SUPPLEMENTAL DATA

Rsf-1, a Chromatin Remodeling Protein, Induces DNA Damage and Promotes Genomic Instability

EXPERIMENTAL PROCEDURES

TP53 mutational analysis — Enriched tumor cells were obtained from fresh surgical specimens after immunosorting using the Ep-CAM antibody conjugated Dynal beads as previously described (1). The genomic DNA was isolated and analyzed for sequence mutations from exons 2 to 11 of the TP53 gene. The PCR primers and method for sequencing were previously reported (2). The nucleotide sequences were analyzed using the Mutation Surveyor program (Soft Genetics LLC, State College, PA). To confirm the mutations, we re-sequenced the exons with TP53 mutations using reverse primers.

Quantitative real-time PCR — PCR reactions were performed using an iCycler (Bio-Rad, Hercules, CA), and threshold cycle numbers (Ct) were obtained using the iCycler Optical system interface software. Mean Ct of the gene of interest was calculated from duplicate measurements and normalized with the mean Ct of a control gene, beta-amyloid precursor gene (APP), for which expression is relatively constant among the SAGE libraries analyzed (3). In cases where no gene expression was observed, a cut-off Ct value of 45 cycles was used. Data were expressed as fold increase or decrease as compared to the gene turned-off or vector control samples. We considered change exceeding two fold (delta Ct > 1) to be significant in this study.

Rsf-1 knockdown using small hairpin RNA — For functional confirmation of Rsf-1 knockdown, two small hairpin RNAs (shRNA1 and shRNA2) were purchased from Sigma. The sequences are CCGGCCAGTTCTGAACATTGTCAGATCTCTTAAAGTTCCAGAACT
(shRNA1) and CCGGCTTCTGACAAAGGTCTACTCGAGTAGAACCCTTTGTCTCAGA (shRNA2). Rsf-1 inducible RK3E cells were transfected with the shRNAs and empty vector (pLKO.1-puro; as the control) at a final concentration of 2 μg/ml using Lipofectamine (Invitrogen) under Rsf-1 gene turned off. Six hours after transfection, the cells were washed and cultured with regular culture medium for overnight. The cells were then harvested on the next day and seeded in 96 wells (4000 cells/well) in Doxy-free (Rsf-1 turned on) for cell growth assays. Cells seeded in Doxy-in (Rsf-1 turned off) were used as the controls.

**Immunofluorescence staining**—Rsf-1 inducible RK3E cells were used to determine whether Rsf-1 expression resulted in genome instability. Rsf-1 gene was turned on in RK3E cells using Dox-free medium. At different time points, cells on chamber slides were fixed with paraformaldehyde and incubated with anti-phospho-CHK2 (pCHK2) antibody (clone ab38461; Abcam, Cambridge, MA), anti-γH2AX antibody (clone ab11174; Abcam, Cambridge, MA) for 2 hrs and followed by Rhodamin-conjugated anti-mouse or anti-rabbit antibody (Jackson ImmunoResearch Lab., West Grove, PA). Cell nuclei were counter stained with DAPI (Sigma).

**Rsf-1 retroviral delivery system**—For the retroviral delivery system, the coding sequence of Rsf-1/V5 was cloned into a retroviral vector, pWZL. The retroviral vectors were transfected into Phoenix retrovirus packaging cells, and virus particles were collected from the culture supernatant 2 days after transfection. Viral transduction was performed by incubating MEF cells with viral supernatant overnight.
Fig. S1. Induction of Rsf-1 expression in RK3E cells. A. Western blot analysis indicates that RK3E cells stably transfected with the pBI-Rsf-1-V5 inducible expression vector showed detectable protein levels as early as 6 hrs after Rsf-1 induction by replacing doxycycline-containing culture medium with regular culture medium. B. Co-immunoprecipitation of Rsf-1 and rat SNF2H using an anti-V5 antibody in immunoprecipitation. Lane 1: silver staining of the pull down complex; Lane 2: two bands corresponding to Rsf-1 and its degraded product detected by anti-Rsf-1 Western blot analysis; Lane 3: rat SNF2H band detected by anti-SNF2H Western blot analysis. C. The number of annexin V positive cells is significantly higher in Rsf-1-on group than in Rsf-1-off group (p<0.001). Cells transfected with empty vector were used as the control. D. The number of annexin V positive cells was not significantly changed after Rsf-1 turned on in RK3E cells transfected with TP53 mutation (R175H) expressing vector (p > 0.05).
Fig. S2. Rsf-1 induces DNA strand breaks and activates DNA damage response. Rsf-1 inducible RK3E cells were used for studying the DDRs induced by Rsf-1 overexpression. A. Western blot analysis shows that induction of Rsf-1 expression in RK3E cells is associated with increased protein levels of phosphorylated γ-histone 2AX (γH2AX), phosphorylated check point kinase-2 (pCHK2), p53, and p21. GAPDH serves as a loading control. B. Upon gel electrophoresis, DNA with strand breaks migrated out of nuclei forming a comet tail like structure whereas the non-damaged DNA remained stationary within the nucleus. At all time points, the percentage of comet-like cells was significantly higher in the Rsf-1 induced group than in the non-induced group (p < 0.001). C. Immunofluorescence staining demonstrates
punctuate immunofluorescence (foci) of both $\gamma$H2AX and pCHK2 in RK3E cells after Rsf-1 induction. Ultraviolet light-irradiated cells (UV) serve as the positive control.
**Fig. S3.** Rescue of Rsf-1 mediated cell growth inhibition by Rsf-1 knockdown with shRNAs. A. Western blot analysis indicates the robustness of two shRNAs in suppressing Rsf-1 protein expression. The empty vector was used as the control. B. Cell growth assay in Rsf-1 inducible RK3E cells transfected with these two shRNAs and the control vector.
**Fig. S4.** A scramble siRNA could not inhibit Rsf-1-induced growth arrest in non-transformed RK3E cells. A, cells treated with scramble siRNA still reveal detectable staining of pCHK2 and γH2AX. B, Western blot analysis shows similar SNF2H levels in both buffer-treated (mock control) and scramble siRNA-treated cells. C, co-events were those cells showing down regulation in both SNF2H and pCHK2 or both SNF2H and γH2AX. D, growth curve analyses in Rsf-1 inducible RK3E cells transfected with scramble siRNA or buffer alone (mock control).
Defects in TP53 and ARF reverse Rsf-1-induced cell growth arrest. Effects of Rsf-1 expression were tested on mouse embryonic fibroblast (MEF) with different genetic backgrounds. A, Western blot analysis shows Rsf-1 protein expression in pWZL-Rsf-1 retrovirus transduced MEF but not in the control virus (pWZL vector only) transduced cells. GAPDH was used as loading control. After virus transduction, MEF with TP53 wt showed remarkable growth inhibition at Day 6. B, MEF cells with different genetic backgrounds were transduced with pWZL-Rsf-1 retrovirus. In contrast to TP53 wt, MEF with TP53-null and ARF-null continued to grow after virus transduction. C, Phase contrast images show cell number in Rsf-1 transduced TP53 wt MEF was significantly reduced as compared to TP53-null and ARF-null MEF.
References:

