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A novel regulation mechanism of DNA repair by damage-induced and RAD23-dependent stabilization of xeroderma pigmentosum group C protein

Jessica M.Y. Ng,1 Wim Vermeulen,1 Gijsbertus T.J. van der Horst,1 Steven Bergink,1 Kaoru Sugasawa,3,4 Harry Vrieling,2 and Jan H.J. Hoeijmakers1,5

1MGC-Department of Cell Biology & Genetics, Centre for Biomedical Genetics, Erasmus Medical Center, Rotterdam, The Netherlands; 2MGC-Department of Radiation Genetics and Chemical Mutagenesis, Leiden University Medical Center, 2333 AL Leiden, The Netherlands; 3Cellular Physiology Laboratory, RIKEN (The Institute of Physical and Chemical Research), and 4Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Wako, Saitama 351-0198, Japan

Primary DNA damage sensing in mammalian global genome nucleotide excision repair (GG-NER) is performed by the xeroderma pigmentosum group C (XPC)/HR23B protein complex. HR23B and HR23A are human homologs of the yeast ubiquitin-domain repair factor RAD23, the function of which is unknown. Knockout mice revealed that mHR23A and mHR23B have a fully redundant role in NER, and a partially redundant function in embryonic development. Inactivation of both genes causes embryonic lethality, but appeared still compatible with cellular viability. Analysis of mHR23A/B double-mutant cells showed that HR23 proteins function in NER by governing XPC stability via partial protection against proteasomal degradation. Interestingly, NER-type DNA damage further stabilizes XPC and thereby enhances repair. These findings resolve the primary function of RAD23 in repair and reveal a novel DNA-damage-dependent regulation mechanism of DNA repair in eukaryotes, which may be part of a more global damage-response circuitry.

[Keywords: mHR23A/B; XPC regulation; ubiquitin/proteasome pathway; DNA damage response; GG-NER]

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all XPC is complexed with hHR23B (van der Spek et al. 1996a), whereas a trace amount copurifies with hHR23A (Araki et al. 2001). Only a minority of hHR23B and hHR23A is bound to XPC, suggesting that both proteins have additional functions (Sugasawa et al. 1996).

HR23 proteins contain a ubiquitin-like [UbL] N terminus and two ubiquitin-associated [UBA] domains (van der Spek et al. 1996b), suggesting a link to the ubiquitin/proteasome-mediated protein degradation pathway. The UbL domain of yeast RAD23 is important for UV survival and for interaction with the 26S proteasome (Watkins et al. 1993; Schaub et al. 1998), whereas the UBA domains enable binding to ubiquitin (Bertola et al. 2001; Chen et al. 2001; Wilkinson et al. 2001; Rao and Sastry 2002). However, the functional relationship between RAD23, NER, and the ubiquitin system is unclear. To investigate the role of HR23 in mammalian NER and other processes and the enigmatic NER–HR23–ubiquitin connection, we have generated mouse mutants for both HR23A and HR23B by gene targeting. Unlike other NER mouse mutants, mHR23B-deficient mice show a severe phenotype including impaired embryonic development and high rates of intrauterine death. Surviving animals display retarded growth, male sterility, and facial dysmorphism, but are NER-proficient (Ng et al. 2002). These data show that mHR23B is essential for normal development of the mouse and suggest an additional role of mHR23B outside NER. Apparently, this role is not or only partly compensated for by mHR23A, whereas HR23A is able to substitute for HR23B in NER. Here we report the generation and analysis of mHR23A−/− mice, the generation of mouse embryonic fibroblasts (MEFs) with a complete mHR23 deficiency, and the use of these double-mutant cells to elucidate the function of mammalian RAD23 homologs in NER.

Results

Generation of mHR23A-deficient mice and cells

To generate a mouse model for mHR23A, we created a targeting construct in which the mHR23A exons III to VI, and part of exons II and VII (encoding residues 55–288 of the mHR23A protein), were replaced by the neomycin-resistance marker. Gene targeting creates an mHR23A allele encoding a severely truncated protein in which >85% of the coding sequence is deleted (even truncating the UbL domain) and thus can be considered a null allele (Fig. 1A). Two correctly targeted clones (obtained at a frequency of 16%; Fig. 1B) were used for blastocyst injections. Heterozygous offspring from matings between germ-line chimeric males and C57BL/6 female mice were intercrossed to generate homozygous mutant mHR23A animals (Fig. 1C), as well as embryonic day 13.5 [E13.5] embryos for isolation of MEFs. Neither the mHR23A mRNA nor the 50-kD mHR23A protein could be detected in mHR23A−/− MEFs (Fig. 1D,E). We conclude that we have generated mHR23A-null mice. The two independent mouse lines were biochemically and phenotypically indistinguishable for all parameters tested, indicating that the findings presented in this study are the result of genuine mHR23A inactivation.

mHR23A−/− animals and MEFs are NER proficient

Because of the expected role of both mHR23 variants [A and B] in NER as suggested by in vitro experiments [Ma-
sutani et al. 1994) we assessed key repair parameters in mHR23A−/− MEFs. As shown in Figure 2A–C, UV survival, UV-induced unscheduled DNA synthesis (UDS), and RNA synthesis recovery after UV exposure were all in the wild-type range, indicating that global as well as transcription-coupled NER are unaffected, mimicking the situation in the mHR23B mutant [Ng et al. 2002]. These data suggest that mHR23A and mHR23B are functionally redundant for NER in vivo, extending our in vitro observations [Sugasawa et al. 1997].

