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The nucleosome-binding protein HMGN2 modulates global genome repair

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Abstract

The HMGN family comprises nuclear proteins that bind to nucleosomes and alter the structure of chromatin. Here, we report that DT40 chicken cells lacking either *HMGN2* or *HMGN1a*, or lacking both *HMGN1a* and *HMGN2*, are hypersensitive to killing by UV irradiation. Loss of both *HMGN1a* and *HMGN2* or only *HMGN2* increases the extent of UV-induced G₂-M checkpoint arrest and the rate of apoptosis. *HMGN* null mutant cells showed slower removal of UV-induced DNA lesions from native chromatin, but the nucleotide excision repair remained intact, as measured by host cell reactivation assays. These results identify HMGN2 as a component of the global genome repair subpathway of the nucleotide excision repair pathway, and may indicate that HMGN2 facilitates the ability of the DNA repair proteins to access and repair UV-induced DNA lesions in chromatin. Our finding that HMGNs play a role in global DNA repair expands the role of these proteins in the maintenance of genome integrity.

Keywords

apoptosis; cell cycle; chromatin; DNA repair; UV irradiation

Introduction

The timely repair of DNA lesions caused by UV irradiation is essential for the survival of cells and for the prevention of cancer. DNA products resulting from UV irradiation, such as cyclobutane pyrimidine dimers (CPDs) and pyrimidine(6–4)pyrimidone photoproducts, are removed from the DNA by a multistep process known as the nucleotide excision repair (NER) pathway. Mutations in the genes coding for components of the NER pathway result in severe genetic disorders, such as xeroderma pigmentosum, Cockayne syndrome, and trichothiodystrophy [1,2]. In the nucleus of eukaryotic cells, the DNA is packaged into chromatin, and repair of DNA lesions therefore occurs within the context of chromatin. The DNA repair rate in chromatin is slower than that of deproteinized DNA [3]. The NER process has been recently linked to various factors that remodel and change chromatin structure. The histone acetyltransferase (HAT) Gcn5 was found to be involved in DNA

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repair as part of the STAGA complex [4] and as part of the FCTC complex, which also contains SAP130, a protein homologous to DDB1 (UV-damaged DNA-binding factor) [5]. Likewise, the HAT CBP/p300 [6] was also linked to the NER process. In addition, the ATP-dependent chromatin-remodeling complexes, such as ACF [7] and SWI/SNF [8,9], have been associated with DNA nucleotide excision repair. The emerging picture reveals that modifying the chromatin at the DNA damage site is an essential step in providing accessibility for the repair complexes [10–12].

The nucleotide repair pathway is subdivided into two subpathways, the transcription-coupled repair (TCR) subpathway, and the global genome repair (GGR) subpathway [1,2,13]. The TCR subpathway is involved in repair of the UV irradiation-induced DNA lesions on the transcribed DNA strands, whereas the GGR subpathway repairs the damage in the entire genome. HMGN1, an architectural protein that remodels chromatin in a nonenzymatic manner, was found to be involved in the TCR subpathway [14]. The HMGN1-mediated enhancement of DNA repair in chromatin was linked to the ability of HMGN1 to bind to the nucleosomes and unfold chromatin [14]. However, the involvement of HMGNs in the GGR subpathway has never previously been shown. Furthermore, most of the published data relate to HMGNs as a family of proteins involved in transcription regulation [15]. The HMGN family comprises structural proteins that specifically recognize the generic structure of the 147 bp nucleosome core particle [16,17]. This family contains several proteins; however, in most species, HMGN1 and HMGN2 are the most abundant family members. Although the overall domain structures of HMGN1 and HMGN2 are very similar, their primary sequences differ by almost 40% [17]. *In vitro* studies have demonstrated that both proteins bind to nucleosomes, reduce the compaction of the higher-order chromatin fibers, and enhance the transcription potential of chromatin templates [15–17]. HMGNs may affect the structure and function of chromatin through several mechanisms. These include competition with H1 for nucleosomal binding sites [18,19], facilitating changes in the levels of histone modifications [20,21], and induction of conformational changes in the nucleosome itself [22].

Here, we investigated whether the involvement of HMGNs in NER is only in TCR or also in GGR, which may indicate that HMGNs' chromatin-unfolding function in NER is transcription-independent. Furthermore, we investigated whether, in addition to HMGN1, other members of the HMGN family play a role in the repair of UV irradiation-induced DNA damage. For this purpose, we used wild-type and gene-targeted chicken lymphoblastoid cells (DT40), which, like other chicken tissues, contain three HMGN proteins: HMGN1a, HMGN1b, and HMGN2 [23,24]. *HMGN1a* has been detected only in chickens, and has a sequence that is partially homologous to the consensus sequence of vertebrate *HMGN1* and *HMGN2*. *HMGN2* is homologous to the other vertebrate *HMGN2*s, whereas the sequence of the chicken *HMGN1b* is homologous to the ubiquitous vertebrate *HMGN1*. In this study, we focused on HMGN2 and used cells that lack *HMGN2* and null cells for both *HMGN1a* and *HMGN2* (as *HMGN1a* is partially homologous to *HMGN2*, and therefore it could, in theory, complement it). As previously described [25,26], the null DT40 cells lack either *HMGN1a* or *HMGN2* or both *HMGN1a* and *HMGN2*, but they still contain *HMGN1b*, a relatively minor component in most chicken cells. The protein profiles of these cells differed slightly; however, all lines had normal proliferation and differentiation rates [25,26].

