Epigenetic and genetic loss of *Hic1* function accentuates the role of *p53* in tumorigenesis

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Summary

The gene hypermethylated in cancer 1 (*HIC1*) is epigenetically inactivated, but not mutated, in cancer. Here we show that cooperative loss of *Hic1* with *p53,* but not *INK4a,* yields distinct tumor phenotypes in mice. Germline deletion of one allele of each gene on the opposite chromosome yields breast and ovarian carcinomas and metastatic osteosarcomas with epigenetic inactivation of the wild-type *Hic1* allele. Germline deletion of the two genes on the same chromosome results in earlier appearance and increased prevalence and aggressiveness of osteosarcomas with genetic deletion of both wild-type genes. In human osteosarcomas, hypermethylation of *HIC1* is frequent only in tumors with p53 mutations. Our results indicate the importance of genes altered only through epigenetic mechanisms in cancer progression in conjunction with genetically modified tumor suppressor genes.

Introduction

The classic hallmark of tumor suppressor genes is the loss of their function through genetic mutations in human cancer (Hanahan and Weinberg, 2000). However, such functional disruption has now also been associated with transcriptional inactivation of defined tumor suppressor genes in association with promoter hypermethylation (Jones and Baylin, 2002). In recent years, there has been a growing list of candidate tumor suppressor genes that appear to be modified only by such epigenetic mechanisms, such as *HIC1* (Chen et al., 2003; Wales et al., 1995) and *RASSF1A* (Dammann et al., 2000). *HIC1* encodes a zinc finger transcriptional factor that represses transcription both dependent and independent of class I histone deacetylases (Deltour et al., 1999, 2002). *Hic1* is essential for mouse development, and homozygous deletion of *Hic1* results in embryonic and perinatal lethality (Carter et al., 2000). Evidence that such a gene may truly play an important role in cancer has come from a study in mice employing germline disruption of one allele of *Hic1*. This maneuver predisposes mice to an age- and gender-dependent spectrum of malignant tumors, suggesting tumor suppressor functions in vivo (Chen et al., 2003). Interestingly, as in human cancers, loss of function of the remaining wild-type *Hic1* allele in cancers from the heterozygous mice appears only in association with hypermethylation of one of the two promoters for the gene, which is sufficient to inactivate it transcriptionally (Chen et al., 2003).

While the above mouse studies indicate a tumor suppressor role for epigenetically silenced *Hic1*, it remains to be further resolved that loss of *Hic1* function, and that for other epigenetically silenced genes, in association with promoter hypermethylation, is a cause rather than a consequence of tumorigenesis. This is especially so since cancer evolves with multiple genetic and epigenetic alterations (Hanahan and Weinberg, 2000; Jones

SIGNIFICANCE

This paper addresses the fundamental question of whether, in cancer, genes altered solely through epigenetic gene silencing can significantly influence tumor progression events driven by classic tumor suppressor genes altered through mutations. Using *Hic1* and *p53* double heterozygous knockout mice, we show that the epigenetically silenced gene, *Hic1,* cooperates with the mutated tumor suppressor gene, *p53,* in determining cancer progression and spectrum. The work provides experimental evidence that epigenetic modification alone can serve as a "hit" in the tumorigenesis pathway, and suggests that human chromosome 17p13 is harboring two tumor suppressors that could function individually or synergistically, each using different mechanisms for inactivation.

and Baylin, 2002). It is thus critical to determine whether loss of *Hic1* function can play a significant role in the progression of cancers in conjunction with defined genetic alterations of powerful tumor suppressor genes. Toward these goals, we have now crossed *Hic1* heterozygous mice to mice carrying the disruption of *p53*, a classic and one of the most powerful and frequently mutated tumor suppressor genes. *HIC1* and *p53* are located on the same chromosome at positions 17p13.3 and 17p13.1, respectively, in humans. The deletion of these regions on at least one chromosome is frequently observed in cancers, including breast and ovarian carcinomas (Cornelis et al., 1994; Konishi et al., 1998; Phillips et al., 1996; Saxena et al., 1992). The intact copy for *p53* is then frequently mutated, and that for *Hic1* is frequently hypermethylated (Chen et al., 2003; Fujii et al., 1998; Narayan et al., 2003; Rathi et al., 2003).

In mice, both *Hic1* and *p53* are also closely located on chromosome 11, with another tumor suppressor gene, *Nf1,* residing in between them. It has been shown that disruption of *Nf1* cooperates with *p53* disruption differently when deleted on separate chromosomes (*trans*) versus on the same chromosome (*cis*) (Cichowski et al., 1999; Vogel et al., 1999). *Cis* $Nf1^{+/-}p53^{+/-}$ mice have far more accelerated tumorigenesis than *trans* $Nf1^{+/-}p53^{+/-}$ mice due to codeletion of the wild-type alleles for each gene in tumors. Therefore, we reasoned that if genetic codeletion of *Hic1* with *p53* would occur in the *cis* $Hic1^{+/-}p53^{+/-}$ mice, it might result in deletion of both wild-type copies of the genes on the opposite chromosome. If loss of *Hic1* function were causal and critical for key steps in tumorigenesis, codeletion of *Hic1* with *p53* might produce far different tumor phenotypes from those seen in $p53^{+/-}$ or *cis* $Nf1^{+/-}p53^{+/-}$ mice. Creating genetic deletion rather than hypermethylation of the wild-type allele of *Hic1* in adult cancer might thus provide even more insight into the true power of the tumor suppressor role of this gene. Our results have borne out all of the above hypotheses and well illustrate how an epigenetically silenced gene can not only cooperate with genetically altered tumor suppressor genes but also play a strong role in determining the course of tumorigenesis.

Results and discussion

Phenotypes of *trans* double heterozygotes

We first generated *trans* double heterozygotes that carried germline disruptions of *Hic1* and *p53* on separate chromosomes. In a 100-week study, as observed before (Chen et al., 2003), $Hic1^{+/-}$ cohort mice in their late life developed a gender-dependent spectrum of malignant tumors in which males developed predominantly carcinomas, while females developed only lymphomas and soft tissue sarcomas (Figure 1A and data not shown). In contrast, *trans* $Hic1^{+/-}p53^{+/-}$ mice did not exhibit significant gender bias of malignant tumors due to the rapid occurrence of lymphomas and sarcomas in the $p53^{+/-}$ background, and there was no acceleration of tumorigenesis for *trans* $Hic1^{+/-}p53^{+/-}$ mice compared to $p53^{+/-}$ mice (data not shown). However, *trans* $Hic1^{+/-}p53^{+/-}$ mice developed, in an age-dependent manner, a higher incidence of osteosarcomas than $p53^{+/-}$ mice, while soft tissue sarcomas and lymphomas had similar incidence between the two groups (Figure 1A). Thirty-five osteosarcomas were identified in 100 *trans* $Hic1^{+/-}p53^{+/-}$ mice (35%) compared to 11 osteosarcomas in 54 $p53^{+/-}$ mice (20%, $p = 0.058$). However, when age is taken

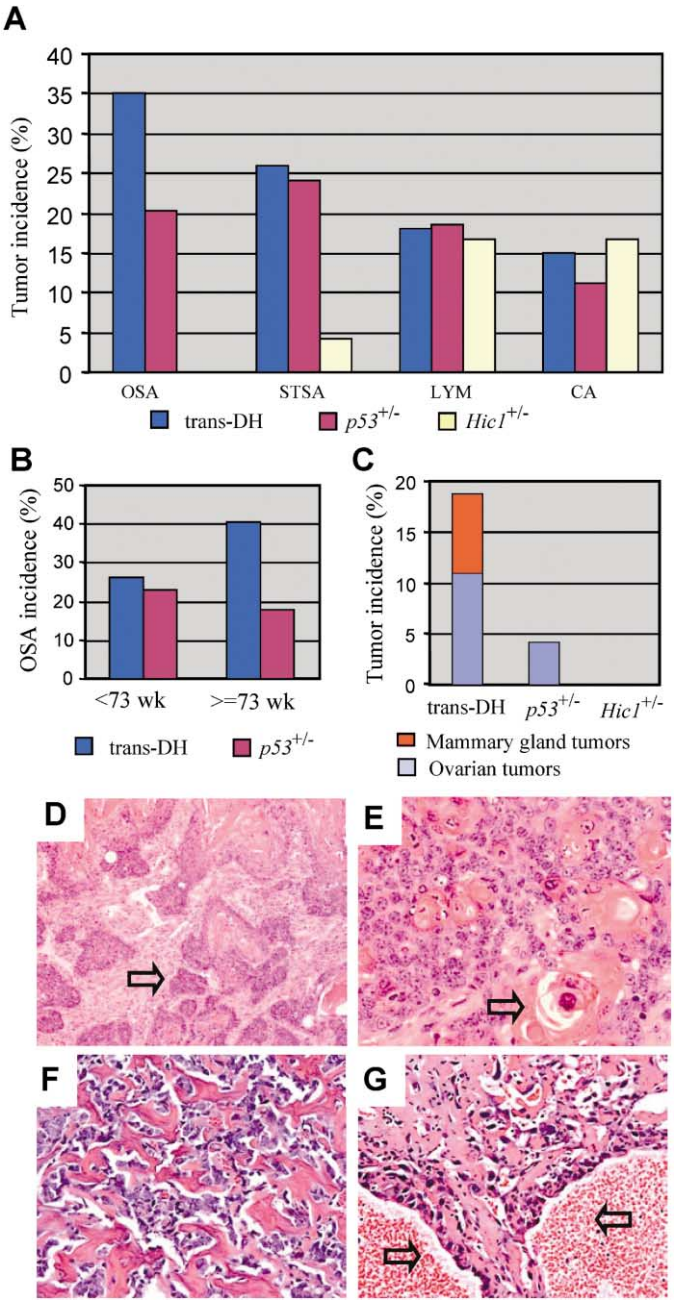

Figure 1. Phenotypes of *trans* $Hic1^{+/-}p53^{+/-}$ mice

A: Malignant tumor incidence. There were 100 *trans* $Hic1^{+/-}p53^{+/-}$ (*trans*-DH), 54 $p53^{+/-}$, and 24 $Hic1^{+/-}$ mice. OSA, osteosarcoma; STSA, soft tissue sarcoma; LYM, lymphoma; CA, carcinoma.

B: Incidence of osteosarcomas with age. For mice younger than 73 weeks, there were ten OSA in 38 *trans*-DH, and six in 26 $p53^{+/-}$ mice. For mice 73 weeks and older, there were 25 OSA in 62 *trans*-DH and five in 28 $p53^{+/-}$ mice.

C: Incidence of mammary gland and ovarian tumors. There were 64 *trans* $Hic1^{+/-}p53^{+/-}$, 24 $p53^{+/-}$, and 14 $Hic1^{+/-}$ females, and there were 5 mammary gland tumors and 7 ovarian tumors in the *trans*-DH, while there was only one ovarian tumor in $p53^{+/-}$ mice.

D–G: Representative histology of an adenosquamous mammary tumor composed of nests and lobules of epithelial cells exhibiting glandular (**D**, arrow, 100×) and squamous differentiation (**E**, arrow, 400×), and a typical osteosarcoma in the *trans*-DH mice demonstrating polygonal to spindle-shaped neoplastic cells producing bone (**F**, 200×). Metastatic osteosarcomas from *trans* $Hic1^{+/-}p53^{+/-}$ mice contained multiple, variably sized, prominent vascular spaces (telangiectatic pattern, **G**, arrows, 400×).

into account, the incidence of osteosarcomas in the *trans* double heterozygotes increased significantly (Figure 1B). Twenty-five osteosarcomas occurred in 62 *trans Hic1*$^{+/-}$*p53*$^{+/-}$ mice (40%) older than 73 weeks, whereas 5 in 28 *p53*$^{+/-}$ mice (18%) developed this tumor for the same age range (p = 0.036). In contrast, both *trans Hic1*$^{+/-}$*p53*$^{+/-}$ and *p53*$^{+/-}$ mice developed similar incidence of osteosarcomas before 73 weeks (Figure 1B). The osteosarcomas in the *trans* mice also had a higher metastasis rate as described further later.

Importantly, we identified five mammary gland and seven ovarian tumors in 64 *trans Hic1*$^{+/-}$*p53*$^{+/-}$ females (19%), versus only a single ovarian hemangiosarcoma in *p53*$^{+/-}$ mice, and none of these tumors in *Hic1*$^{+/-}$ mice (p = 0.025, Figure 1C and data not shown). The mammary tumors included four adenosquamous carcinomas and one myoepithelial tumor, which was positive for smooth muscle actin (data not shown). The adenosquamous carcinomas formed large masses composed of glandular and squamous elements and invaded surrounding tissues, but did not metastasize. Histologically, these carcinomas (Figures 1D and 1E) resembled the pilar mammary gland tumors found in mice engineered for overactivity of the Wnt pathway (Rosner et al., 2002), but appeared distinct from those in mice with conditional *Brca1* knockout in which diverse types of carcinomas occur (Xu et al., 1999), and in mice lacking *p53* and *Brca2* or telomerase in which the acinar type of adenocarcinoma predominates (Artandi et al., 2000; Jonkers et al., 2001). The ovarian tumors of *trans Hic1*$^{+/-}$*p53*$^{+/-}$ mice included two papillary cystadenomas, a choriocarcinoma, a carcinosarcoma, a luteoma, and two hemangiosarcomas. Furthermore, *trans Hic1*$^{+/-}$*p53*$^{+/-}$ mice developed additional tumor types that were not seen in either *Hic1* or *p53* heterozygotes alone, including a metastatic neuroendocrine tumor, an adrenal pheochromocytoma, and a choriostoma in brain (data not shown).

Phenotypes of *cis* double heterozygotes

We then generated mice of the *cis Hic1*$^{+/-}$*p53*$^{+/-}$ setting. As shown in Figure 2A, *Hic1*$^{+/-}$ status did not accelerate tumorigenesis nor alter tumor spectrum in the *p53*$^{-/-}$ background, and thymic lymphoma continued to be dominant (data not shown). However, *cis* double heterozygotes had a distinctly accelerated tumorigenesis compared to cohort *p53*$^{+/-}$ mice by tumor-free survival analysis (p = 0.011, Figure 2A). The *cis Hic1*$^{+/-}$*p53*$^{+/-}$ mice predominantly developed osteosarcomas, in sharp contrast to the predominance of soft tissue sarcomas in *p53*$^{+/-}$ cohorts (Figure 2B). Nineteen osteosarcomas (43%) occurred in 44 *cis Hic1*$^{+/-}$*p53*$^{+/-}$ mice, compared to 2 (10%) in 21 *p53*$^{+/-}$ mice (*p* = 0.007). Osteosarcomas represented 55% of all 35 malignant tumors in *cis Hic1*$^{+/-}$*p53*$^{+/-}$ mice, whereas they were 13% in 16 malignant tumors in *p53*$^{+/-}$ mice (p = 0.005, Figure 2C). The tumor spectrum with predominance of soft tissue sarcomas in our *p53*$^{+/-}$ mice resembled that in *cis Nf1*$^{+/-}$*p53*$^{+/-}$ and their cohort *p53*$^{+/-}$ mice (Cichowski et al., 1999; Vogel et al., 1999).

Metastasis of osteosarcoma

We found a higher incidence of metastatic osteosarcomas in both *trans* and *cis Hic1*$^{+/-}$*p53*$^{+/-}$ mice than that in *p53*$^{+/-}$ mice (Figure 3). In the *trans* mice, this difference was significant for mice older than 73 weeks, as 12 metastatic osteosarcomas (19%) occurred in 62 double heterozygotes compared to only one (4%) in 28 *p53*$^{+/-}$ mice (p = 0.048, Figure 3A). These tumors

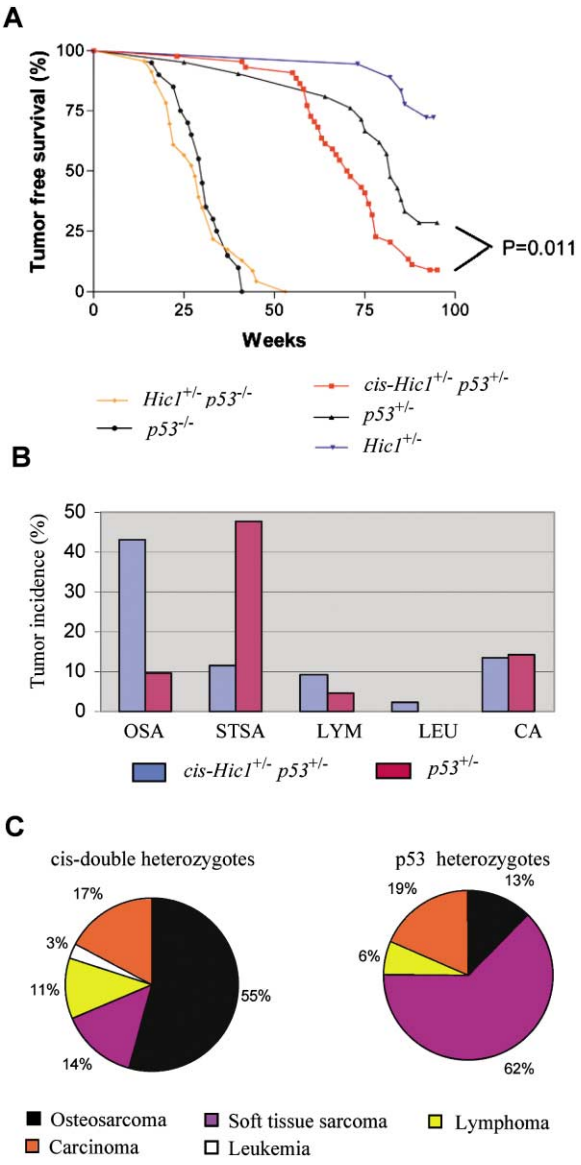

Figure 2. Phenotypes of *cis Hic1*$^{+/-}$*p53*$^{+/-}$ mice

A: Kaplan-Meier analysis of tumor incidence. There were 20 *p53*$^{-/-}$, 23 *Hic1*$^{+/-}$*p53*$^{-/-}$, 44 *cis Hic1*$^{+/-}$*p53*$^{+/-}$, 21 *p53*$^{+/-}$, and 20 *Hic1*$^{+/-}$ mice.

B: Comparison of tumor incidence of *cis Hic1*$^{+/-}$*p53*$^{+/-}$ and cohort *p53*$^{+/-}$ mice. There were 19 OSA and 5 STS in *cis Hic1*$^{+/-}$*p53*$^{+/-}$ mice compared to 2 OSA and 10 STS in *p53*$^{+/-}$ mice. LEU, leukemia; the other abbreviations are the same as in Figure 1A.

C: Comparison of tumor spectrum of *cis Hic1*$^{+/-}$*p53*$^{+/-}$ versus *p53*$^{+/-}$ mice. Thirty-five malignant tumors were identified in *cis Hic1*$^{+/-}$*p53*$^{+/-}$ mice and sixteen in *p53*$^{+/-}$ mice.

in the *trans* mice frequently metastasized to lung, liver, and mesenchymal tissues. Most of these tumors showed classic histological features of osteosarcomas (Figure 1F), but two had telangiectatic patterns with large vessels formed in the distal metastatic sites (Figure 1G), resembling the highly aggressive osteosarcoma counterpart in humans (Spina et al., 1998). Similarly, in the *cis* mice, seven metastatic osteosarcomas occurred in 44 mice compared to none in the 21 control *p53*$^{+/-}$ mice (p = 0.052, Figure 3B). All together, for 144 double heterozygous

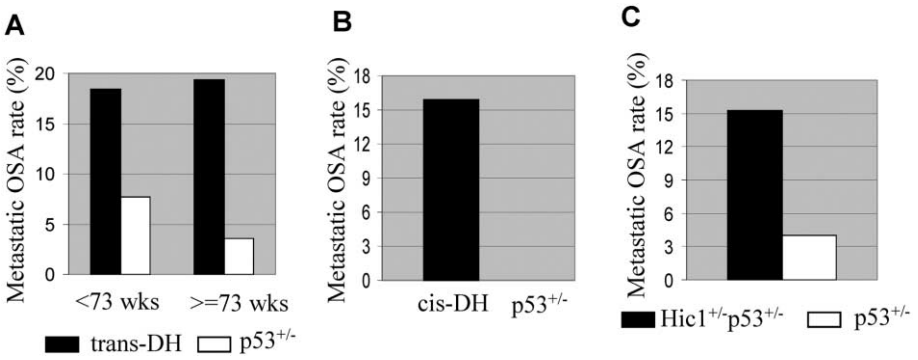

Figure 3. Incidence of metastatic osteosarcomas

A: Metastatic osteosarcomas with age in *trans* *Hic1*$^{+/-}$*p53*$^{+/-}$ versus *p53*$^{+/-}$ mice. There were 7 metastatic osteosarcomas in 38 *trans*-DH mice and 2 in 26 *p53*$^{+/-}$ mice younger than 73 weeks. There were 12 such tumors in 62 *trans*-DH mice and 1 in 28 *p53*$^{+/-}$ mice 73 weeks and older.
B: Metastatic osteosarcomas in *cis* *Hic1*$^{+/-}$*p53*$^{+/-}$ versus cohort *p53*$^{+/-}$ mice. There were 7 metastatic osteosarcomas in 44 cis-DH mice and none in 21 cohort *p53*$^{+/-}$ mice.
C: A combined incidence of metastatic osteosarcomas in both *trans* and *cis* *Hic1*$^{+/-}$*p53*$^{+/-}$ mice compared to that in the combined cohort of *p53*$^{+/-}$ mice.

mice (*trans* plus *cis*), twenty-two (15%) developed metastatic osteosarcomas compared to three (4%) in 75 combined *p53*$^{+/-}$ cohorts ($p = 0.013$, Figure 3C).

Mechanisms for loss of function of *Hic1* and *p53*

By Southern blot, we found that the wild-type allele of *Hic1* was retained in 29 of 30 various tumors analyzed from *trans* *Hic1*$^{+/-}$*p53*$^{+/-}$ mice; for example, osteosarcomas and mammary gland carcinomas (Figures 4A and 4E, and data not shown). Only one ovarian tumor appeared to lose the wild-type *Hic1* allele (data not shown). In contrast, deletion of the wild-type allele of *p53* was observed for 27 out of 30 tumors in *trans* *Hic1*$^{+/-}$*p53*$^{+/-}$ mice (Figures 4B and 4F, and data not shown). Interestingly, the size of the *p53* deletion varied by tumor type and did not always include the targeted *Hic1* allele on the chromosome. Thus, 7 out of 13 osteosarcomas retained the intact targeted *Hic1* allele, while in the rest it was deleted with wild-type *p53* (Figure 4A); in contrast, both the targeted *Hic1* and wild-type *p53* alleles were always deleted simultaneously in the mammary adenosquamous carcinomas (Figures 4E and 4F).

We then analyzed promoter hypermethylation of the remaining wild-type *Hic1* allele in tumors of *trans* *Hic1*$^{+/-}$*p53*$^{+/-}$

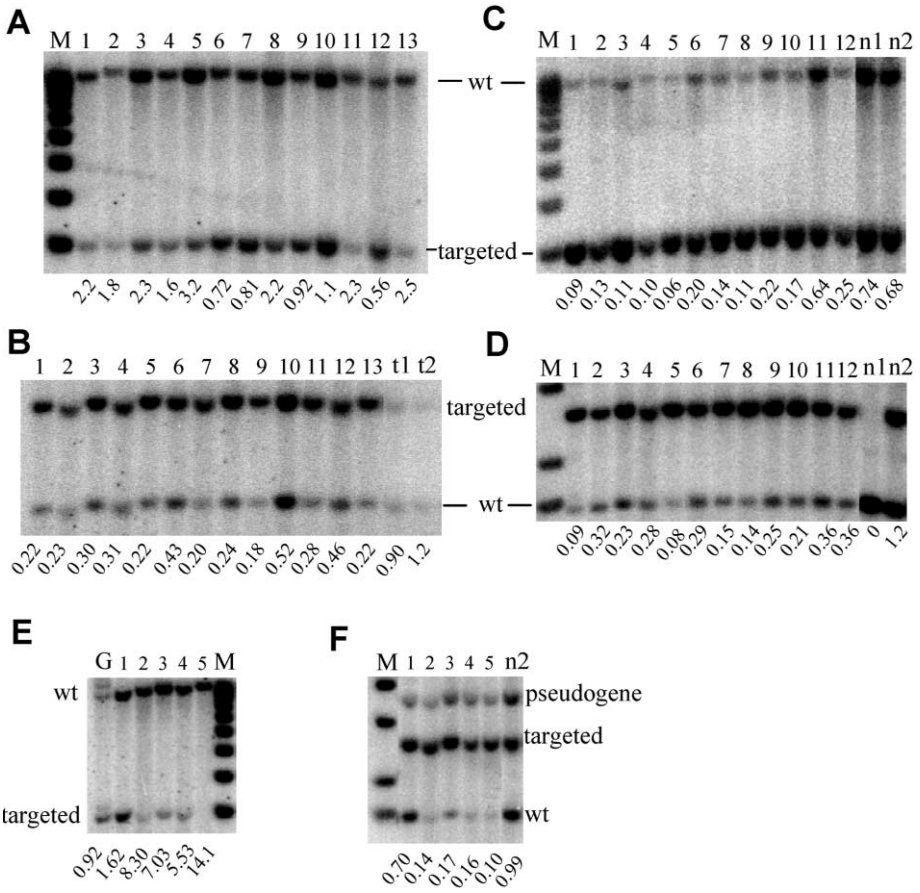

Figure 4. LOH analysis for *Hic1* and *p53*

A–D: Southern blot analysis of osteosarcomas for LOH status of *Hic1* (**A**) and *p53* (**B**) in tumors from *trans* *Hic1*$^{+/-}$*p53*$^{+/-}$ mice, and loss of *Hic1* (**C**) and *p53* (**D**) from *cis* *Hic1*$^{+/-}$*p53*$^{+/-}$ mice. In **C** and **D**, preparations of normal bone from age-matched mice were used as controls from *Hic1*$^{+/-}$ (n1) and *cis* *Hic1*$^{+/-}$*p53*$^{+/-}$ (n2) mice. In **B**, tail DNA of *trans* *Hic1*$^{+/-}$*p53*$^{+/-}$ mice (t1 and t2) was used as controls; for **A**, normal tissue control is illustrated in **E**, which was derived from the same membrane as **A**. Tumor samples are arranged in the same order on gels for **A** and **B**, and on gels for **C** and **D**.
E and F: Southern blot analysis of mammary gland tumors for deletions of *Hic1* (**E**) and *p53* (**F**). Tumor 1 is a myoepithelial tumor, and the rest are adenosquamous carcinomas. In **E**, an age-matched normal mammary gland DNA (G) was used as a control; in **F**, normal bone DNA (n2) was used as a control. The numbers underneath all gels are ratios of wild-type (wt) over targeted alleles. The deletion of the wild-type allele was considered to have occurred if the ratio was less than 0.50 in order to distinguish it from hybridization variation. In contrast, the deletion of the targeted allele occurred if the ratio was higher than 2.0. For **E**, a small portion of the normal tissue control was not completely digested by XbaI. The ratio underneath lane G was calculated for bands with complete digestion. If noncompletely digested bands were taken into consideration, the ratio was 1.18. M, DNA ladder.

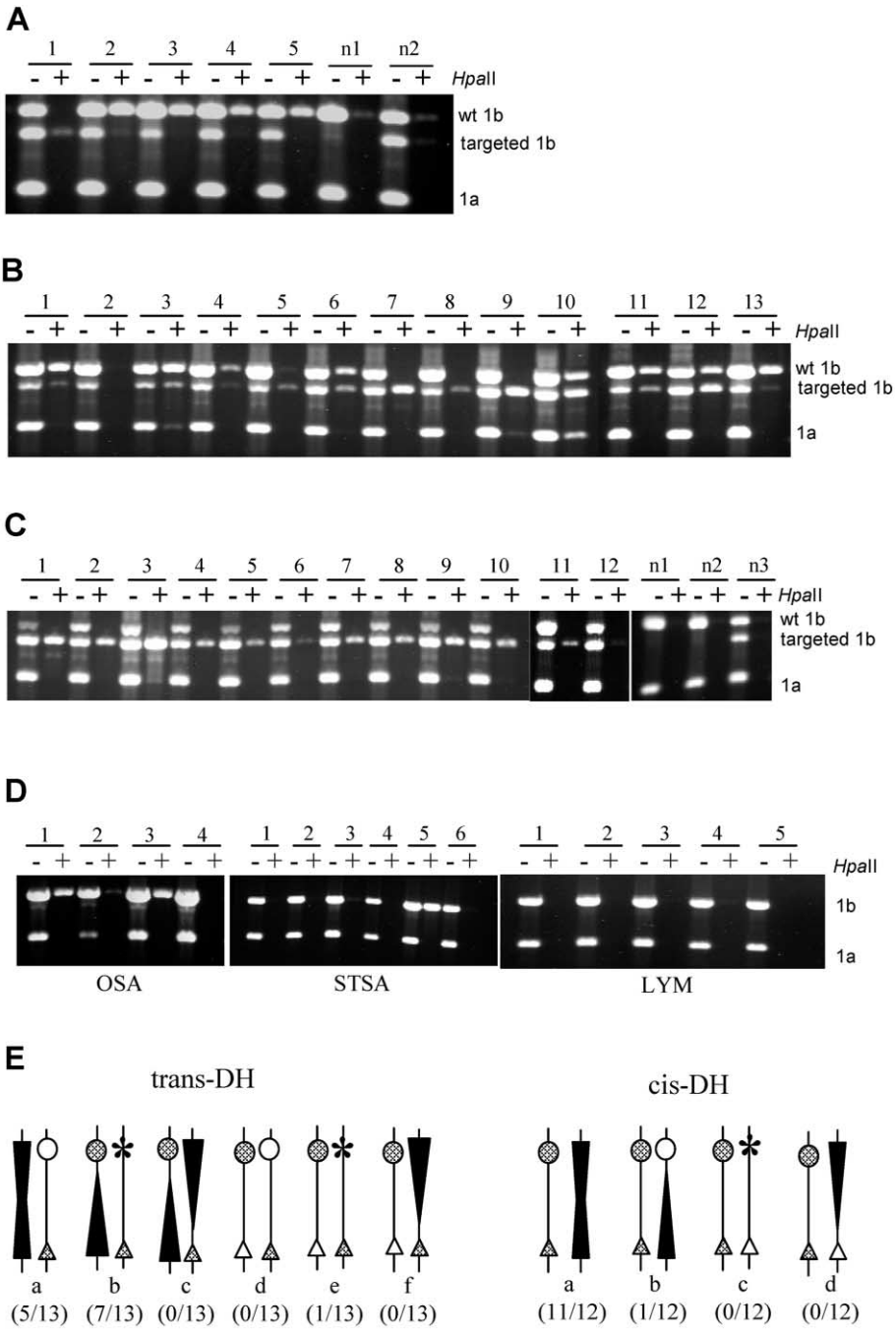

Figure 5. *Hic1* promoter hypermethylation analysis

A: HpaII-PCR assay (Chen et al., 2003) for mammary gland tumors and normal, age-matched mammary glands of wild-type (n1) and $Hic1^{+/-}$ (n2) females. A strong PCR product generated from input DNA digested with HpaII is seen only for densely methylated *Hic1* wild-type (wt) 1b promoter in adenosquamous carcinomas (tumors 2–5). Normal mammary glands had only trace amounts of wt 1b promoter methylation (n1 and n2) seen in aging mice.

B and C: HpaII-PCR assay for osteosarcomas from *trans* $Hic1^{+/-}p53^{+/-}$ (**B**) and *cis* $Hic1^{+/-}p53^{+/-}$ mice (**C**); and normal bone controls from wild-type (n1, n2 in **C**) and *cis* $Hic1^{+/-}p53^{+/-}$ (n3 in **C**) mice. All tumor samples in **A**, **B**, and **C** were arranged in the same order as they appeared in Figures 4E, 4A, and 4C respectively.

D: HpaII-PCR assay for tumors in *p53* heterozygotes. OSA, osteosarcomas; STSA, soft tissue sarcomas; LYM, lymphomas.

E: Summary of allele status of *Hic1* and *p53* in osteosarcomas of *trans* versus *cis* double heterozygotes. Unfilled triangle, wild-type *p53*; grid-filled triangle, germline-disrupted *p53*; unfilled circle, wild-type *Hic1*; grid-filled circle, germline-disrupted *Hic1*; asterisk, hypermethylated *Hic1*; long filled triangle, somatic chromosomal deletion in tumors.

mice. We used a HpaII-PCR assay (Chen et al., 2003) previously designed to distinguish methylation of the *Hic1* downstream 1b promoter in the wild-type allele versus the targeted allele that carries a targeting cassette for the replacement of *Hic1* coding exon 2 (Carter et al., 2000). All four mammary gland adenosquamous carcinomas, but not the myoepithelial tumor, had dense methylation of the wild-type *Hic1* 1b promoter similar to the pattern seen for other carcinomas studied previously (Chen et al., 2003) (Figure 5A). The osteosarcomas also had hypermethylation of *Hic1* promoters. Eight out of 13 showed extensive wild-type *Hic1* 1b promoter hypermethylation, and two also had hypermethylation extending to the upstream 1a promoter (Figure 5B). Consistent with our previous findings (Chen et al., 2003), we found that loss of *Hic1* expression was associated with hypermethylation of the *Hic1* promoter in both mammary gland carcinomas and osteosarcomas examined, while abundant Hic1 expression appeared in tumors without promoter hypermethylation (Figure 6). Interestingly, we also found that two out of four osteosarcomas in control $p53^{+/-}$ mice had dense *Hic1* 1b hypermethylation (Figure 5D), but only one out of eleven soft tissue sarcomas and lymphomas had *Hic1* promoter hypermethylation (Figure 5D). These data indicate that loss of *p53* function, alone, predominantly contributes to soft tissue sarcomas and lymphomas in $p53^{+/-}$ mice, but that concomitant loss of *Hic1* function

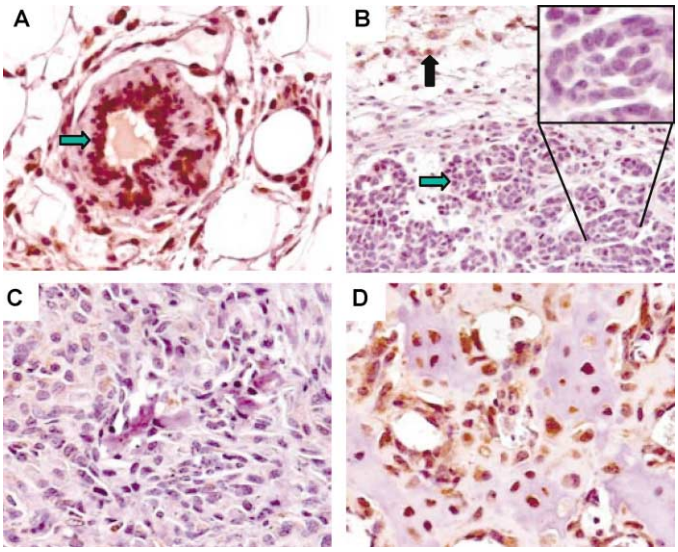

Figure 6. Immunohistochemical analysis of Hic1 expression

A: Strong Hic1 expression (brown stain) in a normal mammary duct surrounded by epithelial cells (green arrow) and fat tissues, which are next to a mammary carcinoma.

B: Loss of Hic 1 expression in a mammary carcinoma with the *Hic1* promoter hypermethylation (nuclei highlighted by a purple stain, green arrow) compared to the adjacent normal stromal tissues with Hic1 expression (brown nuclei stain, black arrow). The inset illustrates an enlarged view of a tumor glandular structure with negative Hic1 stain.

C and D: Loss of Hic1 expression in osteosarcomas (purple nuclei) with Hic1 promoter hypermethylation (**C**), in contrast to strong expression (brown nuclei) in those without hypermethylation (**D**).

through promoter hypermethylation can be associated with development of osteosarcomas in these control $p53^{+/-}$ mice. The germline disruption of one allele of *Hic1* in *trans* $Hic1^{+/-}p53^{+/-}$ mice appears to, then, increase the rate and virulence of osteosarcomas and appearance of the breast neoplasms (Figure 1), by leaving only one *Hic1* allele as a target for the hypermethylation. Together, our results suggest that epigenetic inactivation of *Hic1* cooperates with loss of *p53* function to facilitate the evolution of these tumor types.

In contrast to the above findings for hypermethylation of wild-type *Hic1* in *trans* $Hic1^{+/-}p53^{+/-}$ mice, the wild-type *Hic1* allele was deleted in all but one of 15 osteosarcomas and soft tissue sarcomas analyzed from *cis* $Hic1^{+/-}p53^{+/-}$ mice (Figure 4C and data not shown). While the wild-type *p53* allele was deleted in all tumors in *cis* double heterozygotes (Figure 4D), similar to that in the *trans*, the *p53* deletion in 7 out of 13 osteosarcomas in the *trans* double heterozygotes did not include the targeted *Hic1* allele on the same chromosome, but both wild-type genes were codeleted in 11 out of 12 osteosarcomas in the *cis* double heterozygotes ($p = 0.030$). Interestingly, hypermethylation of the targeted 1b promoter continued to occur (Figure 5C), suggesting that the methylation machinery in the tumors could still affect *Hic1* promoters in the *cis* $Hic1^{+/-}p53^{+/-}$ mice. The above data for different sizes of *p53* deletion in the tumors from the *trans* versus *cis* double heterozygotes, summarized in Figure 5E, indicate that deletion of *Hic1* is not merely driven by deletion of *p53*. Rather, there is a sharp switch from epigenetic to genetic inactivation of *Hic1* in the *cis* double heterozygous mice. The exact molecular basis underling this change is unknown; however, our data suggest that the simultaneous deletions of both wild-type genes in tumors from the *cis* double heterozygotes occur because this is the most powerful and efficient selection event for rapid tumorigenesis.

One possibility that must be considered for all of our above findings in mice is that the large genomic deletions involving *p53* in the *cis* or *trans* $Hic1^{+/-}p53^{+/-}$ mice may involve other genes on the chromosome. The first to be considered is *Nf1* that lies between *Hic1* and *p53*. However, in our *cis* disrupted mice, one wild-type *Nf1* allele is on the chromosome for which the *Hic1* and *p53* genes are knocked out in the germline. No somatic deletions occur for this chromosome in tumors (Figures 4C and 4D). Also, in the *trans* animals, at least one *Nf1* allele is intact on the chromosome that always retains wild-type *Hic1* and germline-deleted *p53*. Therefore, in all of our mice, somatic deletion occurred only on one chromosome (Figure 5E), and *Nf1* is always retained at least heterozygously. Any contribution of *Nf1* heterozygosity to the tumors observed is probably absent or minimal for multiple reasons. First, *Nf1* heterozygotes develop frequent myeloid leukemia and pheochromocytoma (Jacks et al., 1994b) that are rare in our study, and the frequent tumors we have observed, including osteosarcomas and mammary gland and ovarian tumors, do not occur in *Nf1* heterozygotes. Second, about 80% of tumors in *cis* $Nf1^{+/-}p53^{+/-}$ mice are soft tissue sarcomas (Cichowski et al., 1999; Vogel et al., 1999), but we have observed only 13% of such tumors in our *cis* $Hic1^{+/-}p53^{+/-}$ mice with a similar mixed C57BL/6 and 129/Sv genetic background (Figure 2C). Third, we have observed 55% osteosarcomas in *cis* $Hic1^{+/-}p53^{+/-}$ mice, and these tumors seldom develop in *cis* $Nf1^{+/-}p53^{+/-}$ mice. Finally, frequent malignant peripheral nerve sheath tumors occur in *cis* $Nf1^{+/-}p53^{+/-}$ mice, but do not occur in our *cis* $Hic1^{+/-}p53^{+/-}$ mice. All together, *Hic1* has specific roles in tumor suppression in cooperation with *p53* in spite of the fact that *Hic1* and *Nf1* are located at very close loci.

Large chromosomal truncations induced by codeletion of *Hic1* and *p53* may also result in loss of other genes located on the same chromosome but distal from the *Hic1-Nf1-p53* region. One such gene that could influence the tumor spectrum is *Brca1*. While conditional knockout of both alleles of mouse *Brca1* in mammary glands results in spontaneous carcinomas (Xu et al., 1999), heterozygous knockout of *Brca1* in mice does not generate mammary gland tumors (Deng and Scott, 2000). Heterozygous disruption of *p53* can accelerate mammary gland carcinogenesis in the *Brca1* conditional setting (Xu et al., 1999); however, nonconditional $p53^{+/-}Brca1^{+/-}$ double heterozygous mice do not develop spontaneous mammary gland and ovarian tumors or have increased incidence of osteosarcomas (Cressman et al., 1999). The chromosomal loss induced by codeletion of *Hic1* and *p53* on one chromosome in our study might span distal *Brca1* as well; however, similar to *Nf1* discussed above, at least one allele of *Brca1* will remain intact. The heterozygous status of *Brca1* thus excludes any significant contribution of *Brca1* to the phenotypes observed in our studies.

Another gene that could possibly play a role in our findings is *Nf2,* which is located near the centromere of chromosome 11 (Figure 7A). Intriguingly, heterozygous disruption of *Nf2* predisposes mice to a high frequency of metastatic osteosarcomas, and mice double heterozygous for *p53* and *Nf2*, either in *cis* or in *trans*, also exhibit further accelerated formation of osteosar-