In striking contrast to mHR23B−/− animals, mHR23A−/− mice were born with Mendelian frequency and appeared indistinguishable from wild-type and heterozygous littermates for all parameters tested (including morphology, main pathology, and growth rate up to at least 18 mo). mHR23A−/− male and female mice were fertile, and their mating activity and litter size were normal. Apparently, mHR23A is not essential for mouse development, and mHR23B can compensate for any additional functions of mHR23A.

Total mHR23 deficiency is incompatible with animal life

To investigate the effect of a total mHR23 deficiency, we tried to generate mHR23A−/−/mHR23B−/− animals (hereafter referred to as: DKO for “double knockout”) and to obtain corresponding MEFs, by double heterozygous matings. Remarkably, out of 427 newborns analyzed, no DKO s were found (Table 1). This indicates that inactivation of mHR23A aggravates the severe developmental defects caused by a mHR23B deficiency [Ng et al. 2002] to a level

**Figure 2.** Repair characteristics of mHR23A+/+, E13.5 and DKO E8.5 MEFs. [A] UV survival curves of primary mHR23A+/+, mHR23A−/−, and mHR23A+/− E13.5 MEFs. XPC−/− fibroblasts were included as a negative control. Cells were exposed to different doses of UV (254 nm). After 4–5 d, the number of proliferating cells was estimated from the amount of radioactivity incorporated during a 3-h pulse with [3H]thymidine. For each genotype, identical results were obtained with three other cell lines [data not shown]. [B] Global genome repair (UDS) in primary mHR23A+/+, mHR23A−/−, and mHR23A−/− E13.5 MEFs. Cells were irradiated with 16 J/m2 of UV (254 nm) and labeled with [3H]thymidine. Incorporation of radioactivity was measured by autoradiography and grain counting [average of 50 nuclei per cell line; the standard error of the mean is indicated]. XPA−/− fibroblasts were measured as a negative control. For each genotype, consistent results were obtained with three other independent cell lines [data not shown]. [C] RNA synthesis recovery (RRS) after UV exposure of primary mHR23A+/+, mHR23A−/−, and mHR23A−/− E13.5 MEFs. Cells were UV-irradiated [10 J/m2, 254 nm] and allowed to recover for 16 h. After a 1-h pulse labeling with [3H]uridine, cells were processed for autoradiography. The relative rate of RNA synthesis was expressed as the quotient of the number of autoradiographic grains over the UV-exposed nuclei and the number of grains over the nuclei of unirradiated cells [average of 50 nuclei per cell line; the standard error of the mean is indicated]. CSB−/− cells were used as a negative control. For each genotype, three other independent lines were assayed with similar outcomes [data not shown]. [D] UV survival of E8.5 MEF lines of wild-type, XPC−/−, mHR23A−/−/B−/−, mHR23A−/−/B−/−, and mHR23A−/−/B−/−/DKO. [E] UV-induced UDS in wild-type, XPC−/−, and DKO E8.5 MEFs. [F] RNA synthesis recovery after UV irradiation of wild-type, XPC−/−, and DKO E8.5 MEFs. For details for panels D–F, see legends to panels A–C, respectively, and Materials and Methods. Two independent experiments using two other DKO cell lines [before the cultures extinguished; data not shown] showed a similar effect on UDS and RNA synthesis recovery.
incompatible with life. Whereas we obtained phenotypically normal mHR23A−/−B+− mutant mice at Mendelian ratios (71/427 found and 83/427 expected), surprisingly, mHR23A+/−B−/− animals were not born (0/427). However, we were able to isolate E13.5 mHR23A+/−B−/− mutant MEFs, although they showed poor growth. Apparently, loss of even one allele of mHR23A in a completely mHR23B-null background causes lethality in embryogenesis.

To investigate embryonic lethality caused by a complete mHR23 deficiency, we isolated embryos at various stages of development. No DKO embryos were present at E13.5 and E10.5, but growth-retarded mHR23-deficient embryos were observed at E8.5. Importantly, three DKO MEF lines were isolated from E8.5 embryos (3/43; see Table 1). Compared with wild-type and double-heterozygous mutant MEFs, these cells displayed reduced rates of proliferation, which resulted in the loss of two lines. Nevertheless, we succeeded in establishing one DKO cell line after 30 wk of culturing, which permitted functional characterization of a total mHR23 deficiency.

**Table 1. Genotype analysis of DKO (mHR23A−/−B−/) embryos and offspring**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Analyzed</th>
<th>Expected (if Mendelian)</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>E8.5</td>
<td>43</td>
<td>7</td>
<td>3b</td>
</tr>
<tr>
<td>E10.5</td>
<td>14</td>
<td>1.8</td>
<td>0</td>
</tr>
<tr>
<td>E13.5</td>
<td>77</td>
<td>9.1</td>
<td>0</td>
</tr>
<tr>
<td>Newborn</td>
<td>427</td>
<td>41.4</td>
<td>0</td>
</tr>
</tbody>
</table>

-derived from different mHR23A+/−B+/− and mHR23A−/−B+/− intercrosses.

