Cancer-associated mutations in chromatin remodeler hSNF5 promote chromosomal instability by compromising the mitotic checkpoint

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The hSNF5 subunit of human SWI/SNF ATP-dependent chromatin remodeling complexes is a tumor suppressor that is inactivated in malignant rhabdoid tumors (MRTs). Here, we report that loss of hSNF5 function in MRT-derived cells leads to polyploidization and chromosomal instability. Re-expression of hSNF5 restored the coupling between cell cycle progression and ploidy checkpoints. In contrast, cancer-associated hSNF5 mutants harboring specific single amino acid substitutions exacerbated poly- and aneuploidization, due to abrogated chromosome segregation. We found that hSNF5 activates the mitotic checkpoint through the p16INK4a-cyclin/CDK4–pRb–E2F pathway. These results establish that poly- and aneuploidy of tumor cells can result from mutations in a chromatin remodeler.

Carriers of germline mutations are predisposed to various cancers and, consistent with a classic tumor suppressor phenotype, the wild-type allele is either lost or deleted in a large proportion of tumors (Biegel et al. 1999; Sevenet et al. 1999a,b; Taylor et al. 2000). hSNF5 mutations are also associated with a number of neoplasms other than MRTs (Grand et al. 1999; Sevenet et al. 1999a,b; Roberts and Orkin 2004). hSNF5 inactivation studies in mice established its requirement during early embryogenesis and its role as a tumor suppressor (Kloecheld-Weivin et al. 2000; Roberts et al. 2000, Guidi et al. 2001; Roberts et al. 2002).

Several studies found that re-expression of hSNF5 in MRT-derived cell lines caused an accumulation in G0/G1, cellular senescence, and apoptosis (Ae et al. 2002, Betz et al. 2002, Verstege et al. 2002, Zhang et al. 2002, Oruetxebarria et al. 2004). These effects are largely the result of direct transcriptional activation of the tumor suppressor p16INK4a by hSNF5, which appears to be both necessary and sufficient for reduced cell proliferation and induction of cellular senescence and apoptosis (Oruetxebarria et al. 2004). p16INK4a controls the activity of pRb via inhibition of the cyclin D1–CDK4 kinase, which phosphorylates pRb (Lowe and Sherr 2003). Tumor suppressor pRb is a corepressor that is tethered to a broad range of genes by the E2F transcription factors. Hyperphosphorylation of pRb causes its dissociation from E2F, and relieves its antiproliferative activities. In addition to genes required for cell cycle progression from G1 to S phase, E2Fs also regulate genes involved in mitosis, spindle checkpoints, G2/M control, apoptosis, and differentiation (Stevaux and Dyson 2002).

Besides uncontrolled cell proliferation, chromosomal instability, which is characterized by changes in chromosome number or structure, is a hallmark of cancer cells (Rajagopalan and Lengauer 2004). Although still debated, there has been increasing support for the idea that polyploidy can lead to aneuploidy and contribute to the development of cancer (Rajagopalan and Lengauer 2004; Storchova and Pellman 2004). Although gross aneuploidy appears to be rare, chromosomal imbalances are commonly detected in MRTs and other hSNF5-related cancers (Berrak et al. 2002, Mitelman et al. 2003, Rickert and Paulus 2003, Kusaarka et al. 2004). Therefore, we decided to investigate the role of hSNF5 in ploidy control. Our results define a critical function for this chromatin remodeler in the maintenance of numerical chromosomal stability.

Results and Discussion

hSNF5 deficiency in MRT cells leads to polyploidization

In the majority of MRTs, hSNF5 is inactivated due to deletions, truncating nonsense mutations, or frameshift mutations. However, a number of point mutations, resulting in single amino acid substitutions (Fig. 1A), have been identified in tumors (Sevenet et al. 1999a,b). These include proline 48 to serine (P48S), arginine 137 to glycine (R127G), and serine 284 to leucine (S284L). In addition, we also changed serine 289 to alanine (S289A). S284 and S289 are located within one of the most highly con-
IPTG to hSNF5 was expressed (Fig. 1C). As expected, addition of
supplementation, albeit not as pronounced as when wild-type
derived hSNF5 mutants still caused a reduced cell accumu-
lation. 2004; Oruetxebarria et al. 2004). Induction of tumor-
acquired for the assembly of a SWI/SNF complex (Doan et
al. 2004). It should be noted that the induced levels
of hSNF5 fall within the normal physiological range
in MRT cells induces a p16 INK4a-dependent G0/G1 ar-
rest, cellular senescence, and apoptosis (Oruetxebarria et
al. 2004). Examination of the nuclear morphology of cells ex-
pressing wild-type hSNF5, however, the spindles
appeared to be caused by a failure of the mitotic spindle to
connect to the kinetochores, as revealed by confocal mi-
croscopy (Fig. 3B). In contrast, hSNF5-S284L expression exacerbated poly-
and aneuploidization, resulting in ∼25% of cells in the tetra-
plid range and almost 10% of cells that were near oc-
taploid. We note that, because mitotic cells were
obtained by a colcemid block, the karyotypes were de-
rived from cycling cells. Moreover, the presence of octa-
ploid cells demonstrated that a significant portion of tet-
raploid cells did not arrest due to the tetraploidy check-
point but re-entered mitosis.