Although the cells appeared to be normal, it is possible that their stress response was impaired. Therefore, chicken cells disrupted for *HMGN2* or disrupted for both *HMGN1a* and *HMGN2* provide a good model with which to test for functional redundancy among HMGN variants and the possible role of the major HMGN proteins in the cellular response to UV damage.

Here, we show that loss of both *HMGN1a* and *HMGN2*, or of only *HMGN2*, impairs the rate of UV-induced GGR. Loss of *HMGNs* leads to an increase in both the UV-induced rate of apoptosis and in the level of checkpoint arrest. In GGR, *HMGN2* and *HMGN1a* proteins were, for the most part, not redundant in their function, although some additive effects on cell survival, apoptosis and, mainly, checkpoint arrest indicated that there is some level of redundancy. Host cell reactivation assays indicated that *HMGNs* do not affect the integrity of the cellular NER machinery. Thus, *HMGNs* affect the repair of UV-damaged DNA by altering chromatin.

Results

HMGN null mutants are hypersensitive to UV irradiation

To investigate the involvement of *HMGN* variants in the UV response of DT40 cells, we used cells lacking either *HMGN2* (clone D108-1), or *HMGN1a* (clone 8/bsr8), or cells lacking both *HMGN2* and *HMGN1a* (clones Nh43, Bp39, Nh52, and Bp5). The Bp lines were derived by first deleting the *HMGN2* gene, and the Nh lines were derived by first targeting the *HMGN1a* gene [25,26]. We used western analysis to verify that the targeted genes were indeed disrupted (Fig. 1). Interestingly, loss of *HMGN1a* increased the amounts of *HMGN2* (Fig. 1A). In addition, all cells contained *HMGN1b*, which is a minor *HMGN* variant in chicken cells [23].

Wild-type and mutant DT40 cells were irradiated with UV doses ranging from 3 to 12 J·m⁻². Seventy-two hours after the irradiation, the viability of cells was measured by a Trypan blue exclusion assay. As shown in Fig. 2 and Table 1, all of the *HMGN* null mutants were significantly more UV-sensitive than wild-type DT40 control cells. The LD₅₀ (UV dose resulting in 50% survival) for the wild-type cells was 9.4 ± 2.33 J·m⁻², whereas the LD₅₀ for the cell variants lacking *HMGN* was in the range of 2.63 ± 0.51 to 3.69 ± 0.83 J·m⁻². These differences in the LD₅₀ values between the wild-type cells and all of the *HMGN* null cells were found to be significant (non-parametric Mann–Whitney *U*-tests, all *P*-values < 0.01). The level of UV sensitivity of *HMGN2*^{-/-} cells (D108-1) (3.69 ± 0.83 J·m⁻²) was somewhat lower than that of the other null cells, but a statistical analysis showed that D108-1 cells were statistically similar to the other *HMGN* null cells (all *P*-values > 0.127). No major additive or synergistic effect was observed in the sensitivity of the doubly disrupted cell lines. These results may indicate that, for the most part, *HMGN2* and *HMGN1a* function in the same pathway in conferring UV resistance to cells, as disrupting each of them alone was sufficient to reduce the UV tolerance to almost the same level as disrupting both genes. However, these results cannot rule out a partial redundancy between *HMGN2* and *HMGN1a* that contributes to the minor additive effect.

HMGN null mutants have a higher rate of UV-induced apoptosis

The increased rate of mortality in UV-irradiated cells is linked to the activation of the apoptotic pathway [27,28]. To test whether the UV-hypersensitive *HMGN* null cells have a higher apoptosis rate, we UV-irradiated the various cell lines with 6 J·m⁻², and, 48 h following UV irradiation, stained control and UV-irradiated cells with annexin V and propidium iodide (PI). Fluorescein isothiocyanate (FITC)-conjugated annexin V detects translocation of phosphatidylserine across membranes, an early apoptotic event, and PI is used to detect the permeabilization of the plasma membrane, an event that occurs late in apoptosis. Fluorescence-activated cell sorter (FACS) analysis of these cells provided a quantitative measure of the apoptotic events in the cells. The quadrant analysis of the FACS results demonstrated that, after UV irradiation, the late and total apoptosis rates were higher in both *HMGN2*^{-/-} cells (D108-1) and in the *HMGN1a*^{-/-}/*HMGN2*^{-/-} double-knockout clones (Nh43 and Bp5), as compared with the wild-type DT40 cells (Fig. 3 and Table 2)

(independent group *t*-test, $P < 0.05$). In contrast to this, the early apoptotic rates were lower in the *HMGN* null cell lines than in the wild-type DT40 cells (independent group *t*-test, $P < 0.05$). The sum totals of both early and late apoptotic cells were as follows: 33.7% for the wild-type DT40 cells, 41.7% for the D108-1 cells, 47.9% for the Nh43 cells, and 58.6% for the Bp5 cells. These results, taken together, indicate that cells lacking HMGN proteins had a higher apoptosis rate following UV irradiation but also activated the apoptotic pathway faster, and therefore moved faster from early to late apoptosis.

