The tumor suppressor Hic1 maintains chromosomal stability independent of Tp53

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Abstract
Hypermethylated-in-Cancer 1 (Hic1) is a tumor suppressor gene frequently inactivated by epigenetic silencing and loss-of-heterozygosity in a broad range of cancers. Loss of HIC1, a sequence-specific zinc finger transcriptional repressor, results in deregulation of genes that promote a malignant phenotype in a lineage-specific manner. In particular, upregulation of the HIC1 target gene SIRT1, a histone deacetylase, can promote tumor growth by inactivating TP53. An alternate line of evidence suggests that HIC1 can promote the repair of DNA double strand breaks through an interaction with MTA1, a component of the nucleosome remodeling and deacetylase (NuRD) complex. Using a conditional knockout mouse model of tumor initiation, we now show that inactivation of Hic1 results in cell cycle arrest, premature senescence, chromosomal instability and spontaneous transformation in vitro. This phenocopies the effects of deleting Brca1, a component of the homologous recombination DNA repair pathway, in mouse embryonic fibroblasts. These effects did not appear to be mediated by deregulation of Hic1 target gene expression or loss of Tp53 function, and rather support a role for Hic1 in maintaining genome integrity during sustained replicative stress. Loss of Hic1 function also cooperated with activation of oncogenic KRas in the adult airway epithelium of mice, resulting in the formation of highly pleomorphic adenocarcinomas with a micropapillary phenotype in vivo. These results suggest that loss of Hic1 expression in the early stages of tumor formation may contribute to malignant transformation through the acquisition of chromosomal instability.

Introduction
Hypermethylated-in-Cancer-1 (HIC1) was discovered during a screen for highly methylated tumor suppressor genes on chromosome 17p [1]. HIC1 resides immediately telomeric to TP53 at 17p13.3, and encodes a BTB/POZ domain zinc finger transcriptional repressor closely related to the PLZF family of proteins [1–5]. The locus also contains a Tp53 response element, and two major CpG rich promoters that generate different alternatively spliced transcripts [1, 6–8]. Over the last decade, several groups have shown that epigenetic gene silencing and/or loss of heterozygosity of HIC1 is one of the most common events in human cancer [9–12], and this is associated with poor outcomes in a wide variety of tumor types [12–17], including lung cancer [18].

Homozogous deletion of Hic1 with a conventional mouse knockout approach results in mid-gestation embryonic lethality [19], whereas heterozygous mutants develop a range of spontaneous tumors in an age-dependent manner [7]. Furthermore, Hic1 mutant mice demonstrate an accelerated tumor phenotype when crossed into established genetic models of colorectal cancer [20], medulloblastoma [21], and osteosarcoma [22].

The conventional model of Hic1 function is based on the identification of transcriptional targets through a combination of Hic1 re-expression in cancer cells and gene
expression profiling [5, 21, 23–25]. Based on this premise, loss of Hic1 expression through promoter hypermethylation results in aberrant overexpression of lineage-specific genes that promote or maintain the malignant phenotype [5]. In addition, Hic1 also has been shown to attenuate the effect of oncogenic transcription factors complexes that mediate WNT [26] or STAT3 [24, 27] signaling.

One important transcriptional target of Hic1 is the Sirtuin ortholog Sirt1 [28]. Sirtuins belong to the NAD-dependent, trichostatin-insensitive class III histone deacetylases that mediate life span extension in response to caloric restriction in yeast [29]. Importantly, Sirt1 actively deacetylates and inactivates p53 [30], thus establishing a direct link between loss of Hic1 and attenuation of Tp53 signaling [28]. By contrast, mice carrying heterozygous mutations in both Tp53 and Hic1 in cis rapidly develop osteosarcomas in which the wild type copies of both genes are deleted [22], suggesting that loss of both genes predisposes to tumor development that cannot be fully explained through a Hic1-Sirt1-p53 dependent mechanism.

