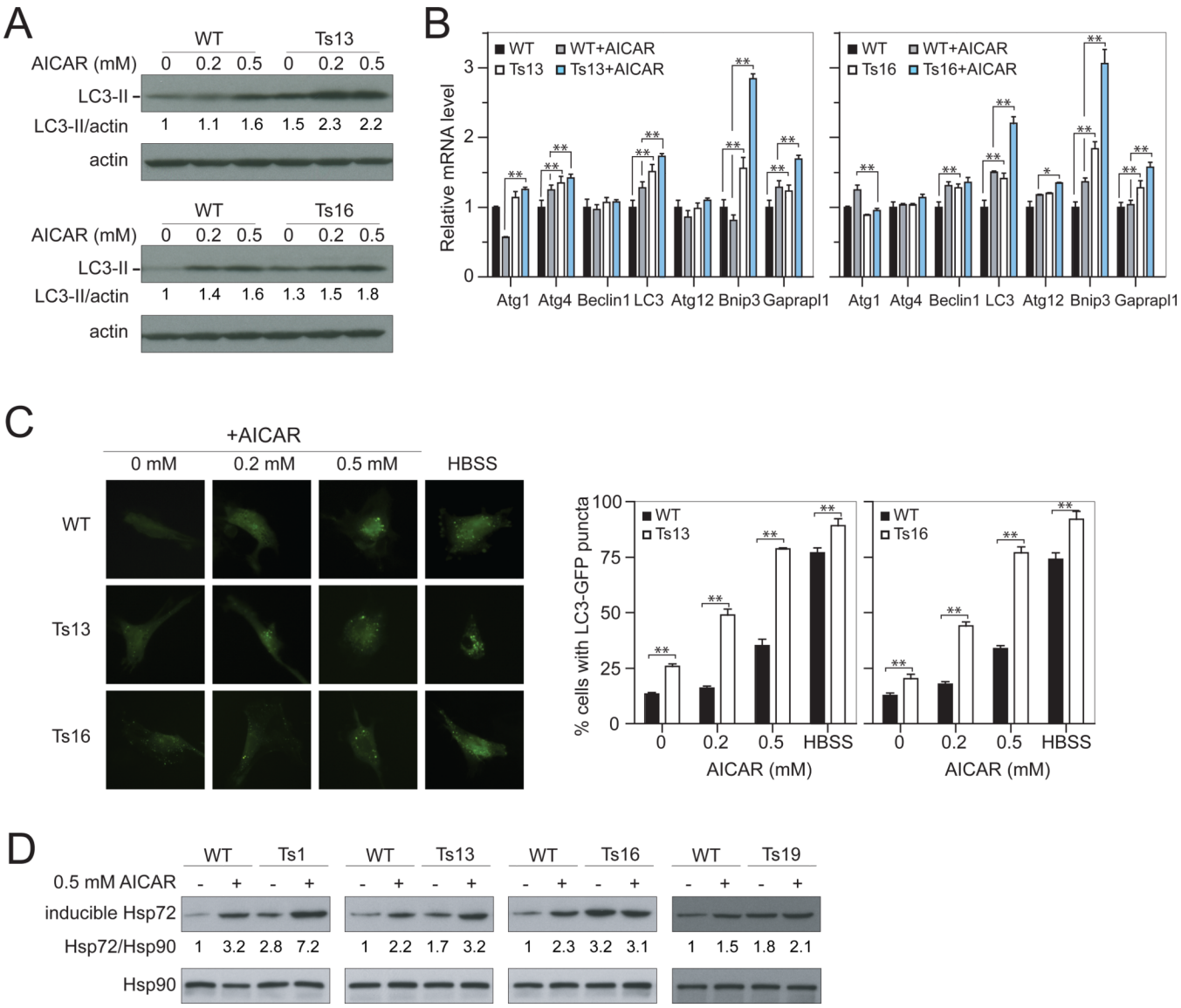

Figure 5. AICAR exaggerates the stressed state of trisomic MEFs

(A) Lipidated LC3-II was analyzed by immunoblotting in wild-type and trisomy 13 and 16 cells after 24 hours of AICAR treatment. Quantifications of the ratio of lapidated LC3II / actin protein normalized to untreated wild-type cells are shown underneath the LC3-II blot.

(B) Quantitative RT-PCR analysis of mRNA abundance of the autophagy genes ATG1, ATG4, Beclin1, LC3, Bnip3 and GAPRAPL1. mRNA levels were quantified in untreated wild-type (black bars) and trisomic (white bars) cells as well as wild-type (grey bars) and trisomic (blue bars) cells treated with 0.5 mM AICAR for 24 hours. RNA levels were normalized to those of the ribosomal RPL19 gene.