Loss of *HMGN* increases the rate of G₂–M checkpoint arrest following UV irradiation

The cellular response to UV radiation is known to involve not only apoptosis but also cell cycle arrest, mainly in the G₁–S or the G₂–M checkpoints. To test whether the UV hypersensitivity of cells lacking *HMGNs* involves increased activation of one of these checkpoints, cells were UV-irradiated (12 J·m⁻²), and, 48 h after irradiation, their cell cycle distribution was measured as follows. The cells were pulsed for 30 min with the thymidine analog bromodeoxyuridine (BrdU), fixed in 70% ethanol, and double-stained with antibodies against BrdU and PI. In FACS analysis, a plot of BrdU levels against PI levels produces a typical ‘horseshoe’ shape (Fig. 4), in which the G₁ and G₀ cells are represented in the lower left corner of the plot, and the G₂–M cells in the right side of the plot. The cells in S-phase are between these two groups, in the arch of the ‘horseshoe’, which is high in BrdU. The results reveal that UV irradiation of cells lacking *HMGN2* (D108-1 cells), or lacking both *HMGN1a* and *HMGN2* (Nh43 cells), decreases the relative number of cells in S-phase as compared with the more moderate decrease in the wild-type DT40 cells. The S-phase population significantly decreased in D108-1 cells, from 40.7% to 21.1%, and in Nh43 cells from 48.2% to 14.0% ($P < 0.05$ by paired *t*-test), as opposed to an insignificant decrease in the number of wild-type cells, from 38.7% to 30.3% (Fig. 4). The decrease in the number of cells in S-phase was associated with a concomitant increase in the population of G₂–M cells for the null mutants after UV irradiation (paired *t*-test, $P < 0.05$), and a significant increase in the G₂–M population of irradiated Nh43 cells in comparison with irradiated wild-type cells (independent *t*-test, $P < 0.05$). This increase in the G₂–M population indicates activation of either the G₂–M checkpoint arrest or one of the mitotic checkpoints. In order to distinguish between these two possibilities, we conducted a western blot analysis with two antibodies, which served as specific markers. We used antibody against phosphorylated Chk1 Ser345 as a marker for activation of G₂–M checkpoint arrest [29]. Antibody against histone H3 phosphorylated on Ser10 was used as a marker for mitotic cells [30]. The western blot analysis (Fig. 4C) indicated that the cells lacking *HMGN2* and also the double-null cells lacking *HMGN1a* and *HMGN2* had a longer arrest time in the G₂–M checkpoint. In the wild-type DT40 cells, there was a sharp drop in the phosphorylation of Chk1 Ser345 10 h after UV irradiation. In contrast, in the D108-1 cell line, the high levels of Chk1 phosphorylation continued to the 24 h point, and in the double-null Nh43 cells, the high level of phosphorylation remained even 48 h after UV irradiation. The number of mitotic cells among the double-null cells was also higher after UV irradiation, indicating activation of mitotic checkpoints. These results explain the significantly higher levels of G₂ and M cells among the *HMGN1a*^{-/-}/*N2*^{-/-} cells (Nh43) that were observed with PI and BrdU double staining (Fig. 4B). It is important to note that the differences between the null cell lines D108-1 and Nh43 and the wild-type DT40 cells cannot be attributed to random mutations that may have accumulated in these cells, but only to the lack of HMGN proteins. The reason for excluding this possibility is that the two null cell lines were independently derived from DT40 cells; Nh43 cells were first disrupted for *HMGN1a* alleles and then for the two *HMGN2* alleles, so they were not derived from the D108-1 (*HMGN2*^{-/-}) cells [25,26].

A decreased rate of CPD removal in the context of chromatin from cells lacking HMGN proteins

The increased rates of apoptosis and checkpoint arrest in cells lacking HMGNs could be due to an impaired ability to repair the damaged DNA. To test this possibility directly, we analyzed the kinetics of CPD removal in *HMGN2*^{-/-} cells, in *HMGN2*^{-/-}/*HMGN1a*^{-/-} cells, and in wild-type cells, following UV irradiation. DNA purified immediately after irradiation (time 0), and 7 and 20 h after irradiation, was slot blotted onto a nylon membrane, probed with antibodies directed against CPDs, and stained with ethidium bromide (Fig. 5A–C). The negative control was DNA from nonirradiated cells. The CPDs and DNA were measured within the linear range, based on a standard curve (data not shown). Following irradiation, there was a gradual decrease in the CPD content of the DNA of all the cells, an indication of active repair of the damaged DNA. However, the removal of CPDs from the chromatin of the *HMGN2*^{-/-} and *HMGN2*^{-/-}/*HMGN1a*^{-/-} cells was significantly slower than from the chromatin of wild-type cells ($P < 0.05$ by nonparametric Kruskal–Wallis test). Thus, 7 h after irradiation, 60% of the CPDs were removed from wild-type DT40 cells, but less than 20% were removed from cells lacking HMGN variants (Fig. 5B). After 20 h, the amount of CPDs present in wild-type cells was ~10% of the initial content, whereas in the *HMGN* null cells, ~40% of the original damage still remained in the DNA. These results, however, could also have been obtained if the *HMGN* null cells had an initially higher susceptibility to UV irradiation. This would result in a higher number of CPDs immediately after UV irradiation, and therefore a slower repair process, by virtue of the cells having more CPD sites to repair. For example, lack of the chromatin architectural factor HMGB1 has been previously found to increase the number of CPDs after UV irradiation [31]. To test this possibility, we analyzed the CPD/DNA ratio at time 0 after UV irradiation in the wild-type DT40 cells and in the null *HMGN* cells, without standardizing these values to 100% (Fig. 5C). The data indicate that although there are small differences between the wild-type DT40 cells and the null cells, these differences are not consistent between the null cell lines, and they are not statistically significant, either between the *HMGN* null cells and DT40 cells, or between the null D108-1 cells and Nh43 cells (non-parametric Kruskal–Wallis test, all P -values > 0.275). These results therefore suggest that HMGNs affect the rate of repair of DNA damage induced by UV irradiation and not the initial number of CPDs formed by UV. The repair kinetics in cells lacking only *HMGN2* were similar to those in cells lacking both *HMGN2* and *HMGN1a*.

