

Hus1 Acts Upstream of Chk1 in a Mammalian DNA Damage Response Pathway

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Summary

The evolutionarily conserved Hus1 proteins function in DNA damage response pathways that serve to maintain genomic stability [1, 2]. Cells lacking mouse *Hus1* are hypersensitive to certain genotoxins [3], and we have explored the molecular basis for this defect by examining how *Hus1* inactivation affects genotoxin-induced signaling events. p53 accumulation and activation in response to DNA damage appeared normal in *Hus1* null cells. Likewise, Hus1 was dispensable for genotoxin-induced Chk2 phosphorylation. In contrast, Chk1 phosphorylation after genotoxic stress was greatly reduced in the absence of Hus1, but was restored in *Hus1* null fibroblasts complemented by infection with a *Hus1*-expressing retrovirus. These results demonstrate that mouse Hus1 is required for a specific subset of DNA damage signaling events and functions to promote genotoxin-induced Chk1 phosphorylation.

Results and Discussion

Hus1^{-/-} mouse embryonic fibroblasts (MEFs) fail to proliferate in culture, but the loss of *p21* allows some *Hus1*-deficient cultures to be established [3], permitting dissection of the role of *Hus1* in mammalian DNA damage responses. We first examined whether Hus1 was required for activation of the DNA damage-inducible transcription factor p53. Following genotoxic stress, p53 undergoes numerous posttranslational modifications, including phosphorylation by kinases Atm and Atr, and consequently becomes stabilized, accumulates to high levels, and transactivates target genes including *p21*, *Mdm2*, and *Perp* [4]. To investigate whether p53 activation was dependent on Hus1, we initially examined p53 protein levels in *Hus1*^{+/+}*p21*^{-/-} and *Hus1*^{-/-}*p21*^{-/-} MEFs after treatment with ionizing radiation (IR) and ultraviolet radiation (UV) (Figure 1A). Immunoblot analyses revealed that p53 was present at relatively low levels in untreated *Hus1*^{+/+}*p21*^{-/-} cells but increased in abundance after exposure to IR or UV. The kinetics and extent of p53 accumulation were similar to those previously

reported by others [5]. Interestingly, in untreated *Hus1*^{-/-}*p21*^{-/-} MEFs, the basal level of p53 was increased relative to that of *Hus1*^{+/+}*p21*^{-/-} cells, perhaps in response to spontaneous genome damage that occurs in *Hus1* null cells. Both IR and UV treatment caused p53 to accumulate in *Hus1*^{-/-}*p21*^{-/-} MEFs, with kinetics similar to those observed in *Hus1*^{+/+}*p21*^{-/-} MEFs. UV did cause a more persistent increase in p53 levels in *Hus1* null cells, possibly reflecting a DNA repair defect or failure of a cell cycle checkpoint, leading to additional damage. Because of the elevated basal level of p53 in *Hus1*^{-/-}*p21*^{-/-} MEFs, subtle effects of *Hus1* inactivation on p53 accumulation cannot be ruled out. Nonetheless, these results suggest that p53 protein accumulation after genotoxic stress does not require Hus1.

To directly assess p53 function in *Hus1* null cells, we made use of the prior observation that *Hus1*^{-/-} mouse embryos contain elevated mRNA levels for the p53 target genes *p21* and *Mdm2* [3] and tested whether the increased expression of these genes reflected p53 activation in the absence of *Hus1*. *Hus1*^{+/+}*p53*^{+/-} and *Hus1*^{+/+}*p53*^{-/-} mice were interbred, and total RNA from the resulting embryos at 8.5 dpc was subjected to Northern blot analysis. The litter shown in Figure 1B contained two *Hus1*^{-/-} embryos (embryos 4 and 7), one of which (embryo 7) was also nullizygous for *p53*. Consistent with previous results, the *Hus1*^{-/-} embryo expressing *p53* (embryo 4) showed elevated expression of *p21* (6.8× elevation in expression level for embryo 4 compared to the mean for all other embryos, except 7) and *Mdm2* (1.6× elevation). This transcriptional response required p53, as *p21* and *Mdm2* were expressed at normal levels in the *Hus1*^{-/-}*p53*^{-/-} embryo (relative expression level of 0.9× for both genes in embryo 7 compared to the mean for all other embryos, except 4). This was unlikely to be an indirect consequence of restoration of normal embryonic development in the *Hus1*^{-/-}*p53*^{-/-} embryo, as the loss of *p53* does not rescue the morphological abnormalities of *Hus1*^{-/-} embryos (R.W. and P.L., unpublished data). These data argue that, in *Hus1* null embryos, p53 retains the capacity to transactivate target genes.