Concomitant with polyploidization we observed cen-
trosome- and spindle amplification, as revealed by γ-tu-
bulin and α-tubulin staining, respectively [Fig. 2E,F]. Fol-
lowing hSNF5-S284L induction, the percentage of mi-
totic cells containing more than one spindle increased
from ∼5% to, respectively, 22% of cells with two
spindles and 11% with more than two spindles. How-
ever, mitotic cells expressing wild-type hSNF5 virtually
always contain one spindle. In summary, these results
revealed that loss of hSNF5 in MRT cells promotes poly-
and aneuploidization, whereas the cancer-associated
S284L substitution acts as a gain-of-function mutation, exacer-
bating chromosomal instability. Collectively,
these observations suggest a critical function for hSNF5
during mitosis.

Mutations in hSNF5 abrogate chromosome segregation

We utilized time-lapse microscopy to determine the cell
cycle stage at which the hSNF5-S284L-induced defect
occurs [Fig. 3A]. Cells expressing hSNF5-S284L enter mito-
sis normally, as judged by rounding up of the cells and
chromosomal condensation [indicated with an arrow]. How-
ever, a significant proportion of these cells subsequently
exited mitosis, as judged by cell flattening and
chromatin decondensation, but abstained from karyoki-
nesis and cytokinesis. Most cells that do not express
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shown], expression of hSNF5-S284L induced multilobed
nuclei, whereas overexpression of wild-type hSNF5 had
no effect. Thus, the S284L substitution mutation can have
a dominant effect, which is not restricted to MRT
cells.

Because multilobed nuclei are a feature of cells that
have undergone endoreplication, we tested whether
hSNF5-S284L might promote polyploidization. The full
karyotypes of G401 MRT-derived cells that either lack
hSNF5 or express wild-type hSNF5 or hSNF5-S284L
were determined by multicolor pg-COBA-FISH analysis [Fig. 2D, Wiegant et al. 2000]. About 90% of Lac-
empty cells or Lac-hSNF5 cells before induction were in
the diploid range of chromosome content, but displayed
frequent numerical chromosome aberrations. The re-
main 10% was near tetraploid. Strikingly, after ex-
pression of wild-type hSNF5 for 96 h, the cell population
became almost perfectly diploid. The disappearance of
aneuploid cells from the cycling population suggested a
role for hSNF5 in mitotic checkpoint control. In con-
trast, hSNF5-S284L expression exacerbated poly-
and aneuploidization, resulting in ∼25% of cells in the tetra-
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provided an orderly connection between metaphase chromosomes and centrosomes. Expression of hSNF5-S289A had similar effects on mitosis as hSNF5-S284L. It will be of interest to investigate whether S284 and S289 might be targets for phosphorylation, regulating the mitotic functions of hSNF5.

hSNF5 is critical for precise ploidy control

We used pq-COBRA-FISH to determine the effects of cancer-associated mutations in hSNF5 on ploidy distribution (Fig. 4A) and on numerical chromosome variation, as determined by the gain or loss of individual chromosomes (Fig. 4B). Examination of cells prior to induction of hSNF5 revealed that more than half displayed general numerical chromosomal aberrations and that ~10% were near tetraploid. Strikingly, after hSNF5 induction virtually all poly- and aneuploid cells were purged and the cell population became almost perfectly diploid. This dramatic effect of hSNF5 expression on numerical chromosome instability was highly significant ($p < 2.0 \times 10^{-6}$), as determined by the Mann-Whitney U-test. Thus, restoration of hSNF5 expression in MRT-derived cells, which lost the hSNF5 gene in the original cancerous lesion, suffices to revert chromosomal instability. In contrast, two different cancer-associated hSNF5 substitution mutants, hSNF5-P48S and hSNF5-P48S.

Figure 2. hSNF5-S284L expression exacerbates polyploidization. (A) DNA staining with DAPI revealed an increased number of multilobed nuclei after hSNF5-S284L expression. (B) Costaining of DNA [blue] and α-tubulin [red]. (C) GFP-hSNF5-S284L but not GFP-hSNF5 induces multilobed nuclei in MRC5 cells. The GFP signal [green] identifies the transfected cells. (D) Representative examples of pq-COBRA-FISH analysis of Lac-hSNF5-S284L cells. Di-, tri-, tetra-, and near octaploid metaphases are shown. Four Y-chromosomes, indicative of octaploidy, are indicated by arrows. (E) Representative examples of Lac-hSNF5-S284L cells with one, two, or more mitotic spindles. (F) Visualization of centrosomes [arrows] by γ-tubulin staining.