To better define the function of Hic1 in suppressing tumor initiation, we developed a conditional deletion mouse mutant to overcome the embryonic lethality of the Hic1 knockout mouse, and bypass the reliance on stochastic promoter methylation of the wild type allele in Hic1 heterozygous mutants [7].

**Results and discussion**

**Growth arrest and premature senescence in Hic1-deficient mouse embryonic fibroblasts**

Using targeted homologous recombination in mouse embryonic stem cells, we introduced loxP sites flanking Exon 2 of the Hic1 locus (Fig. 1a). Mice homozygous for the conditional allele (hereafter Hic1lox/lox) demonstrated no developmental phenotype, were not cancer prone, and were fertile (data not shown). To generate a conditional mutant model in mouse embryonic fibroblasts (MEFs), we crossed the Hic1lox/lox mouse with a line in which a Cre recombinase transgene fused to the estradiol receptor has been introduced into the constitutively expressed ROSA locus (hereafter EsrCre) [31]. Ex-vivo treatment with tamoxifen induces activation and translocation of Cre recombinase resulting in loxP recombination with high efficiency.

Treatment of MEFs generated from Hic1lox/lox EsrCre embryos (hereafter Hic1KO) with tamoxifen, followed by a 48 h incubation period confirmed deletion of Exon 2, which contains the entire coding region of the Hic1 gene (Fig. 1b). Loss of Hic1 protein expression in the same MEF model was also confirmed by Western blot analysis (Fig. 1c).

In contrast to EsrCre MEFs, tamoxifen treated Hic1KO MEFs displayed a marked G2/M arrest (Figs. 1d, e), and premature activation of senescence-associated beta-galactosidase (SA-βGal) (Figs. 1f, g). This was unexpected, since Hic1 is a known tumor suppressor gene, and because its capacity to prevent Tp53 deacetylation through transcriptional repression of Sirt1 would have predicted that Hic1 deletion would phenocopy p53 deletion in this model. Equally surprising was the pattern of gene expression in Hic1KO MEFs 48 h after tamoxifen treatment (Fig. 1h). Although an increase in the expression of the known Hic1 target genes Efna1 [24] and Tbr2 [32] was observed, no changes in Sirt1 were seen (Supplementary Table S1). Unexpectedly, gene expression microarray and gene ontology analysis revealed highly significant enrichment for pathways involved in the regulation of cell cycle, mitosis, and DNA replication consistent with the induction of cell cycle arrest (Figs. 1h, i; Supplementary Table S2). These data are resemble a phenomenon in MEFs known as "tumor suppressor inactivation-induced senescence", best exemplified by inactivation of the breast cancer tumor suppressor gene Brca1, which results in overwhelming DNA damage as the result of replication-dependent double-strand break (DSB) formation, chromosomal instability and Tp53-dependent senescence [33, 34].

**Immortalized Hic1KO MEFs are phenotypically distinct from p53KO MEFs**

To determine whether the effects of Hic1 deletion could be functionally separated from downregulation of Tp53 function through upregulation of Sirt1, we employed a conditional Tp53 knockout allele (hereafter p533lox/lox) to generate MEFs derived from p533lox/lox EsrCre embryos (hereafter p53KO). Embryos heterozygous for the EsrCre allele were used as controls (hereafter EsrCre). Serial passaging of EsrCre, p53KO, and Hic1KO MEFs using a modified 3T3 protocol demonstrated that deletion of Hic1 can replicate the effect of loss of Brca1 function in the MEF model (Fig. 2a). As expected, p53KO MEFs spontaneously immortalized with high efficiency, while control EsrCre cells escaped senescence with similar efficiency but in a much longer timeframe. By contrast, Hic1KO MEFs immortalized with low efficiency after a period of dormancy lasting over 60 days (Fig. 2a) in similar fashion to the Brca1 knockout model [33].

One of the most rigorous tests of malignant transformation in the MEF model is the capacity of immortalized cells to grow as allografts in athymic nude mice [35]. To assess this, early passage immortalized MEF lines from each genotype were injected subcutaneously into the flanks of nude mice and observed. Fast growing Hic1KO tumors rapidly appeared within 3 weeks, whereas tumors derived
from p53KO MEFs developed with a longer latency period (Fig. 2b). No tumors developed from EsrCre MEFs.