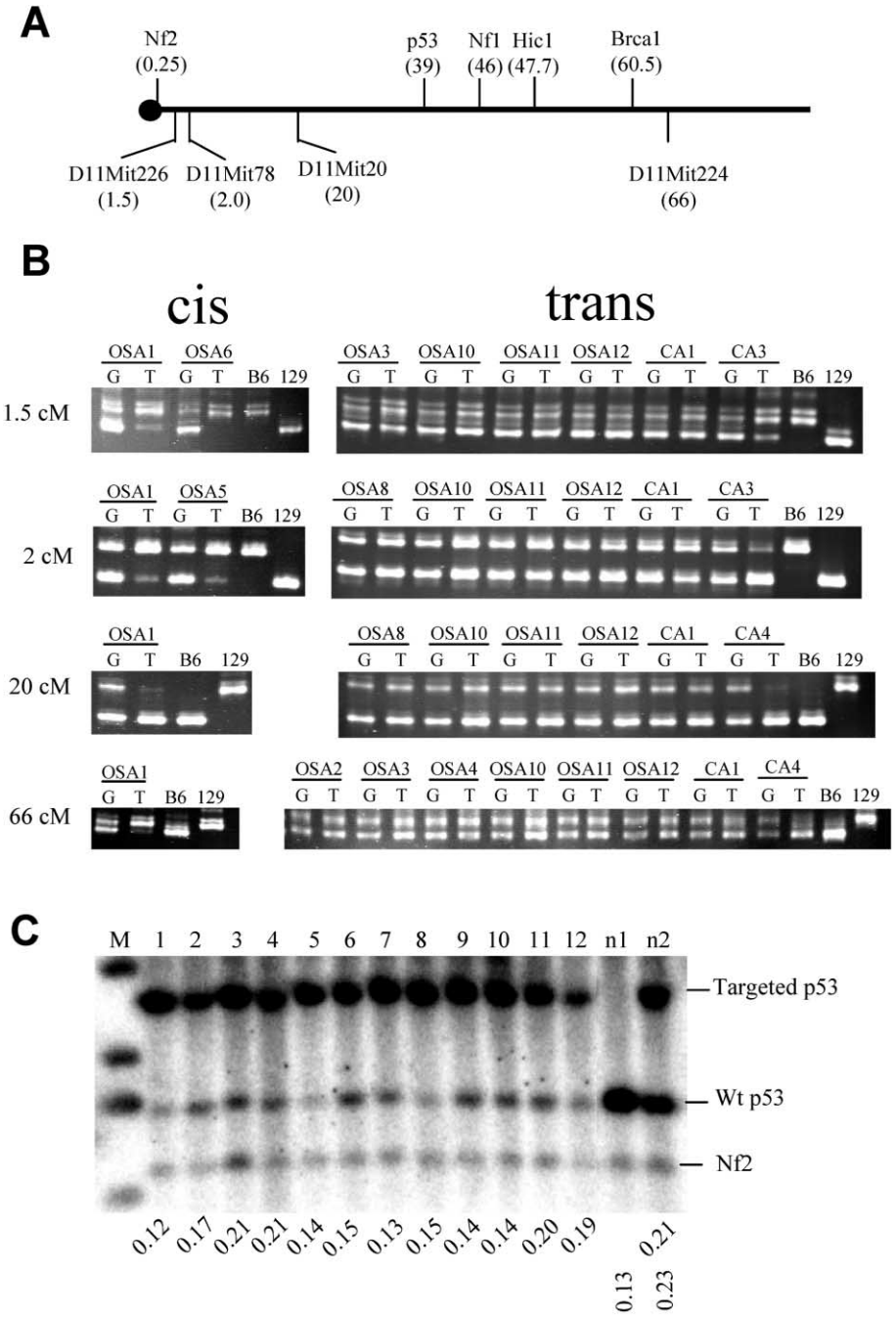

Figure 7. Mapping of chromosomal deletion in tumors

A: Schematic illustration of the genes on mouse chromosome 11 that are important for tumorigenesis, with their meiotic map positions in centimorgan (cM) included in parentheses. Positions for four polymorphism markers are also labeled.

B: PCR polymorphic analysis of allelic loss of *cis* and *trans* tumors at 1.5, 2.0, 20, and 66 cM loci. Only tumors with informative germline polymorphic difference are shown. For *cis* OSAs, loss of the 129/SvJae allele occurs for OSA 6 at 1.5 cM and OSA 5 at 2.0 cM markers; loss of one allele, either 129/SvJae or C57BL/6, occurs for OSA 1 at all four markers. In sharp contrast, no loss of any markers occurs in seven *trans* OSAs. Two mammary gland carcinomas, CA3 and CA4, but not CA1, have lost distal markers. G, germline (from tail DNA); T, tumor; B6, C57Bl/6 control; 129, 129/SvJae control.

C: Southern blot analysis of *Nf2* status in *cis* osteosarcomas. Italic numbers underneath the gel are ratios of *Nf2* over the single copy, targeted *p53* allele. The two vertical numbers under control lanes n1 ($Hic1^{+/-}$) and n2 ($Hic1^{+/-}p53^{+/-}$) are ratios of *Nf2* over the wild-type *p53*, with two copies of *p53* in lane n1 and one copy in lane n2. Loss of one copy of *Nf2* is expected to result in the ratio of *Nf2* over the targeted *p53* to be about 0.11. All of the *cis* tumors appear to have this ratio higher than 0.11, suggesting the retention of at least one copy of *Nf2*.

In **B** and **C**, the tumor number designation is the same as that appearing in Figure 4 for *trans* osteosarcomas, mammary gland tumors, and *cis* osteosarcomas, respectively.

comas, in which *Nf2* is homozygously deleted in nearly all such tumors (McClatchey et al., 1998). Given the phenotypic similarity of *Nf2* mice to our mice, we set out to map the size of chromosomal deletion in tumors, aiming to reveal the potential chromosomal mechanism that leads to loss of heterozygosity (LOH) of *Hic1* and to determine the status of *Nf2*.

We first analyzed a series of polymorphic loci spanning chromosome 11 and identified four simple sequence length polymorphism (SSLP) markers distributing at 1.5, 2.0, 20, and 66 cM along the chromosome (Figure 7A) that were able to distinguish C57BL/6 from 129/SvJae strains. We analyzed germline and tumor pairs for polymorphisms for all osteosarcomas and mammary gland tumors, and found multiple tumors with informative germline polymorphisms in both *cis* and *trans* osteosarcomas (Figure 7B). For tumors, given all of the preceding data presented, we assumed that loss of any marker occurred on the chromosome carrying wild-type *p53*. Three independent *cis* osteosarcomas, in addition to deletion of the *p53* and *Hic1* loci, all had loss of centromeric 129/SvJae markers at 1.5 or 2.0 cM, while OSA1 has deletions of either the 129/SvJae or C57BL/6 markers along the entire chromosome. These data suggest loss of the entire chromosome 11 as a LOH mechanism for the remaining wild-type *Hic1* and *p53* alleles in *cis* tumors, rather than mitotic recombination, in which centromeric

markers would be retained, or interstitial deletion or gene conversion, in which only a limited size of deletion would occur.

In contrast to the above data for the *cis* double heterozygotes, no loss of four markers occurred in any of seven *trans* osteosarcomas with informative germline polymorphisms (Figure 7B), including three osteosarcomas (#3, #8, and #11) that had lost the targeted *Hic1* allele due to *p53* deletion (Figure 4A). Interestingly, we found that two epithelial tumors, namely mammary gland adenosquamous carcinomas CA 3 and 4 in the *trans* double heterozygous mice, carried large chromosomal deletions indicating likely loss of the entire chromosome (Figure 7B). These results suggest that mechanisms of *p53* LOH in *trans* mice are dependent upon tumor types: epithelial tumors tend to have large chromosomal deletion likely involving the entire chromosome similar to the type of loss for the wild-type *Apc* allele in intestinal adenomas in Apc^{Min} mice (Luongo et al., 1994). In contrast, *p53* LOH in the osteosarcomas from the *trans* double heterozygous mice occurs, primarily as interstitial deletions, with the size less than 19 cM toward the centromere or 27 cM toward the telomere. Altogether, our data indicate that in osteosarcomas from the *cis* double heterozygous mice, the additional tumor initiation and/or progression pressure for simultaneous inactivation of both wild-type *Hic1* and *p53* on the wild-type chromosome selects for a LOH mechanism involving entire chromosomal loss.

Within the spectrum of the above dynamics of chromosomal loss, including the loss of an entire chromosome containing wild-type *Hic1* and *p53* in tumors from the *cis* $Hic1^{+/-}p53^{+/-}$ mice, one copy of *Nf2* should remain intact on the targeted allele. To further verify this, we analyzed osteosarcomas directly for *Nf2* status by using Southern blots. As shown in Figure 7C, all tumors indeed retained at least one copy of *Nf2*. Osteosarcomas 1, 5, and 6 appeared to lose one copy of *Nf2*, consistent with the results of the polymorphism analyses presented above (Figure 7B). Retention of at least one copy of *Nf2* in our *cis* osteosarcomas is in contrast to homozygous deletion of *Nf2* in osteosarcomas from $Nf2^{+/-}$ or $Nf2^{+/-}p53^{+/-}$ double heterozygous mice, suggesting that different mechanisms contribute to the development of osteosarcomas in our studies versus those for the same type of tumors in the setting of targeted disruption of *Nf2*.

Hic1 and *INK4a* locus

To explore whether *Hic1* will cooperate with other tumor suppressor genes that impact the *p53* pathway, we crossed *Hic1* heterozygotes to *INK4a* locus knockout mice. The *INK4a* locus encodes two tumor suppressor genes, $p16^{INK4a}$ and $p19^{ARF}$, through alternative codon usage. $P16^{INK4a}$ protein inhibits cyclin D-dependent kinases, Cdk4 and Cdk6, and keeps pRb functionally active for G1 cell cycle control, while $p19^{ARF}$ protein positively regulates p53 function by binding to and sequestering Mdm2 (Sherr, 2001). Loss of the *INK4a* locus (Serrano et al., 1996) or $p19^{ARF}$ alone (Kamijo et al., 1997) strongly predisposes mice to spontaneous soft tissue sarcomas and lymphomas, while loss of $p16^{INK4a}$ alone has milder impact on formation of these tumors (Krimpenfort et al., 2001; Sharpless et al., 2001). We found that *Hic1* heterozygous status did not accelerate tumor formation or significantly alter tumor spectrum in homozygous or heterozygous *INK4a* locus knockout mice, and both *INK4a* heterozygous and *Hic1*/*INK4a* double heterozygous mice developed predominantly soft tissue sarcomas and lymphomas in a 90-week study (data not shown). These results suggest that *Hic1* does not cooperate with the *INK4a* locus. Rather, it is likely that loss of *Hic1* function directly complements loss of *p53* function for maximal effects on tumor progression.