To further confirm these results, we compared by Northern blotting the transcriptional induction of UV-responsive genes in *Hus1*^{+/+}*p21*^{-/-} and *Hus1*^{-/-}*p21*^{-/-} MEFs. As previously described [3], *Hus1*^{-/-}*p21*^{-/-} MEFs did not express wild-type *Hus1* transcripts, but they did express aberrant transcripts at low abundance (Figure 1C). Basal mRNA levels for the p53 target gene *Perp* [6] were elevated in *Hus1*^{-/-}*p21*^{-/-} MEFs, possibly reflecting the greater amount of p53 protein in these cells. Nevertheless, expression of *Perp* was induced by UV to a similar degree and with similar kinetics in *Hus1*^{+/+}*p21*^{-/-} and *Hus1*^{-/-}*p21*^{-/-} MEFs. Together with the other results described above, these findings suggest that p53 can operate normally in the absence of Hus1. mRNA levels for *Fos*, *Kin17*, and *Gadd34*, genes that are induced by UV through p53-independent mechanisms [7], were also upregulated following UV treatment in *Hus1*^{-/-}*p21*^{-/-}

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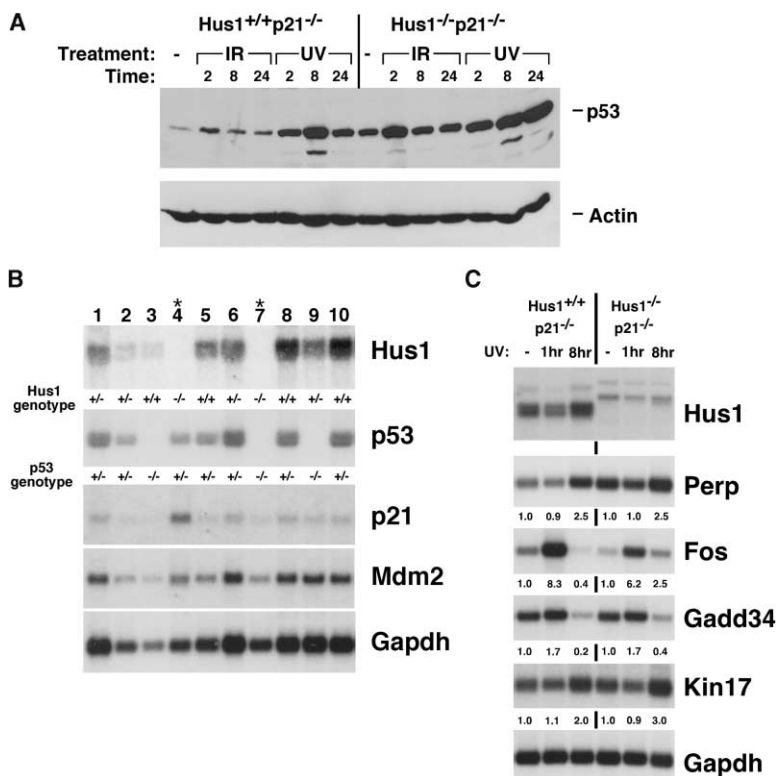


Figure 1. p53 Appears Functionally Normal in the Absence of Hus1

(A) Immunoblot analysis of p53 protein. *Hus1^{+/+}p21^{-/-}* and *Hus1^{-/-}p21^{-/-}* MEFs were mock treated or irradiated with 10 Gy IR or 10 J/m² UV. Cells were harvested at the indicated times posttreatment, and equal amounts of total protein were immunoblotted for p53 protein (upper panel) or β -actin (lower panel).