One cell line established.

mHR23 and DNA damage stabilize XPC

Totally mHR23-deficient cells show an XPC-like repair phenotype

Cell survival experiments revealed that DKO MEFs are remarkably similar to the unique NER phenotype of XPC−/− cells in terms of UV survival (Fig. 2D), deficiency of UV-induced UDS, and proficiency of RNA synthesis recovery after UV exposure (Fig. 2E,F). In contrast, MEFs retaining only one mHR23A or mHR23B allele were NER competent (Fig. 2D). Apparently, one out of four mHR23 copies is sufficient for normal NER activity.

The resemblance to the XPC−/− repair phenotype prompted us to examine the status of the XPC protein in the DKO MEFs. Interestingly, steady-state levels of XPC appeared strongly reduced in DKO MEFs compared with wild-type and mHR23A−/− (Fig. 1E) and mHR23B−/− single KO cells (data not shown), as shown by comparative immunofluorescence (Fig. 3A) and immunoblot analysis of whole-cell extracts (Fig. 3B). Thus, in the absence of both mouse RAD23 proteins, XPC is either hardly expressed at RNA or protein level, or unstable. In view of the direct physical interaction between HR23 proteins and XPC, the latter option seems most plausible.

hHR23B and hXPC-GFP rescue the UV sensitivity of DKO cells

To provide direct evidence that the XPC-like phenotype of DKO cells is specifically caused by the mHR23 defect,
we stably transfected human hHR23B cDNA into DKO MEFs. The UV sensitivity of DKO cells expressing hHR23B was only partly rescued, perhaps because of human–mouse differences (Fig. 4A). Importantly, expression of hHR23B induced an increase in the total amount of endogenous mouse mXPC, as shown by both immunoblot (Fig. 4C, lane 4) and immunofluorescence analysis (Fig. 4D).

Because the absence of mHR23 causes a strong reduction in mXPC, we reasoned that (over)expression of “exogenous” XPC might bypass the repair defect of DKO cells. Therefore, we generated double-mutant MEFs that stably express human hXPC tagged with GFP [and additional His6 and HA tags] (Fig. 4B), to allow direct observation in living cells [Houtsmuller et al. 1999]. Functionality of the hXPC-GFP was demonstrated after microin-
jection and transfection of the cDNA construct in XPC-deficient cells (data not shown). Although hXPC-GFP was undetectable by fluorescence microscopy [Fig. 4E], stable transformants [verified for the presence of hXPC-GFP cDNA by DNA blotting] had largely regained wild-type UV resistance [Fig. 4A], indicating that the repair defect was rescued. Unfortunately, our XPC antibodies [raised against the C terminus of the protein] failed to recognize the hXPC-GFP fusion protein because of interference by the C-terminal tag. However, introduction of hXPC-GFP appeared to restore endogenous mXPC levels as shown by immunoblot [Fig. 4C, lane 5] and immunofluorescence analysis [data not shown]. Apparently, hXPC-GFP has a trans-effect on mXPC stability.

To investigate the stabilizing effect of mHR23B on XPC, we cotransfected hHR23B with hXPC-GFP cDNA into DKO cells. Stably transfected clones exhibited wild-type UV resistance [Fig. 4A] and normalized levels of endogenous mXPC [Fig. 4C, lane 6, data not shown]. In contrast to MEFs expressing only hXPC-GFP, a significant fraction (>40%) of the double cotransfected cells displayed green fluorescent nuclei [Fig. 4F], which, however, decreased to <10% upon culturing. This is probably caused by a level of hXPC-GFP expression still below the detection limit in most cells, because immunofluorescence using anti-HA monoclonals revealed that most cells expressed the tagged transgene [data not shown]. Cells with green nuclei also showed bright foci, corresponding with sites containing a local high DNA concentration visible after DAPI staining (data not shown).

Figure 5. Analyses of XPC-GFP expression after UV irradiation and treatment with proteasome inhibitor CBZ-LLL. [A] Kinetic analysis of living DKO cells expressing XPC-GFP/hHR23B after exposure to 10 J/m2 of UV-C in time over a period of 30 h. Percentage XPC-GFP, the percentage of GFP-expressing fluorescent cells of the total number of cells. [B] Immunoprecipitation study of UV-irradiated DKO cells expressing XPC-GFP/hHR23B in time. XPC-GFP was immunoprecipitated (IP) from WCEs with monoclonal anti-GFP antibodies. The precipitates were analyzed by immunoblotting using polyclonal anti-XPC antiserum. Untreated WCE, and extracts from cells isolated at 45 min, 90 min, 3 h, 6 h, and 9 h after exposure to 10 J/m2 of UV-C. The amount of expressed XPC-GFP was also visualized by immunoblot analysis of the total cell lysate with anti-XPC or anti-GFP antibodies, and with a monoclonal antibody against the p62 subunit of TFIIH as a loading control [data not shown]. XPC-GFP expression was also dose-dependent (4, 8, 12, and 16 J/m2 of UV-C, data not shown). [C] Immunoprecipitation analysis of DKO cells expressing XPC-GFP/hHR23B after CBZ-LLL treatment. IPs of WCEs from cells treated with 10 µM CBZ-LLL for 45 min, 90 min, 3 h, 6 h, and 9 h, were performed as in B. XPC-GFP expression was confirmed by immunoblot analysis of the total cell lysate using anti-XPC or anti-GFP antibodies, and anti-p62 as a loading control [data not shown]. [D] Large magnification of the higher migrating XPC species (arrows) detected in the 3-h samples after UV [left, see panel B] and CBZ-LLL treatment [right, see panel C].