Host cell reactivation reveals the integrity of the NER machinery

Most of the UV-induced damage in DNA is removed by NER, an evolutionarily conserved pathway that repairs the damage in the context of cellular chromatin. To determine whether loss of HMGN affected the activity of proteins in this pathway, we used the host cell reactivation assay [32,33] (Fig. 6). This assay measures the repair of a UV-irradiated plasmid containing the reporter gene for luciferase that was transiently transfected into various cells. The level of the repair of the episomal DNA can be estimated from the levels of luciferase activity in the cellular extracts prepared 48 h after transfection [32,33]. In this assay, the levels of luciferase activity recovered from wild-type DT40 cells were the same as those recovered from DT40 variants lacking both HMGNs, an indication that the UV-irradiated plasmids were repaired at the same rate in these cell types (Fig. 6B,C). Thus, the UV hypersensitivity in the DT40 cells lacking HMGNs is not due to a lack of function in the NER components, but probably to the direct unfolding activity of HMGNs at the damage sites, a finding consistent with previous results obtained with mouse cells lacking HMGN1 [14]. It is important to note that the chromatin structure of the transfected plasmid DNA is different from that of the cellular chromatin [34,35]; therefore, it is conceivable that the effect of HMGN proteins on the repair of the transfected plasmid is different from their effect on the cellular chromatin. However, in the cell line D108-1, which lacks HMGN2,

there was even higher DNA damage repair activity than in the wild-type control (Fig. 6A). One possible explanation for this observation is that D108-1 cells might have an increased expression level of one or more of the genes involved in TCR, which is the major NER subpathway detected by the host cell reactivation.

Discussion

Our main finding is that the nucleosome-binding protein HMGN2 plays a role in the NER GGR subpathway. We found that, in DT40 cells, loss of *HMGN2* or *HMGN2* and *HMGN1a* reduces the rate of CPD removal from chromatin. Taken together with our previous finding, that loss of *HMGN1* from mouse embryonic fibroblasts reduces the rate of transcription-coupled UV repair [14], our present findings indicate that both HMGN1 and HMGN2 play more general roles in repair of UV-induced DNA damage in the context of chromatin. Our previous studies with mouse embryonic fibroblasts lacking HMGN1 [14] did not provide information on GGR, as this repair is not efficient in murine cells. Our present studies reveal that loss of HMGNs reduced the rate of CPD removal not only from transcriptionally active genes (as was shown in mice), but also at the global genomic level. Thus, HMGN proteins affect UV-induced DNA damage removal, both in TCR and in GGR.

Both the higher apoptosis rate and increased checkpoint arrest of *HMGN2*^{-/-} and *HMGN1a*^{-/-}/*HMGN2*^{-/-} cells can be attributed to the lower rate of removal of CPDs from chromatin. It is well established that cells do arrest in various cell cycle checkpoints in response to induced DNA damage. Cells have various mechanisms in place for sensing DNA damage [10] and switching between alternative response pathways [36]. After their arrest at the cell cycle checkpoints, the cells respond either by repairing the DNA damage or, if the damage is beyond repair, by activating an apoptotic pathway [37,38]. HMGNs affect the rate of CPD removal, and in their absence the removal of CPDs is slower. The lower repair rate means that a higher CPD/DNA content will persist in the cell, resulting in more robust cell cycle arrest and a higher rate of activation of the apoptotic pathway following UV irradiation. Interestingly, the *HMGN1a*^{-/-}/*N2*^{-/-} cells (Nh43) were not only arresting in the G₂-M checkpoint, but also significantly accumulated during mitosis. In contrast, the null *HMGN2*^{-/-} cells (D108-1) showed mainly G₂-M arrest. A possible explanation might be that in the *HMGN1a*^{-/-}/*N2*^{-/-} cells, there is a leakage of cells with DNA damage through the G₂-M checkpoint to the mitosis phase, and their arrest in the mitotic checkpoints. This leakage is indicative of a possible role of HMGN1a in activation of the G₂-M checkpoint. Involvement of HMGN1 in activation of the G₂-M checkpoint has also been suggested to occur in mouse cells. In *Hmgn1*^{-/-} mouse embryonic fibroblasts there was no decrease in the level of mitotic cells following γ -irradiation, as opposed to wild-type mouse fibroblasts, which showed a drop of 70% in the number of mitotic cells [39].