Gene expression profiling of immortalized EsrCre, p53KO, and Hic1KO MEFs revealed a highly divergent transcriptional signature (Fig. 2c, d; Supplementary Tables S3, S4, S5). When compared with p53KO cells, Hic1KO MEFs upregulated gene sets strongly associated with DNA synthesis, senescence, oxidative and nutrient stress (Fig. 2e). As expected, expression of Cdkn1a (which encodes p21) was markedly downregulated in p53KO cells while immortalization in response to deletion in Hic1 was associated with dramatic loss of Cdkn1c gene expression (which encodes p57) (Supplementary Table S5). Genomic and RT-PCR sequencing also showed that immortalized Hic1KO MEFs retained intact Tp53 and Cdkn2a genes (data not shown), both of which are commonly inactivated in MEFs that spontaneously escape senescence [36]. To further define the status of Tp53 in this model, we treated WT or immortalized Hic1KO MEFs with doxorubicin to induce DNA damage. As shown in Fig. 2f, this resulted in robust upregulation of Tp53 and phosphorylated Tp53 expression. Using a Tp53-responsive reporter system, we further showed...
that both EsrCre and Hic1KO MEFs were able to induce a p53-dependent transcriptional response when compared to p53KO MEFs (Fig. 2g).

Taken together, these data show that immortalization following the loss of Hic1 occurs independent of Tp53. Although we cannot exclude the possibility that attenuation of Tp53 function plays a role in maintenance of the immortalization phenotype in Hic1KO MEFs, the weight of evidence strongly suggests that a significant component of the tumor suppressive activity of Hic1 in the context of tumor initiation outside the previously described Hic1-Tp53-Sirt1 regulatory loop [28].

Loss of Hic1 in MEFs leads to chromosomal instability

Chromosomal instability is a cardinal feature of tumors with defective HR, typified by cancers with mutations in BRCA1 and BRCA2 [37]. Considering the similarities between Hic1KO and Brca1 mutant MEFs, we asked whether loss of Hic1 function in immortalized MEFs would result in chromosomal instability. Quantitative anaphase analysis of immortalized MEF lines in Fig. 1c when compared with control MEFs. Gene ontology analysis of differentially expressed genes when comparing immortalized p53KO vs. Hic1KO MEFs. The analysis was performed by an observer with no a priori knowledge of the cellular phenotype. Western blot analysis of lysates from wild type (WT) or Hic1 KO MEFs showing the expression of p53 phosphorylated at serine 15 (pSer15-p53, Cell Signaling Technology, Danvers, MA, USA, #9284S), p53 (Santa Cruz Biotechnology, Dallas, TX, USA, sc-6243), phosphorylated-γH2AX (γH2AX, Novus Biologicals, Littleton, CO, USA #NB100-74435), total H2AX (Cell Signaling Technology, 2595 S) and Actin [66, 67]. Cells were treated with vehicle or doxorubicin (Dox; 1 µM, 6 h).

A Venn diagram depicting differentially expressed genes in the immortalized MEF lines shown in Fig. 1c when compared with control MEFs. The analysis was performed by an observer with no a priori knowledge of the cellular phenotype. Array data are available through GEO, GSE104394. Western blot analysis of lysates from wild type (WT) or Hic1 KO MEFs showing the expression of p53 phosphorylated at serine 15 (pSer15-p53, Cell Signaling Technology, Danvers, MA, USA, #9284S), p53 (Santa Cruz Biotechnology, Dallas, TX, USA, sc-6243), phosphorylated-γH2AX (γH2AX, Novus Biologicals, Littleton, CO, USA #NB100-74435), total H2AX (Cell Signaling Technology, 2595 S) and Actin [66, 67]. Cells were treated with vehicle or doxorubicin (Dox; 1 µM, 6 h). Activity of a p53-responsive luciferase reporter (Qiagen, Hilden, Germany, #CCS-004L) in MEFs 24 h after treatment with doxorubicin, 1 µM, for 6 h. n = 4 independent cell lines performed, each performed in triplicate, mean + SEM, *P < 0.05