(B) Expression of p53 target genes in *Hus1* embryos. Total RNA was prepared from ten individual 8.5-dpc embryos from a timed mating between *Hus1^{+/+}p53^{+/-}* and *Hus1^{+/+}p53^{-/-}* mice, and Northern blotting was performed with the indicated radiolabeled probes. Genotypes were deduced from expression levels. Asterisks highlight the two *Hus1^{-/-}* embryos.

(C) UV-responsive gene expression in *Hus1* MEFs. Poly(A)⁺ mRNA was prepared 1 or 8 hr after UV irradiation (5.0 J/m²) or 1 hr after mock treatment of *Hus1^{+/+}p21^{-/-}* and *Hus1^{-/-}p21^{-/-}* MEFs and was subjected to Northern blotting with the indicated probes. Signal intensity from Northern blots was quantitated by PhosphorImager. Values were normalized based on results for *Gapdh*, and relative expression levels are indicated below each lane.

MEFs, although some minor differences were noted with respect to the extent or timing of induction relative to results for *Hus1^{+/+}p21^{-/-}* MEFs. These results suggest that transcriptional responses to UV-induced DNA damage are not grossly abnormal in the absence of *Hus1*.

Another candidate DNA damage response protein that might function downstream of Hus1 is Chk2 (also known as Cds1), a mammalian homolog of the fission yeast Cds1 and budding yeast Rad53 protein kinases. Chk2 becomes phosphorylated and activated by IR in an Atm-dependent manner and also becomes phosphorylated in UV- and hydroxyurea (HU)-treated cells in an Atm-independent fashion [8–12]. Because phosphorylation of Chk2 alters its mobility in SDS-PAGE gels, it was possible to monitor the phosphorylation state of Chk2 by immunoblotting and to ask whether Hus1 was required for genotoxin-induced Chk2 phosphorylation (Figure 2). Chk2 protein from untreated *Hus1^{+/+}p21^{-/-}*

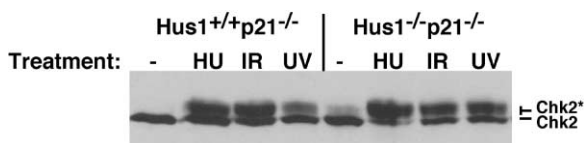
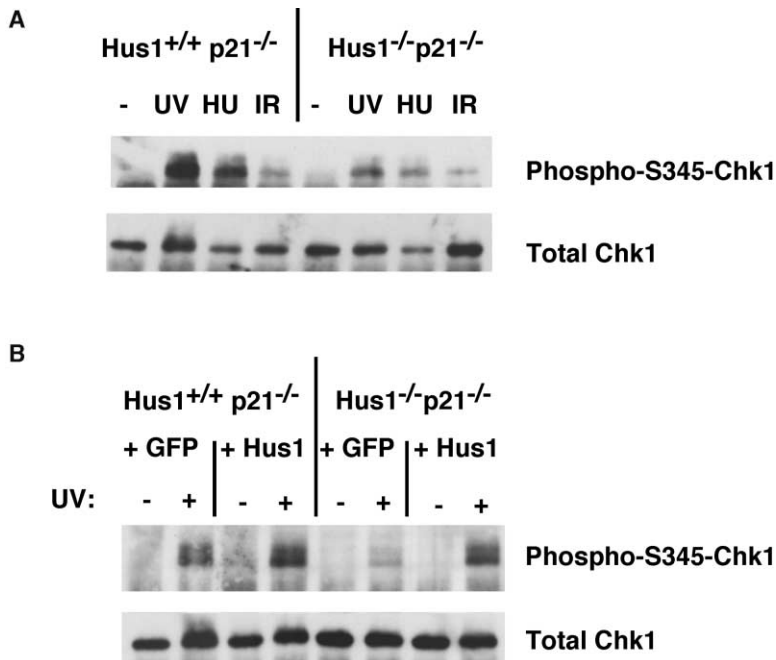


Figure 2. Genotoxin-Induced Chk2 Phosphorylation in the Absence of Hus1

Total-cell lysates were prepared from *Hus1^{+/+}p21^{-/-}* and *Hus1^{-/-}p21^{-/-}* MEFs 8 hr after mock treatment (–) or irradiation with 10 Gy IR or 10 J/m² UV or after 24 hr of incubation in 250 μ M HU. Equal amounts of total protein were immunoblotted for Chk2 protein. “Chk2*” indicates the position of phosphorylated Chk2 with reduced gel mobility.

cells migrated in SDS-PAGE gels as a single band. After treatment of *Hus1^{+/+}p21^{-/-}* MEFs with HU, UV, or IR, the electrophoretic mobility of Chk2 was reduced, corresponding to its phosphorylation following genotoxic stress. Some Chk2 protein with reduced gel mobility was observed in untreated *Hus1^{-/-}p21^{-/-}* MEFs. HU, UV, and IR treatment of *Hus1^{-/-}p21^{-/-}* MEFs all caused the appearance of significant amounts of Chk2 with reduced gel mobility, implying that normal Chk2 phosphorylation follows genotoxic stress in *Hus1*-deficient cells. Comparison of the kinetics of the UV- and IR-induced Chk2 mobility shift also failed to reveal differences between *Hus1^{+/+}p21^{-/-}* and *Hus1^{-/-}p21^{-/-}* cells (data not shown). These results suggest that Hus1 is dispensable for genotoxin-induced Chk2 phosphorylation.

We next investigated a role for Hus1 in genotoxin-induced phosphorylation of the Chk1 protein kinase, an essential component of the G₂/M DNA damage and DNA replication checkpoints [13–15]. In response to genotoxic stress, Chk1 is activated by phosphorylation on serines 317 and 345 by the upstream kinase Atr [15, 16]. Consistent with the possible involvement of Atr, Chk1, and Hus1 in a common pathway, *Hus1*-deficient cells are hypersensitive to UV and HU [3], genotoxins that potentially induce Chk1 phosphorylation by Atr [15, 16]. *Atr* and *Chk1* are both essential genes in the mouse [14, 15, 17, 18], their inactivation leading to chromosomal fragmentation, apoptosis, and peri-implantation embryonic lethality. Interestingly, *Hus1^{-/-}* embryos display similar phenotypes, including widespread cell death and spontaneous chromosomal abnormalities, but die significantly later in development than *Atr* or *Chk1* null animals [3].



Initial examinations of Chk1 protein levels revealed that some *Hus1*^{-/-}*p21*^{-/-} MEF cultures contained greatly elevated Chk1 protein levels (data not shown). Because differences in total Chk1 levels would complicate the comparison of Chk1 phosphorylation in *Hus1*^{+/+}*p21*^{-/-} and *Hus1*^{-/-}*p21*^{-/-} cells, we focused on MEF cultures that expressed equal amounts of total Chk1 protein. Chk1 from HU-, UV-, or IR-treated *Hus1*^{+/+}*p21*^{-/-} or *Hus1*^{-/-}*p21*^{-/-} MEFs was immunoprecipitated either with antibodies that recognize all forms of Chk1 (total Chk1) or antibodies specific for S345-phosphorylated (p-S345) Chk1 and was then detected by immunoblotting. The total Chk1 immunoprecipitations showed that the *Hus1*^{+/+}*p21*^{-/-} and *Hus1*^{-/-}*p21*^{-/-} MEFs contained similar amounts of total Chk1 protein and that the level of Chk1 did not change after UV or IR treatment (Figure 3A). Chk1 levels were reduced in HU-treated cells regardless of *Hus1* genotype. Immunoprecipitations with anti-p-S345 Chk1 antibodies suggested that little p-S345 Chk1 was present in untreated cells, but that p-S345 Chk1 accumulated in UV-, HU-, and to a lesser extent, IR-treated *Hus1*^{+/+}*p21*^{-/-} cells. The p-S345 Chk1 signal was specific, as immunoprecipitation of p-S345 Chk1 was blocked by preincubating the phospho-specific antibody with the phosphorylated peptide antigen, but not the unphosphorylated peptide (data not shown). Significantly, the appearance of p-S345 Chk1 was reduced, though not completely ablated, in UV-, HU-, and IR-treated *Hus1*^{-/-}*p21*^{-/-} MEFs. These data indicate that genotoxin-induced Chk1 phosphorylation is partially dependent on Hus1.