In considering the possible molecular mechanisms whereby HMGNs affect the rate of CPD removal from damaged DNA, we note that the DT40 cells lacking HMGN2 and HMGN1a, as well as the murine cells lacking HMGN1 [14], repair irradiated plasmids with the same efficiency as wild-type cells. Thus, the host cell reactivation assays suggest that the known NER factors are functional and normally expressed in the cells lacking HMGNs. Further support for this conclusion comes from microarray analysis, which could not detect changes in the transcription levels of NER-related genes between *Hmgn1*^{+/+} and *Hmgn1*^{-/-} mouse cells [14]. These results suggest that the impaired UV repair is not due to a significant change in one of the components of the NER repair complex, and that the loss of HMGN does not have significant effects on the transcription levels of genes coding for these components. Most likely, the effects of HMGNs are related to their ability to induce structural changes in chromatin, the substrate of the NER factors. The chromatin structure of transiently transfected plasmids is different from that of 'native' cellular chromatin [34,35].

Therefore, the NER machinery could efficiently repair the damage to the transfected plasmids but not that to the cellular chromatin. Interestingly, D108-1 cells lacking HMGN2 were even more efficient in repairing the DNA damage than the wild-type DT40 cells in the host cell reactivation assay. To explain why the same cells had an impaired DNA repair rate in the southwestern analysis (Fig. 5), we need to stress that the host cell reactivation assay measures predominantly TCR, with a small contribution from GGR, whereas the southwestern assay quantifies mainly GGR. The simplest explanation could therefore be that the HMGN2 null cells (D108-1) have higher expression of a TCR-specific protein or proteins, which therefore do not contribute to the repair demonstrated in the CPD-removal southwestern assay, which mainly detects GGR. A recent study has shown that, during TCR, HMGN1 is recruited to the damage site by association with Cockayne syndrome A protein, which also interacts with the UV-stalled hyperphosphorylated RNA polymerase II [40]. As the recruitment of HMGN1 takes place after the incision complex is assembled, this work suggests that, in TCR, HMGN1 may be involved in establishing epigenetic conformation post-repair, or additional remodeling beyond that needed for preincision complex activation. This role of HMGN in TCR may differ from that in early chromatin unfolding, which we presume HMGNs to be involved in during the NER pathway. These possibly two different modes of action of HMGNs, which may specify their different modes of involvement in TCR and GGR, may explain the conflicting results obtained with the host cell reactivation assays and the southwestern whole genome analysis. We suggest that, in GGR, HMGNs affect the ability of the NER proteins to access and repair the damaged site in cellular chromatin.

Our findings demonstrate that the UV sensitivity of *HMGN2*^{-/-} cells is very similar to that of cells lacking *HMGN1a* and even to the double-knockout *HMGN1a*^{-/-}/*HMGN2*^{-/-} cells. The similarity in the response level is also demonstrated in the rate of CPD removal. Despite partial compensation of HMGN levels in *HMGN1a*^{-/-} cells by over-expression of HMGN2 protein, which could be detected by western blotting (Fig. 1), we could not observe an increase in UV resistance relative to the double-disrupted *HMGN1a*^{-/-}/*HMGN2*^{-/-} cells, suggesting a lack of redundancy. On the other hand D108-1 cells were somewhat less sensitive to UV in the survival curve assay, they demonstrated a lower level of apoptosis relative to the double null cells, and also had weaker cell cycle arrest at G₂-M. Although the differences in the cell survival curve and the apoptosis assay did not reach statistical significance, the overall implication from these three assays is that HMGN2 and HMGN1a also have a level of redundancy. Thus, the results indicate that HMGN1a and HMGN2 could be active in the same pathway in GGR, probably consecutively, but that they may also be capable of partially compensating for each other.

HMGN2 and HMGN1, which are nonhistone chromatin architectural proteins, form part of a growing list of chromatin modifiers found in recent years to be involved in DNA repair [10,12,41,42]. HMGA1 was reported to inhibit the removal of CPDs [43,44], and HMGB proteins inhibited cisplatin-induced DNA interstrand cross-link removal by NER [44,45] but enhanced the removal of UV-induced DNA adducts *in vivo* [46]. HMGB1 was also found to be involved in mammalian base excision repair [47] and in enhancing the initial thymine dimer levels after UV irradiation [31]. Some of the modifiers are suggested to function as chromatin accessibility factors that remodel or unfold the damaged site and make it accessible to the repair complex. These modifiers include the following: ATP-dependent chromatin-remodeling factors such as ACF [7,48]; HATs such as p300 [6], Tip60 [49], and the TFTC complex [5]; and other proteins such as Gadd45 [50] and p53 [51]. Our studies establish a role for all HMGN variants in the repair of UV-induced DNA damage.