DNA damage causes accumulation of hXPC-GFP

The hXPC-GFP/hHR23B DKO cell line provided a convenient tool to monitor the effect of DNA damage on XPC levels and mobility in living cells. Interestingly, UV irradiation [5, 10, and 15 J/m2] strongly increased the percentage of green cells and the intensity of the GFP signal. Kinetic analysis of UV exposure revealed a time-dependent reversible accumulation of XPC-GFP in the majority of the cells [Fig. 5A]. In addition, immunoprecipitation studies using an anti-GFP antiserum showed a significant increase in time after UV irradiation of the amount of precipitated fusion protein [Fig. 5B]. Monitoring individual cells in time after UV irradiation corroborated these findings [Fig. 6A]. Because this phenomenon was specific for DKO cells transfected with hXPC-GFP/hHR23B [data not shown], these results indicate that XPC levels are responsive to UV in an HR23-dependent fashion.

To investigate whether XPC accumulation is specific for NER-type DNA damage or just stress-related, cells were exposed to different kinds of genotoxic agents. N-acetoxy-2-acetylaminofluorene (NA-AAF, 50 and 100 µM), which induces bulky adducts processed by NER, elicited a very potent UV-like response in all cells within 6–8 h [Fig. 6B]. In contrast, γ-rays [6 and 10 Gy] and mitomycin C [MMC, 1.2 and 2.4 µg/mL], inducing mainly strand breaks and interstrand cross-links, respectively [which are dealt with by other repair pathways], failed to provoke detectable XPC accumulation [data not shown]. Also, heat shock [41°C, analyzed for up to 12 h] failed to boost fluorescence. The possibility that UV and
NA-AAF evoke a general accumulation of protein was ruled out because cells expressing GFP alone do not exhibit a significant increase in fluorescence after genotoxic insults. This indicates that lesions specifically recognized by the NER pathway enhance the level of hXPC-GFP in an HR23-dependent manner.

One of the direct consequences of UV- and NA-AAF-induced DNA damage is a temporary block of transcription. To investigate whether hXPC-GFP accumulation requires transcription or is induced by a DNA-damage-independent blockage of transcription, mRNA synthesis in DKO cells expressing hXPC-GFP/hHR23B was reversibly arrested by incubation with 5,6-dichloro-1β-D-ribofuranosyl-benzimidazole (DRB, 100 μM) and α-amanitin (10 μg/mL). No induction of XPC-GFP fluorescence was observed; instead, preincubation with DRB (2–3 h) prior to UV treatment prevented UV-induced XPC-GFP accumulation (data not shown). Consistent with this result,
no enhanced XPC fluorescence was found in cells treated with the translational inhibitor cycloheximide (30 and 50 µg/mL), demonstrating the requirement for de novo RNA and protein synthesis. However, hHR23B-mediated XPC-GFP accumulation is strictly dependent on the presence of DNA damage, because in unchallenged conditions the steady-state level of XPC remains low.

**Regulation of hXPC-GFP levels involves proteasome-dependent proteolysis**

To further examine the HR23-dependent XPC stabilization, DKO cells expressing hXPC-GFP/hHR23B were incubated with the proteasomal proteolysis inhibitor N-CBZ-LEU-LEU-LEU-AL [CBZ-LLL, 5 and 10 µM; Wiertz et al. 1996]. Similar to UV irradiation and NA-AAF, all cells displayed a striking XPC-GFP accumulation in time [Fig. 6C], which was reversible upon drug removal [data not shown]. Longer exposure to CBZ-LLL further increased XPC-GFP levels, as demonstrated by immunoblot analysis [Fig. 5C]. These findings indicate that degradation of XPC-GFP occurs via ubiquitin/proteasome-dependent proteolysis and suggest that the NER-damage-specific accumulation of XPC-GFP is due to inhibition of degradation in combination with de novo mRNA and protein synthesis.

Ubiquitination is an important step in targeting proteins to the 26S proteasome for proteolysis [Pickart 2002], and Saccharomyces cerevisiae RAD23 was found to be involved in translocating ubiquitinated RAD4 [the yeast ortholog of XPC] to the proteasome [Lommel et al. 2002]. Therefore, we examined whether XPC is also subject to [poly]ubiquitination. Immunoprecipitation of XPC-GFP [using anti-GFP] and subsequent immunoblot analysis with XPC antibodies from cells exposed to UV-C light or CBZ-LLL revealed a clear time-dependent accumulation of XPC-GFP [Fig. 5B,C]. Interestingly, in addition to the expected XPC-GFP protein band, a number of slower-migrating XPC species were identified [Fig. 5D], consistent with the idea that they are [poly]ubiquitination products of XPC-GFP. Unfortunately, none of the different anti-ubiquitin anti-sera used was sensitive enough to detect those species above background, precluding unequivocal assessment of the identity of these XPC modifications.

**Application of local UV damage to hXPC-GFP-expressing cells**

Two mechanisms may explain the transient stabilization of hXPC-GFP. The binding of XPC to DNA damage per se might protect it from proteolysis. Alternatively or in addition, DNA damage may trigger a specific response such as XPC modification that inhibits degradation. To explore the mechanism by which hXPC-GFP is stabilized, we used a recently developed method for induction of DNA damage in a restricted part of the nucleus. For this purpose, a monolayer of DKO cells expressing hXPC-GFP/hHR23B was covered with a UV-light-shielded isopore polycarbonate filter (pore diameter ∼5 µm). Upon UV irradiation, only at the position of pores is UV damage induced, as detected with antibodies that specifically recognize CPD and 6–4PP lesions. These locations attract all NER proteins tested thus far, including XPC [Volker et al. 2001; W. Vermeulen, unpubl.]. Cells were fixed at different time points after UV to allow simultaneous immunostaining with antibodies against various proteins and GFP fluorescence microscopy [Fig. 7]. Nonirradiated nuclei and undamaged regions within partly irradiated nuclei serve as internal controls. Very rapidly (<2 min) after UV exposure, GFP fluorescence [data not shown] and anti-HA immunostaining revealed high local accrual of hXPC-GFP [His6HA] in part of the nuclei, which colocalized with XPA [Fig. 7A] and the p62 subunit of TFIIH [data not shown]. These findings demonstrate that in living cells, the GFP-tagged XPC protein translocates very rapidly to sites containing UV lesions. If XPC stabilization only occurs when bound to the damage, we expect an increase in fluorescent signal predominantly at the damaged sites. On the other hand, with an [additional] overall stabilization of hXPC, it is expected that in time a concomitant increase of fluorescence over the entire nucleus [in addition to the damaged area] would be observed in comparison to undamaged nuclei. The increase of hXPC-GFP [Fig. 7A] initially occurs only at the locally damaged sites, but after 2 h also in the remainder of locally damaged nuclei, a clearly higher signal is noted when compared to unexposed nuclei in the vicinity [Fig. 7B]. These findings suggest an overall intranuclear stabilization of hXPC-GFP triggered by binding to lesions.

**High levels of XPC mediate a transient enhancement of DNA repair**

To investigate the biological significance of DNA-damage-induced stabilization of XPC, we tested the DNA repair capacity [UV-induced UDS] in DKO cells expressing XPC-GFP/hHR23B prechallenged with UV light. The mean UDS level [as determined by 1 h of [H]-thymidine pulse-labeling immediately after a dose of 16 J/m²] 5 h after UV irradiation [10 J/m²] was increased 1.5-fold compared with cells assayed in parallel that were not preirradiated [Fig. 8A]. UV-induced XPC-GFP accumulation was confirmed microscopically [data not shown] just prior to the UDS assay. The increase in UDS is not derived from the additional effect of NER still dealing with lesions remaining from the first UV dose, because in a separate UDS experiment without the second UV irradiation, no significant UDS was observed [data not shown]. The 1.5-fold increase in UDS is an underestimation because not all cells respond to the UV challenge [see also Figs. 5A, 6A]. When corrected for the nonresponding cell fraction, UDS levels were more than twofold enhanced. These data suggest that UV-induced accumulation of XPC-GFP caused a concomitant increase in GG-NER. Enhanced repair by increased levels of XPC was confirmed by microinjection of XPC-GFP cDNA into homopolykaryons of wild-type human fibroblasts.
Microinjected cells expressing XPC-GFP (Fig. 8B, top right panel) exhibit a higher UDS compared with neighboring, noninjected monokaryons (Fig. 8B). Interestingly, when a cocktail of XPC-GFP and hHR23B cDNA was injected, UDS in the majority of the cells was significantly lower and injection of this cocktail appeared highly toxic (data not shown). These data indicate that large amounts of stabilized XPC (as a result of overexpressed hHR23B) may reduce cell viability and suggest that the XPC level is under a delicate control.