Fig. 2 Immortalization of MEFs lacking Hic1 results in a phenotype distinct to MEFs lacking p53. a Growth of MEFs (shown as cumulative population doublings) following tamoxifen treatment using the 3T3 protocol [65]. Data shown as mean±SEM. Sample size was determined by the number of available immortalized MEF lines. Cell lines were checked for Mycoplasma and genotype every 6 months. b Kaplan–Meier survival analysis of athymic nude mice injected with 1 × 106 immortalized MEFs with the genotypes indicated. **P < 0.005, log-rank analysis. 1 × 106 MEFs were resuspended in 50 µl media+50 µl Matrigel and injected subcutaneously in the right flank and observed for 26 weeks, until the tumor reached 800 mm3 measured by an observed blinded to the MEF genotype. c Principal component (PC) analysis of gene expression in immortalized MEFs generated from embryos with the genotypes indicated compared to control MEFs. Detailed bioinformatic methods are described in Supplementary Information. Array data are available through GEO, GSE104394. d A Venn diagram depicting differentially expressed genes in the immortalized MEF lines shown in Fig. 1c when compared with control MEFs. e Gene ontology analysis of differentially expressed genes when comparing immortalized p53KO vs. Hic1KO MEFs. The analysis was performed by an observer with no a priori knowledge of the cellular phenotype. f Western blot analysis of lysates from wild type (WT) or Hic1 KO MEFs showing the expression of p53 phosphorylated at serine 15 (pSer15-p53, Cell Signaling Technology, Danvers, MA, USA, #9284S), p53 (Santa Cruz Biotechnology, Dallas, TX, USA, sc-6243), phosphorylated-γH2AX (γH2AX, Novus Biologicals, Littleton, CO, USA #NB100-74435), total H2AX (Cell Signaling Technology, 2595 S) and Actin [66, 67]. Cells were treated with vehicle or doxorubicin (Dox; 1 µM, 6 h). g Activity of a p53-responsive luciferase reporter (Qiagen, Hilden, Germany, #CCS-004L) in MEFs 24 h after treatment with doxorubicin, 1 µM, for 6 h. n = 4 independent cell lines performed, each performed in triplicate, mean + SEM, *P < 0.05
Chromosomes was seen in Hic1KO MEFs (Fig. 3e, f). Histologic analysis of the MEF allograft tumors described in Fig. 2b showed that Hic1KO MEFs formed aggressive, pleomorphic sarcomas with some features of skeletal muscle differentiation resembling adult pleomorphic rhabdomyosarcoma (Fig. 3h). By contrast, p53KO MEFs formed sarcomas more consistent with pediatric rhabdomyosarcoma, with a more regular nuclear morphology. Consistent with our in vitro findings, Hic1KO MEF nude mouse allograft tumors exhibited marked nuclear pleomorphism, as well as numerous anaphase bridges and multinucleated giant cells (Fig. 3i). These histological findings are consistent with marked chromosomal instability [41–43].

**Cooperation between inactivation of Hic1 and oncogenic KRas in vivo**

Our data suggest that loss of Hic1 in the setting of the replication stress and impending senescence induced by culture of MEFs [44] can lead to chromosomal instability and spontaneous transformation. To test the functional importance of this observation in vivo, we made use of a