Figure 3. hSNF5-S284L induction causes an abortive cell cycle. (A) Time-lapse microscopy of hSNF5-S284L-expressing cells, which enter mitosis but exit prior to cell division. Arrows indicate condensed chromatin. (B) Failure of microtubule–kinetochore association in cells expressing hSNF5-S284L. Kinetochore were identified with CREST [red] antibodies and mitotic spindles with α-tubulin [green] antibodies.
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Figure 4. Restoration of wild-type hSNF5 expression, but not of cancer-associated mutants, reverts chromosomal instability. (A) Histogram depicting the frequency of cells with a given chromosome number as determined by metaphase analysis using pg-CO-BRA-FISH. The total number of chromosomes per cell was determined either before or after the induction of hSNF5. [B] Individual chromosome gains or losses.

R127G, failed to generate a diploid cell population. Expression of either hSNF5-S284L (p < 3.6 × 10^{-4}) or hSNF5-S289A (p < 2 × 10^{-2}) strongly promoted polyploidization and aneuploidy. In conclusion, our analysis of both loss-of-function and gain-of-function mutations revealed the critical role of hSNF5 in ploidy control.

hSNF5 activates the mitotic checkpoint through the p16^{INK4a}–cyclinD/CDK4–pRb–E2F pathway

Our results suggested that re-expression of hSNF5 tightens the mitotic checkpoint such that cell cycle progression of cells with an abnormal ploidy is blocked. To karyotype these noncycling cells, we used the drug calcyclin A to induce premature chromosome condensation of interphase cells [Bezrookove et al. 2003]. Indeed, we found that the karyotypes of noncycling hSNF5-expressing cells displayed a significantly higher degree of chromosome gains and losses than those of mitotic cells (p < 10^{-2}) [Fig. 5A,B]. These results suggest that the reduced accumulation of hSNF5-expressing cells is caused by selective arrest and senescence of aneuploid cells. After hSNF5 induction, only diploid cells remain cycling.

Next, we considered the pathway through which hSNF5 controls cellular ploidy. Whole-genome expression profiling of Lac-hSNF5 cells prior to and after hSNF5 induction revealed, among other findings, changed expression of many E2F targets, including mitotic control genes. This is illustrated by the representa-
hSNF5 cells (Lac-hSNF5/CDK4R24C) from a pREP4-derived vector (Rane et al. 2002). Mutants were generated using QuickChange mutagenesis (Stratagene). mRNA expression analysis was performed as described (Oruetxebarria et al. 2004). All primer sequences will be provided upon request. Gene expression profiling was performed using Affymetrix U133A GeneChips, and data analyses were performed using Omniviz software and the Ease program. An extensive description of these experiments will be reported elsewhere.

Immunofluorescence and time-lapse microscopy

Cells were grown on cover slips, fixed with 4% paraformaldehyde, and permeabilized in PBS with 0.1% Triton X-100, followed by standard indirect immunofluorescence. Nuclei were visualized by DAPI staining. Antibodies used: anti-Flag, F3165 (Sigma); anti-β-tubulin, T5168 (Sigma); anti-β-tubulin, T6557 (Sigma); anti-CREST was a gift from H. Clevers (Hubrecht Laboratory, Utrecht, The Netherlands) (Fodde et al. 2001). To quantify centrosome- and spindle amplification, ∼1000 mitotic cells for each condition were analyzed. Cells for time-lapse microscopy were grown on glass-bottom culture dishes (MatTek). Two hours before transfer to the 37°C microscope, medium was changed to HEPES-buffered DMEM without Phenol red (21063-029, Life Technology).

pq-COBRA-FISH and cytogenetic analysis

Detailed cytogenetic analysis using pq-COBRA-FISH was performed essentially as described (Wiegant et al. 2000). ULS reagent was provided by Kreatech Biotechnology. Interphase cells were karyotyped following Cal-lyculin A-induced chromosome condensation (Bezrookoove et al. 2001). To quantify centrosome- and spindle amplification, ∼1000 mitotic cells for each condition were analyzed. Cells for time-lapse microscopy were grown on glass-bottom culture dishes (MatTek). Two hours before transfer to the 37°C microscope, medium was changed to HEPES-buffered DMEM without Phenol red (21063-029, Life Technology).

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Figure 5. Expression of p16INK4a-insensitive CDK4R24C blocks hSNF5-induced mitotic checkpoint activation. Individual chromosome gains or losses as determined by karyotyping after a colcemid block (A) or calyculin A-induced premature chromosome condensation (B) of interphase cells. (C) Selection of E2F targets and mitotic controllers, regulated by hSNF5 identified by whole-genome expression profiling. Gene symbols according to unigene convention, known E2F targets, and fold changes in expression following hSNF5 are indicated. (D) Cell accumulation of Lac-hSNF5 cells stably expressing CDK4R24C in the presence (filled circles) or absence (open circles) of hSNF5. (E) Histogram depicting the frequency of cells with a given chromosome number. (F) Individual chromosome gains or losses. (G) RT–PCR analysis of gene expression. The same mRNA isolates were used to detect expression of the indicated genes.
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*Genes Dev.* 2005, 19:
Access the most recent version at doi:10.1101/gad.335805