Discussion

Involvement and function of HR23A and HR23B proteins in NER

The aim of this work was to shed light on the enigmatic role of the two mammalian RAD23 orthologs [HR23A and HR23B] in NER and to reveal their biological impact by the generation of mHR23A and mHR23B mouse mutants. Because single mutants failed to exhibit any detectable NER defect, the most logical interpretation is that for NER, these proteins are functionally redundant despite their ~40% amino acid sequence divergence and size difference [Masutani et al. 1994]. Indeed, double-mutant cells, which we were able to establish, notwithstanding early embryonic lethality and poor growth properties during adaptation to in vitro culturing, exhibited an NER-deficient phenotype. The ability of each of the HR23 genes to rescue this defect unequivocally established their involvement and fully overlapping function in NER in vivo. DKO cells carried a selective impairment of the GG-NER subpathway and apparently normal TC-NER, as deduced from the fact that recovery of RNA synthesis after UV irradiation is unaffected. This is strikingly similar to the repair phenotype of XPC, which hitherto was unique among the NER mutants, but deviates from the S. cerevisiae RAD23 prototype mutant and its Schizosaccharomyces pombe equivalent, which carry a combined GG-NER and TC-NER deficiency [Verhage et al. 1996; Lombaerts et al. 2000]. This unresolved mammalian–yeast difference is also registered for XPC (Venema et al. 1990) and its yeast counterpart RAD4, underlining the parallels between HR23 and XPC.

HR23 proteins control proteasome-mediated degradation of XPC

The remarkable correspondence with XPC mutants prompted us to examine the XPC status in DKO cells. The virtual absence of XPC in immunoblots and immunofluorescence (Fig. 3) directly explained the HR23 phe-nocopy of XPC: in the absence of HR23, the XPC protein is apparently unstable. This function of HR23 reveals a
Figure 8. Enhanced DNA repair correlates with high levels of XPC in UV-induced UDS in DKO cells expressing XPC-GFP/hHR23B. (A) Histogram of UV-induced UDS in DKO cells expressing XPC-GFP/hHR23B. Five hours after exposure to 10 J/m² of UV-C, cells were subsequently irradiated with 16 J/m² of UV-C and labeled with [3H]thymidine for 1 h (white columns, mean of UDS level is 16 ± S.E.M. 0.6). Asterisks indicate the mean values of the UDS levels. Incorporation of radioactivity was measured by autoradiography and grain counting (130 fixed squares counted per cell line; each square represents ~30% of the nucleus surface). UV-induced UDS of wild-type (mean 17 ± S.E.M. 0.8) fibroblasts were measured as a control (black columns, mean of UDS level is 16 ± S.E.M. 0.6). A further perspective to the growing number of links emerging between RAD23/HR23 and the ubiquitin system (see above). Although our anti-ubiquitin antibodies were not sensitive enough to directly demonstrate ubiquitination of XPC, we were able to detect a minor XPC fraction migrating as discrete higher-molecular-weight protein bands in immunoblot analyses. This suggests that the steady-state levels of this type of intermediate are very low and that XPC targeted for degradation is rapidly processed.

The results derived from the CBZ-LLL experiments are also relevant in relation to controversies on the functional implications of the RAD23/NER-19S/26S proteasome connection. In the absence of proteasome function, yeast conditional 19S proteasome mutants were reported to display enhanced repair, suggesting that proteins involved in (regulation of) NER are degraded by the 26S proteasome [Lomel et al. 2000]. Consistent with this data, evidence was reported for RAD4 ubiquitination and degradation via the 26S proteasome, involving RAD23 [Lomel et al. 2002], although the specific function in DNA repair remained unresolved. In addition, in vitro studies using GST-ΔUb l rad23 [RAD23 lacking the UbL domain] indicate that the UbL domain targets [multianubiquitinated] protein substrates for proteolysis [Ortolan et al. 2000]. On the other hand, overexpression of tagged wild-type RAD23 caused stabilization of artificial substrates in vivo by preventing multianubiquitination [Chen and Madura 2002]. Physiological levels of RAD23 were speculated to facilitate substrate degradation, but might also transiently stabilize [Chen and Madura 2002].

In contrast, other investigators [Russell et al. 1999; Gillette et al. 2001] showed that the 19S regulatory complex represses NER independently of RAD23 and apparently independently of proteolysis. Finally, interactions between HR23B and the 26S proteasome were also observed in human cells [Hiyama et al. 1999]. Our studies of the HR23A/B double mutant clearly demonstrate that XPC is intrinsically unstable and is degraded by the 26S proteasome and that HR23 proteins partly stabilize XPC in vivo. Consistent with this finding, we were able to alleviate the NER defect caused by HR23 deficiency, by forced overexpression of exogenous hXPC compensating for the short half-life of endogenous XPC in the absence of HR23. The severity of the NER defect in DKO cells correlating with the low cellular XPC content suggests that XPC stabilization is the main in vivo NER function of HR23 proteins.

Effect of DNA damage on XPC/HR23

Interestingly, both UV and NA-AAF induced a dramatic accumulation of XPC-GFP [Fig. 6A,B], causing strong nuclear fluorescence within a few hours. This phenomenon clearly depends on [helix-distorting] lesions that are substrates for GG-NER and is not due to a general stress-related response or to any type of DNA damage. XPC-GFP induction occurred with grossly similar kinetics when proteasome-mediated proteolysis is inhibited, providing further evidence for the idea that protein stabilization is responsible for the XPC-GFP increase. Interestingly, very recently a twofold increase in the interaction of RAD23 with ubiquitinated proteins was observed upon treatment with the genotoxin 4-NQO [Chen and Madura 2002].