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**Fig. 3** Deletion of Hic1 results in chromosomal instability. a Representative confocal photomicrographs showing anaphases in MEFs with the genotypes indicated. Cells were stained for tubulin (Red) and DNA (DAPI) (Blue) as described [66]. Scale bar = 5 μm. Anaphases in cell culture were scored as described previously by an observer blinded to the MEF genotype [66]. b–d Quantitative analysis of aberrant anaphase events in MEFs with the genotypes from the same experiment shown in Fig. 1a, n = 3 (ESRCre), 6 (p53KO) and 4 (Hic1KO) cell lines, average of 55 anaphases per cell line. Mean ±SEM. *P < 0.05; **P < 0.01, one-way ANOVA with Bonferroni correction. Sample size was chosen by the number of available MEF lines. e Examples of karyotypes from immortalized MEFs with the genotypes shown. Karyotyping was performed as previously described [70], f, g Quantification of chromosome number and the number of marker chromosomes, n = 4 ESRCre, n = 6 p53KO and n = 5 Hic1KO cell lines, average of 15 metaphase cells per genotype. **P < 0.01, one-way ANOVA with Bonferroni correction. h Representative photomicrographs of hematoxylin and eosin (H&E) stained sections from formalin-fixed, paraffin-embedded nude mouse allograft tumors with the genotypes indicated. Scale bar = 20 μm. i Representative high-powered photomicrographs of H&E stained sections of Hic1KO nude mouse allograft tumors. Scale bar = 5 μm. MAR marker chromosomes.
conditional mouse model of lung adenocarcinoma in which an oncogenic mutant KRasG12D allele is knocked into the endogenous KRas locus downstream of a loxP-STOP-loxP cassette (hereafter KRas) [45]. When treated with an inhaled recombinant adenovirus expressing Cre recombinase (Ad5Cre), these mice develop multiple lung adenocarcinomas within 6–8 weeks [46]. We chose this model based on (i) the ability of mutant KRas to trigger replication stress and senescence in the absence of a cooperating mutations in Tp53 or p16 [47, 48]; (ii) the cooperating effect in this model on tumor progression due to combined deletion of Tp53 [49]; (iii) the prevalence of HIC1 hypermethylation in human lung adenocarcinoma [50]; and (iv) the clinical significance of HIC1 methylation in non-small cell lung cancer [18].

We crossed KRas and Hic1Δlox/Δlox mice to generate wild type, Hic1KO, KRas, and KRas × Hic1KO experimental cohorts, and administered inhaled Ad5Cre at 6–8 weeks of age. Wild type and Hic1KO mice treated with Ad5Cre showed no abnormalities and did not develop lung lesions by 12 months of age (data not shown). As shown in Fig. 4a, KRas × Hic1KO mice treated with inhaled Ad5Cre had a shorter lifespan compared to KRas littermates, consistent with a reduced tumor latency. Consistent with this observation, quantitative histologic analysis showed a marked increase in tumor size (Fig. 4b, c). Immunohistochemical staining for Pancytokeratin, Surfactant Protein C and Ki67 revealed an increase in tumor size (Fig.4b, c). Immunohistochemical staining for Pancytokeratin, Surfactant Protein C and Ki67 revealed an increase in tumor size (Fig. 4b, c) and pleomorphic features (Fig. 4d), both of which are indicative of spontaneous DSB formation. Although we cannot exclude the possibility that deregulation of Hic1 target genes may contribute to the micropapillary lung adenocarcinoma phenotype seen in KRas × Hic1KO mice, these data support our findings in the MEF model, and demonstrate that loss of Hic1 in the setting of oncogenic KRas activation in the adult airway epithelium leads to chromosomal instability and a distinct mouse lung adenocarcinoma phenotype.

A novel role for Hic1 as a tumor suppressor

Using a conditional mutant mouse model, we have identified a new and unexpected function for Hic1 as tumor suppressor by maintaining chromosomal stability in the setting of sustained DNA replication stress. Although our model may not be broadly applicable to the models in which the functions of Hic1 have been described previously, our results do suggest that loss of Hic1 function in the early phases of tumor initiation may have a major impact on the subsequent tumor phenotype. These data support the notion that the acquisition of chromosomal instability during the early phases of tumor evolution can have a major effect on tumor phenotype and genotype through deletion of tumor suppressors, amplification of oncogenes, and transcriptional deregulation [53–55]. Our results are also consistent with the idea that chromosomal instability can drive tumor evolution, as well as genomic, epigenetic and phenotypic heterogeneity [53–55].