HIC1 promoter hypermethylation and *p53* mutations in human osteosarcomas

Finally, given all of our findings in the mouse studies, and particularly those for osteosarcomas, we wondered whether collaboration between loss of *Hic1* and *p53* function might be relevant to development of cancer in humans. We thus extended our study to human osteosarcomas by examining the relationship of *HIC1* promoter hypermethylation to *p53* mutations in this disease. Osteosarcomas are uncommon and aggressive tumors, occurring mostly in children and young adults (Spina et al., 1998). Somatic *p53* mutations in human osteosarcomas range from 19% to 38%, depending on exon regions examined and detection methods used (Gokgoz et al., 2001; Miller et al., 1996; Overholtzer et al., 2003; Radig et al., 1996). The presence of *p53* mutations correlates with high levels of genomic instability in osteosarcomas (Overholtzer et al. 2003). Recently, hypermethylation of *HIC1* has been found in 17% of pediatric osteosarcomas (Rathi et al., 2003).

We analyzed *HIC1* hypermethylation in 44 osteosarcomas that had previously been analyzed for *p53* mutations by single-strand conformation polymorphism analysis (SSCP) and PCR/ligase-detection reaction (PCR/LDR) (Gokgoz et al., 2001; Overholtzer et al., 2003). We identified *HIC1* hypermethylation in 8 out of 21 tumors (38%) with *p53* mutations, but only in 2 of 23 tumors (9%) without *p53* mutations ($p = 0.020$, Figure 8). In an additional set of nine osteosarcomas in which missense *p53* mutations were screened by immunohistochemistry and verified with *p53* gene chip and sequencing in this study, we also found frequent *HIC1* hypermethylation that occurred in 4 out of 8 tumors with *p53* missense mutations, but not in the one without (Figure 8C). Altogether, *HIC1* hypermethylation occurred in 12 out of 29 osteosarcomas (41%) with p53 mutations compared to 2 out of 24 tumors (8%) without p53 mutations ($p = 0.007$). Therefore, of 14 tumors with *HIC1* hypermethylation, 12 (86%) occurred in those with p53 mutations. These results are consistent with our findings in the above mouse study, and suggest that loss of *HIC1* function may complement *p53* mutations in the development of a subset of human osteosarcomas.

Insight of *HIC1* as a tumor suppressor gene and genetic versus epigenetic inactivation

Although it has long been speculated that other tumor suppressor genes exist on human chromosome 17p13 in addition to *p53* (Cornelis et al., 1994; Konishi et al., 1998; Phillips et al., 1996; Saxena et al., 1992), we demonstrate that *HIC1* qualifies as such a gene using mouse models. *Hic1* and *p53* can function as tumor suppressors individually, or work synergistically, each using different mechanisms for their loss of function. When germline disruptions of both genes are placed on the same chromosome, the mice exhibit accelerated tumorigenesis accompanied by loss of the entire chromosome containing wild-type copies of both genes. These phenotypic and molecular features are strikingly similar among three sets of compound knockouts on mouse chromosome 11, namely *cis* $Hic1^{+/-}p53^{+/-}$, *cis* $Nf1^{+/-}p53^{+/-}$, and *cis* $Nf2^{+/-}p53^{+/-}$. Selection pressure to inactivate two genes on the same chromosome in these

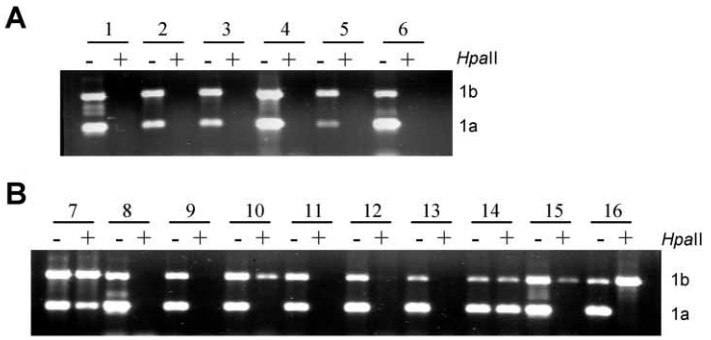

Figure 8. *HIC1* promoter hypermethylation analysis of human osteosarcomas

A and B: Examples of HpaII-PCR analysis of human osteosarcomas without *p53* mutations (**A**) and with *p53* mutations (**B**).

C: Summary of *p53* mutations and *HIC1* hypermethylation data. The *p53* mutation status of 44 osteosarcoma samples determined in previous studies with SSCP and PCR/LDR is listed in the 2nd and 5th columns, labeled SSCP; nine osteosarcomas screened with IHC and verified with GeneChip are listed in the 8th column, labeled IHC. Two osteosarcomas with wild-type p53 by SSCP (number 1 and 5) had *HDM2* amplification. The numbers given for the tumors listed in the table are not the same as those numbers shown for example tumors in **A** and **B**. *, stop codon.

C

Tumor	P53 status (SSCP)	HIC1 methylation	Tumor	P53 status (SSCP)	HIC1 methylation	Tumor	P53 status (IHC)	HIC1 methylation
1	Wild type	-	24	R273H	+	45	S241F	-
2	Wild type	-	25	V173M	-	46	C238S	+
3	Wild type	-	26	1bp deletion (codon 83)	-	47	R273H	-
4	Wild type	-	27	R248Q	-	48	R273H	-
5	Wild type	-	28	GGT insertion (codon 107-108)	+	49	R175H	-
6	Wild type	-	29	G245S	-	50	M237I	+
7	Wild type	-	30	L43 ⋆	-	51	S241Y	+
8	Wild type	-	31	P250L	+	52	G245A	+
9	Wild type	-	32	M237I	-	53	Wild type	-
10	Wild type	-	33	C238Y	-			
11	Wild type	-	34	R273H	-			
12	Wild type	-	35	L43 ⋆	-			
13	Wild type	+	36	T43A	-			
14	Wild type	-	37	15bp deletion (codon 202-206)	+			
15	Wild type	-	38	T220C	-			
16	Wild type	-	39	T220C	-			
17	Wild type	+	40	E285K	-			
18	Wild type	-	41	1bp deletion (codon 298)	+			
19	Wild type	-	42	E343 ⋆	+			
20	Wild type	-	43	ATGgt-ATGat (Intron 5 splice site)	+			
21	Wild type	-	44	G245C	+			
22	Wild type	-						
23	Wild type	-						

settings results in the loss of the entire chromosome (Cichowski et al., 1999; Eden et al., 2003; McClatchey et al., 1998; Vogel et al., 1999). The striking difference, and novel situation, for *Hic1*, as opposed to the studies for *Nf1* or *Nf2*, is that such a *cis* double heterozygous scenario converts the usual mechanism of epigenetic inactivation of this gene to one of chromosomal deletion.

The switch of mechanism for *Hic1* inactivation is intriguing. One possibility is that the clonal evolution of the tumors would simply reflect the random collision of targeted allele events with inactivation events on the opposite chromosome that would most readily facilitate and select for tumorigenesis. The choice for *Hic1* inactivation between the *trans* and *cis* double heterozygous mouse tumors is clearly nonrandom. In the default state, *Hic1* is epigenetically inactivated, probably as a result of local chromatin events. Thus, in the *trans* setting, changing the wild-type *Hic1* inactivation route to deletion would not provide additional selection advantage for clonal expansion, since the nearby *p53* gene has already been knocked out by the targeting event. In the *cis* setting, however, deletion of *Hic1* would clearly have more selection advantage than promoter hypermethylation, because the deletion can simultaneously include the wild-type *p53* on the same chromosome, whereas promoter hypermethylation inactivates only *Hic1*. We cannot, obviously, fully rule out that additional mechanisms could be at play between the *cis* and *trans* double heterozygotes. For example, the meiotic recombi-

nation events necessary to derive the *cis* double heterozygotes might have influence on this choice; however, the mice involved in the study were not the mice harboring the original meiotic recombination, but rather later offspring. Thus, the recombined allele has been through germline transmissions, and the wild-type chromosome is universally derived from parents that have never undergone the original meiotic recombination process.