(C) The extent of autophagy was quantified by determining the number of LC3-GFP puncta in cells. Typical images are shown as examples for LC3-GFP puncta formation in trisomy 13 and 16 and wild-type cells after AICAR treatment (left). Incubation in HBSS induces acute starvation and served as a positive control. 24 hours after AICAR treatment, the number of cells that harbor more than 4 LC3-GFP puncta was determined (right). *$P<0.05$, **$P<0.005$, *t* test.

(D) Wild-type and trisomic MEFs were treated with AICAR at the indicated doses and levels of inducible Hsp72 were determined by immunoblotting. Quantifications of the ratio of inducible Hsp72/Hsp90 protein normalized to untreated wild-type cells are shown underneath the Hsp72 blot.

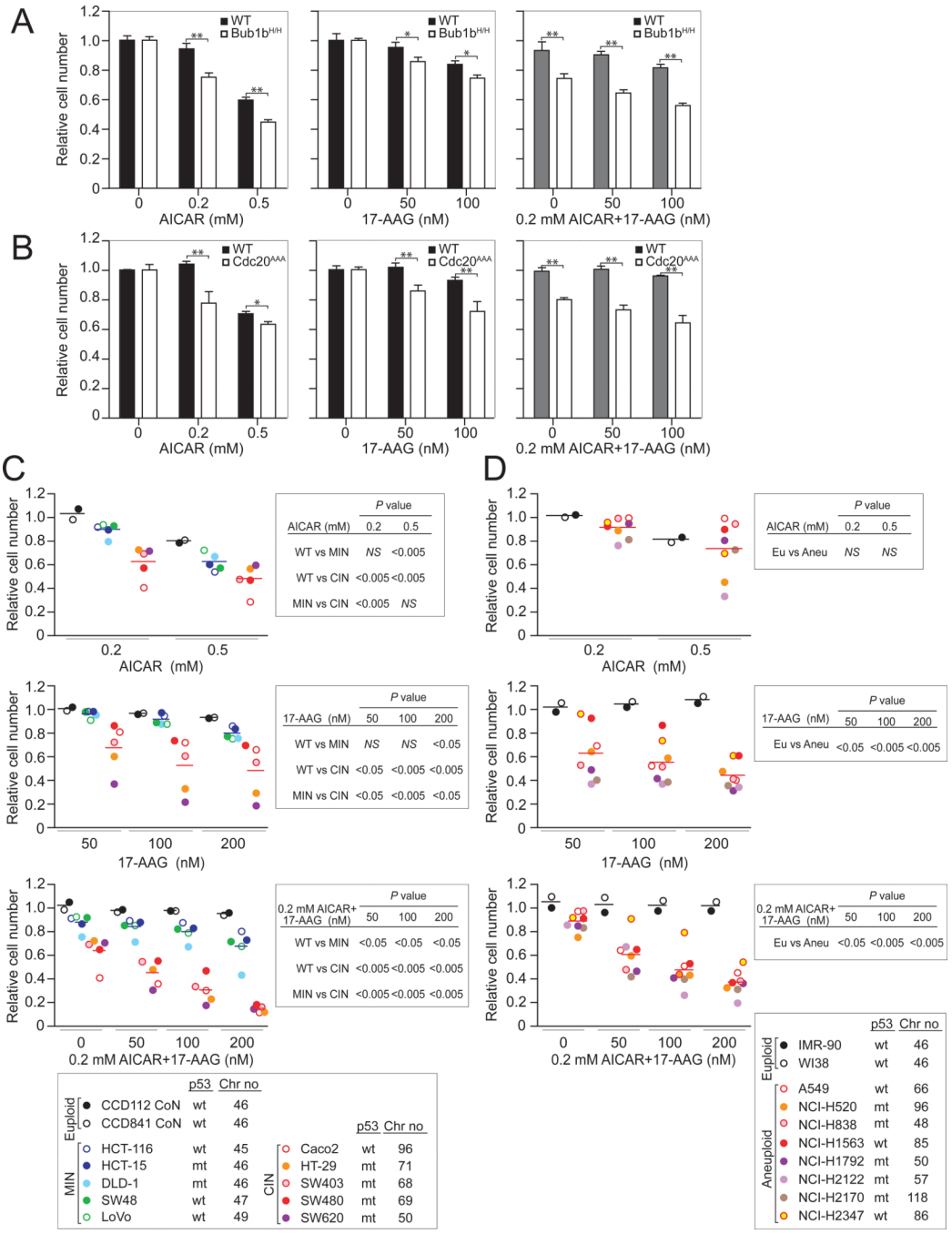

Figure 6. AICAR and 17-AAG inhibit the proliferation of MEFs with decreased chromosome segregation fidelity and of aneuploid human cancer cells

(A, B) Wild-type (filled bars) and Bub1b$^{H/H}$ cells (open bars; A), or wild-type (filled bars) and Cdc20^{AAA} cells (open bars; B) were treated with the indicated concentrations of AICAR, 17-AAG or both, and cell number was determined after 3 days. *P<0.05, **P<0.005, t test.