To verify that the Chk1 phosphorylation defect was due specifically to the absence of Hus1, we tested whether reintroduction of *Hus1* into *Hus1*^{-/-}*p21*^{-/-} MEFs would restore genotoxin-induced Chk1 phosphorylation. *Hus1*^{+/+}*p21*^{-/-} and *Hus1*^{-/-}*p21*^{-/-} MEFs were infected with retroviruses expressing either *Hus1* (pBP2-

Figure 3. Reduced Genotoxin-Induced Chk1 Phosphorylation in *Hus1*-Deficient Cells

(A) Immunoprecipitation analysis of Chk1 phosphorylation. Total-cell lysates were prepared from *Hus1*^{+/+}*p21*^{-/-} and *Hus1*^{-/-}*p21*^{-/-} MEFs 2 hr after mock treatment (-) or irradiation with 20 Gy IR or 50 J/m² UV or after 24 hr of incubation in 1.0 mM HU. Immunoprecipitations were performed with an antibody specific for phospho-S345-Chk1 (upper panel) or total Chk1 (lower panel), followed by immunoblotting with anti-Chk1.

(B) Immunoprecipitation analysis of Chk1 phosphorylation in reconstituted *Hus1* null cells. Total-cell lysates were prepared from the indicated cell pools 2 hr after mock or UV (50 J/m²) irradiation and were subjected to immunoprecipitation with an antibody specific for phospho-S345-Chk1 (upper panel) or total Chk1 (lower panel), followed by immunoblotting with anti-Chk1.

Hus1) or *GFP* (pBP2-*GFP*) as a control, and pools of cells with stably integrated virus were generated. *Hus1*^{-/-}*p21*^{-/-} MEFs infected with pBP2-*Hus1* expressed full-length *Hus1* transcripts and showed genotoxin sensitivity similar to that of *Hus1*^{+/+}*p21*^{-/-} MEFs (data not shown). Chk1 S345 phosphorylation was induced by UV in *Hus1*^{+/+}*p21*^{-/-} cells carrying the control pBP2-*GFP* retrovirus as expected (Figure 3B). *Hus1*^{+/+}*p21*^{-/-} cells infected with pBP2-*Hus1* showed an even greater extent of UV-induced Chk1 phosphorylation, suggesting that Hus1 protein levels may be limiting in *Hus1*^{+/+}*p21*^{-/-} cells. Consistent with our findings in parental *Hus1*^{-/-}*p21*^{-/-} MEFs, UV-induced Chk1 phosphorylation was impaired in *Hus1*^{-/-}*p21*^{-/-} cells containing pBP2-*GFP*. However, restoration of *Hus1* expression by pBP2-*Hus1* complemented this defect and allowed for considerable UV-induced Chk1 phosphorylation. Similar results were obtained with individual stable clones of *Hus1*^{-/-}*p21*^{-/-} cells infected with pBP2-*Hus1*. These results place Hus1 upstream of Chk1 and demonstrate that Hus1 plays an important role in enabling Chk1 phosphorylation following genome damage.