Experimental procedures

Cells

The DT40 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The DT40-derived cell lines, null for *HMGN1a*, *HMGN2*, or both *HMGN1a* and *HMGN2*, were generated by targeted gene disruption in the laboratory of J. B. Dodgson, who gave them to us as a gift [25,26]. Cells were cultured in suspension at 37 °C under 7.5% CO₂ in DMEM (Gibco BRL; catalog number 11960-044), supplemented with 10% fetal bovine serum, 5% chicken serum, 10 µg·mL⁻¹ gentamicin, 0.5 µg·mL⁻¹ amphotericin B (Fungizone), 4 mm L-glutamine, and 50 µM 2-mercaptoethanol.

Western blotting

Whole cell lysates were resolved on 15% SDS/PAGE (for HMGN2) or on 18% SDS/PAGE (for HMGN1a and HMGN1b). Western blotting was performed using a Bio-Rad semidry transfer cell. The proteins were transferred to poly(vinylidene difluoride) membranes and detected with antibody against hHMGN2 (0.25 µg·mL⁻¹) for HMGN2, and with antibody against hHMGN1 (0.1 µg·mL⁻¹) for HMGN1a and HMGN1b. The bound antibodies were detected with secondary antibodies and an ECL kit from Amersham.

Survival after UV irradiation

Cells (1×10^6) were washed once with NaCl/P_i and plated at 0.5×10^6 cells·mL⁻¹ in 60 mm tissue culture dishes. The cells were irradiated with UVC from a 254 nm germicidal lamp while being gently agitated, at doses of 3–12 J·m⁻², resuspended in DMEM, and cultured at 37 °C under 7.5% CO₂ for 72 h; their survival was determined by a Trypan blue exclusion assay (0.2%). All experiments were performed in triplicate.

Apoptosis

Cells irradiated with a UVC dose of 6 J·m⁻² were labeled using the annexin V–FITC Apoptosis Detection Kit I (BD Pharmingen), according to the manufacturer's recommendations, with slight modifications. Briefly, 48 h following irradiation, the cells were rinsed twice with chilled $1 \times$ NaCl/P_i and centrifuged at 196 *g* at room temperature for 8 min; the pellet was then resuspended in 500 µL (1×10^6 cell·mL⁻¹) of $1 \times$ annexin V Binding Buffer. Nonirradiated cells were used as controls. Then, cells (200 µL) were stained with 10 µL of PI and 5 µL of annexin V–FITC, and incubated for 15 min at room temperature in the dark. The samples were brought to a volume of 800 µL with Binding Buffer, and run on a FACS Calibur (BD Biosciences, San Jose, CA, USA). A minimum of 10 000 cells was acquired for each sample. CELL QUEST pro software (BD Biosciences) was used for both acquisition and analysis. The experiments were performed in triplicate, and the averages were statistically analyzed by paired or independent group *t*-tests as indicated in Table 1.

Cell cycle analysis

Cells grown at 5×10^6 cells in 90 mm Petri dishes were UV-irradiated at 12 J·m⁻². Forty-eight hours after irradiation, the cells were pulsed with 20 µM BrdU for 30 min. Cells were washed with NaCl/P_i, resuspended, fixed with chilled 70% ethanol, and stored at –20 °C. For analysis, the cells were incubated with 3 mL of 2 M HCl for 30 min, and 6 mL of 0.1 M sodium borate (pH 8.5) was then added. The cells were washed twice with NaCl/P_i containing 0.5% Tween and 0.5% BSA (NaCl/P_i/T/B). The cells were stained with FITC-conjugated antibody against BrdU for 60 min at room temperature. This was followed by a 20 min treatment with 200 µg·mL⁻¹ RNase A, and overnight incubation with 20 µg·mL⁻¹ PI. Samples were run on a FACS Calibur (BD Biosciences). A minimum of 20 000 cells was

acquired for each sample. CELLQUEST software was used for both acquisition and analysis.

Western blot assays for cell cycle analysis

Cell lysates were prepared from chicken cells at various time intervals after irradiation with $12 \text{ J}\cdot\text{m}^{-2}$. The time intervals were as follows: 30 min, 4, 10, 24, 48, and 72 h; a nonirradiated control was included. The extracts were resolved on SDS/PAGE and subjected to western blotting against phospho-Chk1 Ser345 (Cell Signaling; $0.1 \mu\text{g}\cdot\text{L}^{-1}$) and phospho-H3 Ser10 (Upstate Biotech; $0.04 \mu\text{g}\cdot\mu\text{L}^{-1}$) (the gels used were 12% and 15%, respectively). The bound antibodies were detected with secondary antibodies and an ECL kit from Amersham. Standardization of protein loading was performed by Coomassie Blue staining. All experiments were performed in triplicate.

Southwestern analysis of photoproduct levels

DNA was extracted from cells at various times after UV irradiation at a dose of $12 \text{ J}\cdot\text{m}^{-2}$ and slot-blotted onto Hybond- N+ membranes (GE Lifesciences, Pittsburgh, PA, USA). The DNA was cross-linked by a 15 min incubation in an 80°C vacuum-oven. The relative levels of CPD dimers were assessed using mouse monoclonal antibody against CPD (TDM-2; gift from T. Tadokoro, Department of Dermatology, Osaka University, Japan). The relative level of DNA loaded on each blot was determined by staining with $0.5 \mu\text{g}\cdot\text{mL}^{-1}$ ethidium bromide in $1 \times$ Tris/acetate/EDTA (40 mM Tris/acetate; 2 mM disodium EDTA). The CPD/DNA ratio was determined using densitometry of the CPD blot and IMAGEQUANT software (Molecular Dynamics). The tests were performed in a linear range according to the calibration curve.