(C) Cells were treated with the indicated concentration of AICAR (top) or 17-AAG (center) or both compounds (bottom). Cell number was determined 3 days after the addition of compound and is shown as the percentage of the untreated control. Primary euploid cells

(black symbol), MIN colon cancer cell lines (blue, green symbols) and aneuploid CIN colon cancer cells (red, purple symbols) were analyzed.
(D) Cell number of euploid (black symbols) and aneuploid lung cancer cells (red, purple symbols) was determined after 3 days of treatment with the indicated compounds and is shown as the percentage of the untreated control. The data presented are the mean and the *P* value results of *t* test. NS, not significant.

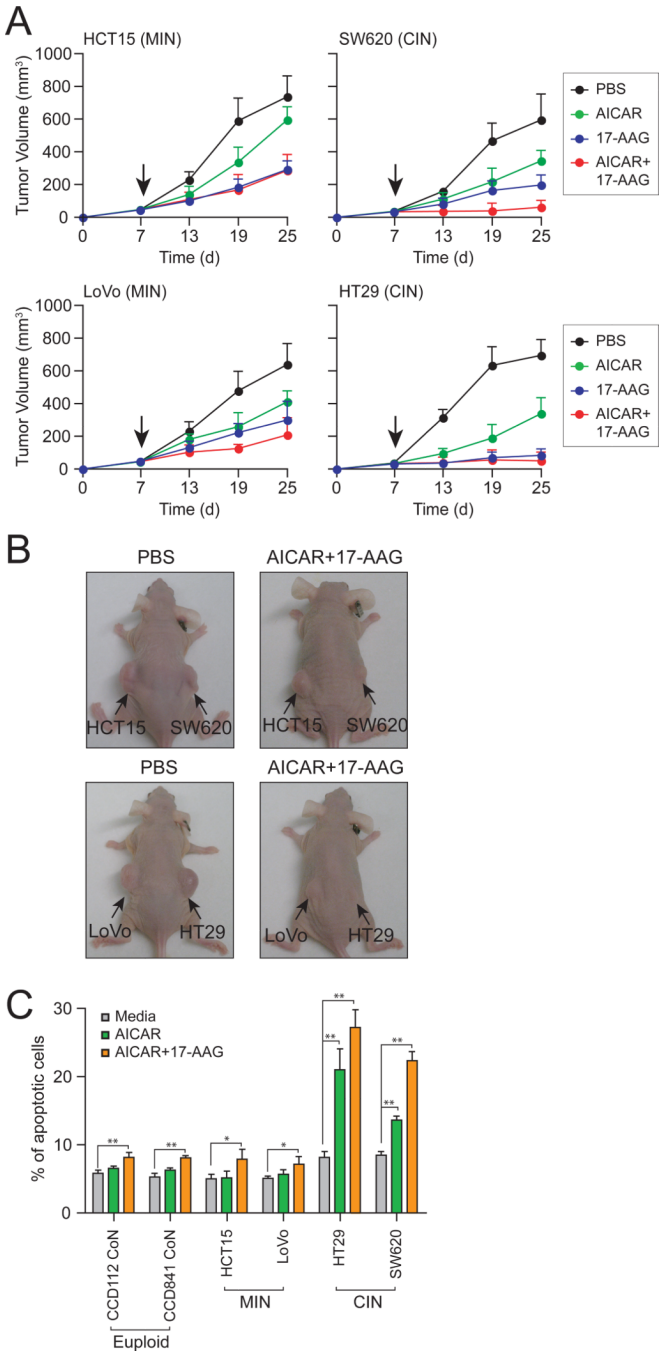

Figure 7. AICAR and 17-AAG inhibit growth of human colon cancer cells in xenografts

(A) Mice were implanted with 4 ×10^6 MIN cells on the left flank and with the same number of CIN cells on the right flank. Seven days after injection (indicated by the arrow) mice were treated with daily i.p. injections of AICAR, 17-AAG, both or PBS. Tumor volume (mm^3) was measured at the indicated time points and shown as mean tumor volumes.

(B) Mice treated with PBS (left) or AICAR+17AAG (right) 25 days after transplantation.

(C) Quantification of the percentage of annexin V-FITC positive, PI negative cells in wild-type, MIN and CIN cell cultures 24 hours after AICAR or AICAR+17-AAG treatment. *P<0.05, **P<0.005, t test.