Fission yeast appear to utilize a single branching pathway to respond to a variety of genotoxins. Consequently, all known downstream DNA damage responses, including activation of Chk1 and Cds1, are defective in *hus1*⁻ strains [19]. In contrast, we found that loss of mouse *Hus1* led specifically to defects in genotoxin-induced signaling to Chk1, but not Chk2 or p53. These findings are consistent with accumulating evidence that mammalian cells contain at least two parallel pathways that respond to particular genotoxins but also interact through extensive cross-talk [1]. One mammalian DNA damage response pathway is activated principally by double-stranded DNA breaks (DSB) and involves Atm and Chk2. The results presented here identify a role for Hus1 in a second pathway that centers on

Atr and Chk1 and responds primarily to other types of DNA damage, such as lesions caused by UV, and DNA replication blocks. The existence of two partially independent DNA damage signaling cascades in mammalian cells may promote effective repair by tailoring responses toward particular DNA lesions and may also provide redundancy if one pathway was to fail.

The finding that Hus1 is required for optimal genotoxin-induced Chk1 phosphorylation suggests that Hus1 assists Atr in targeting its substrate. In addition to Chk1 [15, 16], other Atr substrates include Brca1 [20], Chk2 [12], p53 [21, 22], and Rad17 [23]. These proteins all contain consensus Atr phosphorylation sites, can be phosphorylated by Atr in vitro, and typically show reduced genotoxin-induced phosphorylation in cells overexpressing dominant-negative Atr. Chk1 is not the only Atr substrate that requires Hus1 for optimal phosphorylation after genome damage, as uv-induced Rad17 phosphorylation is also Hus1 dependent (L. Zou, D. Cortez, and S.J.E., unpublished data). Meanwhile, the apparently normal Chk2 phosphorylation and p53 activation observed in *Hus1* null cells might suggest that Hus1-independent mechanisms promote the phosphorylation of these substrates by Atr. However, another possibility is that redundant mechanisms obscure a role for Hus1 in p53 and Chk2 activation by Atr. For instance, the inability of *Hus1*-deficient cells to respond properly to UV might lead to additional genome damage, including DSB that could activate Atm in a parallel DNA damage response pathway and thereby cause phosphorylation and activation of p53 and Chk2. Notably, p53 and Chk2 can both be efficiently phosphorylated by Atm, whereas Chk1 is a poor Atm substrate [16, 24]. Precedent for such a mechanism comes from fission yeast. *Schizosaccharomyces pombe* Chk1 ordinarily responds specifically to DNA damage, but in strains lacking *Cds1*, defects in the HU response lead to abnormal Chk1 activation by HU [25].

There are several possibilities as to how Hus1 might promote DNA damage-induced Chk1 phosphorylation by Atr. Hus1 could act upstream of Atr, possibly by regulating the subcellular localization or catalytic activity of Atr. Although genotoxin-induced changes in Atr kinase activity have not been reported, Atr is known to relocate to discrete nuclear foci following genotoxic stress [20]. However, studies in *S. pombe* suggesting that the Atr homolog Rad3 can be activated in the absence of Hus1 argue against this model and imply that Hus1 acts downstream of Rad3 [26]. In light of such results, another possibility is that Hus1 acts to recruit substrates to Atr. Such a proposition is consistent with the predicted structural similarity between Hus1, as well as its binding partners Rad1 and Rad9, and proliferating cell nuclear antigen (PCNA), a trimeric sliding clamp that encircles DNA ([27] and references cited therein). PCNA has no known enzymatic activity and acts to tether proteins with limited DNA binding activity to their sites of action on DNA [28]. By analogy, the Hus1/Rad1/Rad9 complex could localize to sites of genome damage and subsequently recruit downstream signaling proteins, which could then be efficiently phosphorylated and activated by Atr. The relationship between Hus1 and Atr could be complex, however, and might involve interactions at multiple levels, as well as possible roles in paral-

lel signaling pathways. Experiments to further delineate mammalian DNA damage signaling pathways may resolve these intricacies and shed light on how these pathways induce effective responses to a variety of threats to genomic integrity.

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Note Added in Proof

The data referred to as “L. Zou, D. Cortez, and S.J.E., unpublished data” are now in press: Zou, L., Cortez, D., and Elledge, S.J. (2002). Regulation of ATR substrate selection by RAD17-dependent loading of Rad9 complexes onto chromatin. *Genes Dev.*, in press.