Infliction of local DNA damage [Fig. 7] revealed a very novel mechanism of NER regulation. In view of the involvement of the proteasome in XPC degradation indicated by the CBZ-LLL treatment, these observations put a functional perspective to the growing number of links emerging between RAD23/HR23 and the ubiquitin system (see above). Although our anti-ubiquitin antibodies were not sensitive enough to directly demonstrate ubiquitination of XPC, we were able to detect a minor XPC fraction migrating as discrete higher-molecular-weight species consistent with a set of [poly]ubiquitinated XPC modifications. This suggests that the steady-state levels of this type of intermediate are very low and that XPC targeted for degradation is rapidly processed.
rapid (within minutes) accumulation of XPC-GFP in the damaged regions, which is found for all NER factors and is caused by actual engagement in DNA repair events [Volker et al. 2001; Hoogstraten et al. 2002, W. Vermeulen, unpubl.]. However, the additional enhancement of XPC-GFP over the entire nucleus [2 h postirradiation] supports the argument against the possibility that only lesion-bound molecules are protected from proteolysis. It is possible that participation of XPC/HR23 in NER events triggers its stabilization and that the subsequent dissociation of XPC/HR23 from the damage [when repair is accomplished] provides a pool of modified/stabilized protein that distributes over the entire nucleus. In view of the fact that XPC is involved in the initial lesion-sensing step, the DKO cells stably expressing functional GFP-tagged XPC allow direct visualization of the effect of DNA damage in living cells.

A novel regulation mechanism of GG-NER

The level of XPC appears to modulate GG-NER. In the absence of HR23, XPC concentrations are strongly reduced, and as a consequence GG-NER is severely affected. Conversely, elevated amounts of XPC, both by UV irradiation of DKO cells expressing XPC-GFP/hHR23B and by microinjection of XPC-GFP in control cells, augmented the repair capacity by 1.5- to 2-fold [Fig. 8]. These findings are in line with the observations made in yeast, where overexpression of RAD4 confers accelerated rates of NER [Lommel et al. 2000]. The maximal twofold increase is consistent with our previous in vivo findings that under uninduced conditions, maximal NER capacity is associated with damage-dependent immobilization of 40%–45% of the ERCC1/XPF [Houts Muller et al. 1999] and TFIIH [Hoogstraten et al. 2002] NER complexes. Thus, these factors become limiting when NER stimulation exceeds approximately twofold. A unifying model for all our findings on HR23, XPC, and proteolysis is depicted in Figure 9. As the main initiator of GG-NER [Sugasawa et al. 1998], XPC constitutes an ideal focal point for the regulation of the entire pathway, which involves HR23. Absence of HR23 proteins reveals that XPC on its own is highly unstable because of proteolysis via the 26S proteasome. Under normal conditions, HR23 complex formation with XPC results in a significant reduction of XPC proteolysis and consequently in increased steady-state levels of the protein complex. This correlates with proficient GG-NER. As mentioned above, a protecting role of Rad23/HR23 via inhibition of polyubiquitination was already postulated from various other studies [Schauber et al. 1998; Lommel et al. 2000, 2002; Ortolan et al. 2000]. Under conditions of a high level of DNA damage, involvement in NER stimulates the protective role of HR23. Particularly after prolonged higher damage load, this would lead in normal cells to gradual up-regulation of XPC and consequently the entire GG-NER pathway. This rheostat model for adapting XPC levels to the amount of damage provides a novel type of regulation of DNA repair capacity in eukaryotes. Intriguingly, overexpression of exogenous hXPC costabilized endogenous mXPC at the same time (Fig. 4). One interpretation of this trans-effect is that the pathway responsible for degradation of mXPC becomes saturated by...
high production of exogenous hXPC driven by the strong SV40 promoter. This points to a quite specific proteolysis mechanism that keeps XPC concentrations low in the absence of DNA damage.

**Down-regulation of XPC**

Why are XPC levels not constitutively high? At first sight, it would seem only advantageous to allow GG-NER always to operate at maximal capacity instead of time-consuming up-regulation as revealed here for XPC. However, several observations indicate that RAD4 and its mammalian equivalent XPC are highly toxic when expressed at too high levels in homologous and heterologous systems. Even low-copy RAD4 expression in *Escherichia coli* interfered with growth, and only defective derivatives of the gene could be propagated (Siede and Eckardt-Schupp 1986, Wei and Friedberg 1998). These findings and the results of XPC microinjection (see Results) suggest that the protein interferes with a vital process, likely DNA metabolism, by its ability to bind to a wide range of (aberrant) DNA structures. XPC detects helix distortions on the basis of disrupted base pairing but also binds to regular mismatches, the normal substrate for mismatch repair (Sugasawa et al. 2001). This highlights a fundamental dilemma of damage sensors: some normally occurring DNA conformations resemble DNA lesions, which may lead to disruption of important cellular processes. Thus, titrating XPC to low levels may reduce untargeted repair interfering with other DNA transactions.