Several lines of evidence support a potential role for Hic1 in maintaining chromosomal stability independent of Tp53. In a human cell line model, Dehennaut et al. showed that HIC1 promotes the response to DNA double strand breaks through an ATM-SIRT1-HDAC4 dependent mechanism [56]. Importantly, this was dependent on the interaction between HIC1 and MTA1, a component of the nucleosome remodeling and deacetylase (NuRD) complex. Interestingly, this complex is required for the effective DNA repair during S phase [57–59]. By contrast, Paget et al. recently showed that in response to repairable DNA damage, HIC1 was more important in mounting a transcriptional response to DSB than directly promoting DNA repair [60]. Although our data do not resolve this apparent contradiction, our findings are consistent with both papers in that Hic1 is involved in mediating DNA repair and/or in triggering cell death in response to DNA damage.

Interestingly, HIC1 is also known to interact with the tumor suppressor gene ARID1A [61], a component of the SWI/SNF chromatin remodeling complex that also has important roles in maintaining genome stability during DNA replication [62, 63]. This concept is strengthened by the observation that mouse embryonic stem cells lacking SWI/SNF protein Brg1 undergo cell cycle arrest associated with chromatin bridge formation and a defective decatenation checkpoint [64]. Taken together, these observations suggest that genome-wide interactions between Hic1 and both the NuRD and SWI/SNF complexes may play a role in
maintaining chromosomal instability during DNA replication.

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Fig. 4 Effects of Hic1 deletion (Hic1KO) on the development of lung tumors induced by the conditional activation of an activating KRas\(^{G12D}\) (KRas) mutant in the airway epithelium of adult mice. Mice from each genotype were anesthetized using Avertin at a dose of 0.5 mg/gram of body weight, and administered 5 \( \times \) 10\(^8\) PFU Ad5CMVCre virus (Viral Vector Core Facility, University of Iowa, Iowa City, IA) by intranasal inhalation at 8 weeks of age and observed for 9 months or until ethical endpoint. A Kaplan–Meier survival analysis of KRas and KRas \( \times \) Hic1KO mice following inhalation of adenoviral Cre recombinase. \( n = 12 \) (KRas) and 9 (KRas\( \times \) Hic1KO). Sample size was based on previous published studies [71]. B Quantitative analysis of lung tumor area and size in mice from the same experiment depicted in Fig. 4a. \( n = 6 \) per genotype, ***\( p < 0.001 \), *\( p < 0.05 \), unpaired \( t \)-test. Mouse lungs were inflated with 10% buffered formalin and fixed overnight before paraffin embedding. For quantitation of tumor burden and number, sections were scanned using the Aperio Scanscope XT (Leica Biosystems, Buffalo Grove, IL, USA) and analysed using Aperio Imagescope software by a blinded observer as described [71]. Sample size was chosen based on previous studies [71]. C Representative photomicrographs of hematoxylin and eosin (H&E) stained sections of lungs from the experiment depicted in Fig. 4a. Scale bar = 5 mm. D Representative high-powered photomicrographs of hematoxylin and eosin (H&E) stained sections of lungs from the same experiment. Scale bar = 20 \( \mu \)m. E Representative photomicrographs of sections from the same tumors stained with immunoperoxidase (brown) for Hic1, and counterstained with hematoxylin (blue). Immunohistochemistry was performed as described [71]. F Immuno-staining for Proliferating cell nuclear antigen (Pcna, Dako, Troy, MI, #M087901-2) or phospho-\( \gamma \)H2AX (\( \gamma \)H2AX, Abcam, Cambridge, UK) in the same tumors shown in Fig 4d. Immunohistochemistry and quantification of staining was performed as described [71]. Scale bar = 100 \( \mu \)m. G Quantitative analysis of Pcna and \( \gamma \)H2AX staining from the same experiment depicted in Fig. 4f. \( n = 5 \) per genotype, 5 fields of view at 40\( \times \) counted per animal. **\( p < 0.01 \), unpaired \( t \)-test.
Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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