Another intriguing finding in our study is the promoter hypermethylation on the targeted *Hic1* allele, in both *trans* and *cis* tumors. In both settings, there is no selection pressure to methylate the targeted allele, as *Hic1* coding sequence has been knocked out. There are at least three reasons why this may occur. First, the *Hic1* promoter may be vulnerable for hypermethylation. This is evident in that certain normal aging tissues can also bear a small degree of hypermethylation even in the wild-type *Hic1* allele, such as mammary glands (Figure 5A and data not shown). This is similar to the hypermethylation of other genes in tissues such as normal aging colon in humans (Issa et al., 1994). Second, insertion of the targeting cassette carrying bacterial *lacZ* and *neo* sequences on the locus may promote methylation of the bacterial and adjacent cellular sequence. This phenomenon has been well documented in many transgenic studies (Engler et al., 1991; Hertz et al., 1999; Rose et al., 2000). Third, hypermethylation of the targeted allele could be a secondary event. In the clonal selection model for loss of *Aprt* function, Rose et al. (2000) have found that hypermethylation of the *Aprt* promoter on the targeted allele carrying the *neo* expression cassette can occur in a passive way that is dependent upon the promoter hypermethylation of the wild-type *Aprt* allele. Homology-dependent *trans* allelic methylation or transvection then may render hypermethylation of promoters on the targeted allele. Transvection might thus provide a source for promoter hypermethylation of the targeted *Hic1* allele in the *trans* tumors, but could not apply directly to the *cis* tumors where the wild-type *Hic1* is deleted.

Finally, functional cooperation of *HIC1* and *p53* in tumor suppression is interesting, as the *HIC1* promoter contains a consensus p53 binding site (Wales et al., 1995), and overexpression of p53 can transactivate silent endogenous *HIC1* in cultured human cancer cell lines (Guerardel et al., 2001; Wales et al., 1995). We therefore speculate that HIC1 might be involved in a certain feedback regulation for p53 in tumor suppression. One previous example for the feedback regulation of p53 function is MDM2. P53 can transactivate MDM2, whereas MDM2 binds the p53 N terminus, promotes ubiquitin-mediated degradation of p53, and thus inhibits its function in controlling G1 cell cycle arrest and apoptosis (Levine, 1997). Other tumor suppressor genes can act through this pathway by neutralizing MDM2-mediated inhibition of p53; for example, the *Nf2* gene product, merlin, promotes degradation of MDM2 and therefore stabilizes p53 (Kim et al., 2004). Interestingly, in our mouse studies described above, *Hic1* heterozygosity did not cooperate with the *Ink4a* locus, in which $p19^{Arf}$ blocks Mdm2 interaction with p53, and two human osteosarcomas with MDM2 amplification did not show HIC1 hypermethylation (Figure 6C), suggesting that HIC1 may not act through the MDM2 pathway.

In summary, we have found that *Hic1* plays a crucial role, by cooperating with *p53*, in controlling osteosarcoma and mammary gland carcinoma formation. Although loss of *Hic1* function is most often achieved through an epigenetic mechanism during tumor development in humans and in our mouse models, *Hic1* can be genetically inactivated if the germline disruptions of this gene and *p53* are introduced into the same chromosome in mice. This study not only provides further evidence that *Hic1* is a tumor suppressor gene, but also shows that loss of function mediated predominantly by epigenetic events for given genes can effectively cooperate with genetic disruption of powerful tumor suppressor genes, such as *p53*, to drive tumorigenesis.

Experimental procedures

Mouse colonies

We bred $Hic1^{+/-}$ mice (on about 50% C57Bl/6 and 50% 129Sv/Jae) to $p53^{+/-}$ mice (on C57Bl/6, Jackson Lab) that carry the deletion of *p53* exons two to six (Jacks et al., 1994a) to generate *trans* $Hic1^{+/-}p53^{+/-}$ mice as well as $p53^{+/-}$ and $Hic1^{+/-}$ cohorts. For study of *cis* double heterozygous knockouts, we bred *trans* $Hic1^{+/-}p53^{+/-}$ mice with either wild-type or $p53^{-/-}$ mice generated by mating $p53^{+/-}$ mice. Six meiotic recombinants ($Hic1^{+/-}$p53$^{-/-}$ and *cis* $Hic1^{+/-}p53^{+/-}$) were identified at a frequency of 1.2%. These six recombinants were then bred to $p53^{-/-}$, $p53^{+/-}$, and $Hic1^{+/-}$ mice to produce $Hic1^{+/-}$ $p53^{-/-}$, *cis* $Hic1^{+/-}$ $p53^{+/-}$, and their littermate cohorts, $p53^{-/-}$, $p53^{+/-}$ and $Hic1^{+/-}$ mice. Therefore, all mice in the *trans* and *cis* colonies had mixed C57Bl/6 and 129Sv background, with *cis* mice having more C57Bl/6 background than *trans*. The use of mice in this study was approved by the Institutional Animal Care and Use Committee of Johns Hopkins University.

Mouse tumor analysis

We monitored mice closely for tumors or illness, and harvested and processed tumors as described previously (Chen et al., 2003). Skeletons of mice with limb paralysis but without overt bone tumors were formalin fixed and examined with radiography for possible small bone tumors. For Kaplan-Meier analysis of tumor-free survival, mice with histologically confirmed tumors or killed for severe illness were scored as death. Log-rank test was used for calculating statistical significance. For measuring metastasis rate, we performed careful histological examination for osteosarcomas and other tumors in all major organs of each mouse to compare with histology of the primary tumor. Immunohistochemical staining of paraffin-embedded tissues was carried out as previously described (Chen et al., 2003).

Molecular analysis of mouse tumors

Tumor DNA was extracted as described (Chen et al., 2003). For normal bone controls, surgically normal femurs were removed and flushed with saline to remove bone marrow. The cancellous parts of femurs were pooled, crushed, and then used for DNA extraction. For normal mammary gland controls, surgically normal whole mammary fat pads were dissected for DNA extraction. *Hic1* deletion and methylation status were analyzed as described (Chen et al., 2003). Southern blot analysis of *p53* was done as described (Jacks et al., 1994a), except that genomic DNA was digested with StuI only so that the *p53* pseudogene was detected at a higher molecular weight, 3.6 kb. Quantitation of blot signals was performed using a PhosphorImager, and after subtracting background, intensity of each band was used for calculating ratios of the wild-type over targeted allele.

Mapping chromosomal deletion

For PCR polymorphism analysis, we first verified primers (from Jackson Lab) to be able to distinguish C57BL/6 versus 129/SvJae strains, and then applied them for analyzing tumor and tail DNA. For Southern blot analysis of *Nf2*, we PCR synthesized a short *Nf2* probe that covers the unique sequence for the PstI-EcoRI region around exon 2, which detects a 1.1 kb fragment of *Nf2* in StuI-digested genomic DNA (McClatchey et al., 1997). The membrane was simultaneously probed with *p53* and *Nf2* probes and analyzed as above.

Human osteosarcoma analysis

We analyzed a total of 53 human osteosarcoma samples for *p53* mutations and *Hic1* promoter hypermethylation. Forty-four of these human samples had been collected and analyzed previously for *p53* mutations by SSCP and PCR/LDR (Gokgoz et al., 2001; Overholtzer et al., 2003), and nine additional samples were collected at Memorial Sloan-Kettering Cancer Center using a protocol approved by the MSKCC Institutional Review Board. These latter

samples were initially screened for p53 missense mutations by immunohistochemistry (IHC) with antibody DO7 (DAKO, Carpinteria, CA; 1:500; 0.2 μg/ml) as previously described (Lonardo et al., 1997). Cells with moderate to intense nuclear staining were scored positive, and samples containing more than 20% IHC positive tumor cells were considered positive for potential p53 missense mutations. This cut-off percentage was chosen according to previously established thresholds (Lonardo et al., 1997). The IHC positive samples were then verified with p53 GeneChip analysis (Affymetrix, Santa Clara, CA) on DNA extracted from frozen tissue. Mutations with GeneChip report scores >11 were considered genuine (Ahrendt et al., 1999; Wen et al., 2000; Wikman et al., 2000). We confirmed those mutations that had only borderline GeneChip scores (namely, 12–15) by direct sequencing. The concordance of GeneChip and sequencing analysis has been previously demonstrated to be better than 80% (Ahrendt et al., 1999; Wen et al., 2000; Wikman et al., 2000). For analysis of *HIC1* promoter hypermethylation, HpaII-PCR assay was performed with 35 cycle PCR using the same conditions as previously described (Chen et al., 2003).

To ensure, for detection of p53 mutations, the concordance of IHC with molecular assays for osteosarcomas, we found, in a separate study of 15 osteosarcomas that are not available for Hic1 methylation analysis, that only one out of eight IHC negative osteosarcomas carried a *p53* mutation as detected by SSCP and PCR/LDR. Also, of seven IHC positive osteosarcomas, five were indeed positive for *p53* mutations by SSCP and/or PCR/LDR (M.O., unpublished data). Therefore, the overall concordance is similar to that found for other types of tumors (Baas et al., 1994; Hsia et al., 2000; Lonardo et al., 1997).

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