Host cell reactivation

The host cell reactivation assay was performed as previously described [32,33]. Briefly, plasmids containing the reporter gene for luciferase were irradiated with UV, at energy levels of 250, 500, 1000 and $2000 \text{ J}\cdot\text{m}^{-2}$. The wild type and various null mutant cells were transfected with $2 \mu\text{g}$ of either control or irradiated plasmid, using DMRIE-C (Invitrogen). Forty-eight hours later, the transfected cells were harvested. Extracts were examined for luciferase activity levels. Each dose was assessed as independent triplicates.

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Abbreviations

BrdU	bromodeoxyuridine
CPD	cyclobutane pyrimidine dimer
FACS	fluorescence-activated cell sorter
FITC	fluorescein isothiocyanate

GGR	global genome repair
HAT	histone acetyltransferase
NER	nucleotide excision repair
PI	propidium iodide
TCR	transcription-coupled repair

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Fig. 1. Loss of HMGN variant expression in DT40 clones with disrupted HMGN genes. (A) Western blot analysis of HMGN2 in whole cell lysates fractionated by 15% SDS/PAGE. The cell lines tested are identified at the top of each lane, and the location of the HMGN2 protein is indicated by an arrow. The bottom panel shows Coomassie blue staining of a similar gel to demonstrate equal loading of cell lysates (shown from top are the core histones H3, H2B, H2A, and H4). (B) Western blot analysis of HMGN1a and HMGN1b in whole cell lysates fractionated by 18% SDS/PAGE. The cell lines tested are identified at the top of each lane, and the locations of the HMGN1a and HMGN1b proteins are indicated by arrows. The bottom panel shows Coomassie blue staining of a similar gel to demonstrate equal loading of cell lysates (shown from the top are the core histones H3, H2B, H2A and H4).

Fig. 2. Loss of *HMGN* variants leads to UV hypersensitivity in DT40 cells. Shown are survival curves of wild-type and mutant *HMGNDT40* cells 72 h after irradiation with various doses of UV. Each data point represents the mean of three independent measurements ($\pm$ standard deviation).

Fig. 3.

Higher UV-induced apoptosis rate in *HMGN2*^{-/-} and *HMGN1a*^{-/-}/*HMGN2*^{-/-} cells. Early and late apoptosis rates were measured by annexin V and PI double staining. Wild-type DT40 cells, knockout *HMGN2*^{-/-} (clone D108-1) cells and double-knockout *HMGN1a*^{-/-}/*HMGN2*^{-/-} (clones Nh43 and Bp5) cells were irradiated with UV at 6 J·m⁻². The apoptosis rate was measured 48 h after irradiation. Shown is a dot plot of the cell population as detected by FACS and analyzed by quadrant statistics. The bottom left rectangle represents live and nonapoptotic cells, which are negative for both annexin V and PI; the bottom right rectangle represents early apoptotic cells, which are annexin-positive, but PI-negative. The top right rectangle represents late apoptotic and dead cells (annexin V-positive and PI-positive); the top left rectangle includes dead cells (only PI-positive). This experiment was repeated three times, and the averages are summarized in Table 1.

Fig. 4. UV-induced G₂-M arrest in *HMGN2*^{-/-} cells and G₂-M and mitotic arrest in *HMGN1a*^{-/-}/*HMGN2*^{-/-} cells. Cells (1×10^6 cells·mL⁻¹) were irradiated with 12 J·m⁻². Forty-eight hours later, the cells were labeled with BrdU, fixed, incubated with FITC-conjugated antibody against BrdU, stained with PI, and analyzed by FACS. The results indicate that cells lacking *HMGN2* and, to an even greater extent, cells lacking both *HMGN1a* and *HMGN2* have a lower rate of transition to S-phase after UV irradiation, and consequently show greater accumulation at G₂-M. The bar graph (B) represents the averages of three experiments such as the one depicted in the dot plots of Fig. 5A. The letters a–d indicate the columns with significant statistical differences as determined by paired *t*-test (one-tailed, *P* <

0.05). The letters e and f indicate the columns with significant statistical differences as determined by independent *t*-test (one-tailed, $P < 0.05$). (C) The wild-type cell line DT40, *HMGN2*^{-/-} cells (D108-1) and *HMGN1a*^{-/-}/*N2*^{-/-} cells (Nh43) were analyzed for the levels and kinetics of G₂-M checkpoint and mitotic checkpoint activation after UV irradiation. The cells were lysed at various time intervals after UV irradiation at 12 J·m⁻². Whole cell extracts were resolved by SDS/PAGE and analyzed by western blot. The antibody used to detect the levels of cells arrested in the G₂-M checkpoint was antibody against phospho-Chk1 Ser345. The antibody used to detect accumulation of cells in mitosis was antibody against phospho-H3 Ser10. Equal loading of proteins was demonstrated by Coomassie staining of a similar gel.

Fig. 5.

Decreased CPD removal rate in cells lacking *HMGN* variants. (A) Shown is a southwestern analysis of the CPD removal rates in the DT40 cells lacking *HMGN2* (D108-1) or both *HMGN2* and *HMGN1a* (Nh43) as compared with that of wild-type DT40 cells. DNA was extracted from cells that were not irradiated and from cells immediately after UV irradiation, and 7 and 20 h after irradiation with a dose of $12 \text{ J} \cdot \text{m}^{-2}$. One microgram of DNA was loaded per slot in a slot blot system, and transferred to a Hybond-N+ membrane. The membrane was incubated with monoclonal antibody against CPD. The CPD levels were normalized against the DNA levels by staining the membranes with ethidium bromide. Note the absence of signal in DNA samples that were not exposed to UV. The CPD/DNA ratio was determined using densitometry of the CPD blot. (B) A bar graph representing the averages ($\pm$ standard error) of three repetitions of the experiment described in (A). The CPD/DNA averages are presented as percentage of the initial level of CPD/DNA detected at time 0 (immediately after UV irradiation). DT40 cells have a significantly more efficient CPD-removal rate, 7 h and 20 h after irradiation, than D108-1 and Nh43 null cells ($P < 0.05$ by nonparametric Kruskal–Wallis test). (C) A bar graph presenting the averages ($\pm$ standard error) of the CPD/DNA ratio at time 0 after UV irradiation of three repetitions of the experiment described in (A) and (B). A nonparametric Kruskal–Wallis test showed that the wild-type DT0 cells and the null D108-1 and Nh43 cells were not statistically different from each other (all $P > 0.275$).

Fig. 6.

Intact NER of a luciferase reporter plasmid in DT40 cells lacking HMGN variants. Shown are host cell reactivation assays of wild-type DT40 cells and cells lacking HMGN variants. (A) Null D108-1 cells (*HMGN2*^{-/-}) in comparison with wild-type DT40 cells. (B) The null cell line Nh43 (*HMGN1a*^{-/-}/*HMGN2*^{-/-}) in comparison with wild-type DT40 cells. (C) The null cell line Bp5 (*HMGN1a*^{-/-}/*HMGN2*^{-/-}) in comparison with wild-type DT40 cells. Luciferase expression plasmids were irradiated with various doses of UV and then used for transfection of cells. Cell extracts prepared 48 h after transfection were examined for luciferase activity, an indicator of DNA repair potential of the cells [32]. Each point of irradiation was checked independently in triplicate.

Table 1

LD₅₀ values of UV-irradiated wild-type DT40 cells and DT40-derived null HMGN cell lines. The LD₅₀ values [in J·m)² ± standard deviation] of the wild-type DT40 cells and the derived null *HMGN2* cells (D108-1), null *HMGN1a* cells (8bsr8) and *HMGN1*^{-/-}/*N2*^{-/-} double-null cells (Bp5 and Nh43) were calculated on the basis of the experiments presented in Fig. 2 (*n* = 3).

DT40	D108-1	8bsr8	Bp5	Nh43
9.40 ± 2.33	3.69 ± 0.83 ^a	2.83 ± 0.35 ^a	3.03 ± 0.80 ^a	2.63 ± 0.51 ^a

^aSignificant difference of *HMGN* null cells from the wild-type cells as determined by nonparametric Mann–Whitney *U*-tests (*P* < 0.01).

Apoptosis levels following UV irradiation of cells lacking *HMGNs* and wild-type DT40 cells. The various cell lines were irradiated at 6 J·m⁻², and 48 h later they were double-labeled with PI and annexin V (see explanations in legend to Fig. 3 and Experimental procedures). After labeling, the cells were subjected to FACS quadrant analysis. Early apoptotic cells were positively stained with annexin V-FITC, and late apoptotic cells were positive for annexin V-FITC as well as PI (see more details in Results).

Table 2

Cell line	Apoptosis before UV irradiation (%)			Apoptosis 48 h after UV irradiation (%)		
	Early	Late	Total	Early	Late	Total
DT-40	2.9 ± 0.7	2.2 ± 0.4	5.1 ± 1.2	10.4 ± 0.9 ^a	23.3 ± 3.3 ^a	33.7 ± 3.6 ^a
D108-1	1.3 ± 0.1	3.3 ± 0.5	4.5 ± 0.5	5.2 ± 1.5 ^{a,b}	36.5 ± 1.1 ^{a,b}	41.7 ± 2.4 ^{a,b}
Nh43	2.1 ± 0.5	6.5 ± 3.4	8.6 ± 3.9	5.4 ± 0.7 ^{a,b}	42.6 ± 2.5 ^{a,b}	47.9 ± 3.1 ^{a,b}
Bp5	1.3 ± 0.3	2.5 ± 0.6	3.9 ± 0.9	6.7 ± 0.4 ^{a,b}	51.9 ± 2.0 ^{a,b}	58.6 ± 2.0 ^{a,b}

^aEarly and late phases in each cell line showed a significant difference when compared before and after UV irradiation. This difference was tested using a paired *t*-test, and was shown to be significant ($P < 0.05$).

^bThere were significant differences in early, late and total apoptosis levels after UV irradiation between null *HMGN* cell lines and the wild-type DT40 cells. These differences were tested using independent group *t*-tests, and shown to be significant ($P < 0.05$).