**Other targets for HR23**

Next to XPC, several other proteins have been found to associate with HR23 and may be subject to a similar mechanism of transient stabilization. An interesting HR23 binding partner is 3-methyladenine (3Me-A) DNA glycosylase [MAG or MPG], an enzyme that initiates base excision repair [BER] of a number of DNA alkylated base damages [Memisoglu and Samson 2000]. Binding of HR23 in vitro elevates the rate of excision of specific substrates [Miao et al. 2000], which opens the possibility that this branch of BER is regulated in an analogous fashion to GG-NER. Consistent with this model, the human small ubiquitin-related modifiers SUMO-1 and SUMO-2/3 physically interact with the human BER initiator thymine-DNA glycosylase and actively modulate its enzymatic turnover in base release assays [Hardeland et al. 2002]. All SUMOs are known to modify target proteins covalently by an enzymatic pathway analogous to ubiquitin conjugation [Jenzenberger and Jentsch 2002].

Moreover, multiple engagements between RAD23/HR23 and cell cycle regulation are apparent: [1] RAD23 has a partially redundant role with and binds to RPN10 in the G2/M transition [Lamberton et al. 1999]; [2] RAD23 is involved in spindle assembly and S-phase checkpoints together with the RAD23-like protein DD1 in PDS1-dependent mitotic control [Clarke et al. 2001]; and [3] RAD23, together with DSK2, has a role in spindle pole duplication [Biggins et al. 1996]. The link with spindle pole duplication was recently strengthened by the discovery of the centrosome factor CEN2 as the third component of the XPC/HR23 complex [Araki et al. 2001]. Furthermore, the damage-signaling tumor-suppressor protein p53 is partly regulated by hHR23A via inhibition of the CREB (cyclic AMP-responsive element binding) protein, which acts as a coactivator of p53 transcription [Zhu et al. 2001]. Finally, HR23 proteins themselves appear regulated in a cell-cycle-dependent manner with specific degradation during S phase [Kumar et al. 1999].

The principal mechanism for regulation of XPC and GG-NER by HR23 may also be applicable to many other HR23 partners and corresponding processes (such as cell cycle control and BER). These mechanisms represent important pathways in the total network of genomic maintenance, which might be functionally connected via HR23. A common regulatory mechanism, such as binding of HR23 to primary damage sensors (XPC and MAG), would enable coordinated control of major cellular DNA-damage-response pathways, including DNA repair, cell cycle progression, and checkpoints. This response is in its effect reminiscent (but with important mechanistic differences) to the well-known SOS system in *E. coli*. Whereas the *E. coli* SOS system mainly works via RecA/LexA regulon at the transcriptional level, the HR23-dependent regulation acts even within the posttranslational stage (for review, see Sutton et al. 2000). However, XPC transcription may also be under p53 control [Adimoolam and Ford 2002; Amundson et al. 2002], extending the parallel with the *E. coli* paradigm. In addition, the multiple engagements of HR23 proteins provide an explanation for the deviant, compared with other NER mutants, phenotype of *mHR23B*-deficient mice and embryonic lethality of *DKO*.

In conclusion, the generation of mammalian cells deficient in *mHR23* [starting from *mHR23A* and *mHR23B* single-mutant mice] allowed the elucidation of the main NER function of RAD23, revealed an ingenious DNA damage-controlled mechanism of GG-NER regulation, and clarified an important link between NER and the ubiquitin system. The multiple engagements of HR23 proteins may connect stress-response mechanisms including cell cycle control to known damage sensors like XPC. Finally, the cell line expressing GFF-tagged XPC will be useful for rapid screening and detection of genotoxic or carcinogenic agents.

**Materials and methods**

**Construction of mHR23A targeting vector**

An Ola129 mHR23A targeting construct was generated by converting the BglII site in exon II of clone pG7M23Ag1 (containing a 4-kb genomic *EcoR*I fragment subcloned in pgEM7) into a ClaI site, which (because of a ClaI site in the polylinker) allowed deletion of sequences downstream of the BglII site in exon II (clone pG7M23Ag7). Next, the remaining *EcoR*I site was

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*mHR23 and DNA damage stabilize XPC*
removed by filling in the overhangs with Klenow, resulting in clone pG7M23Ag9. After changing the BstXI site into an Sall site, the 3-kb Xbal–Sall fragment was cloned into Sall-digested pGEM5, resulting in clone pG5M23Ag17. Next, the 3’ arm of the construct, consisting of a Klenow-blunted 1.5-kb Smal-Xbal fragment starting at the Sall site in exon VII, was inserted into the blunted Ndel site of pG5M23Ag17 (giving pG5M23Ag20), followed by insertion of a Neo marker cassette in antisense orientation in the Clal site (giving pG5M23Ag24). Finally, the Nol–Nsel insert of pG5M23Ag24 was recloned into a pGEM-9ZdI-based vector containing a 2.8-kb thymidine kinase (TK) marker cassette (giving pG5M23Ag30).

**ES cell culture and transfection**

The Ola129-derived ES cell line E14 was electroporated with the mHR23A targeting construct and cultured on dishes treated with gelatin as described previously [Ng et al. 2002]. G418 (Geneticin, Gibco; final concentration, 200 µg/mL) was added 24 h after electroporation, and cells were maintained under selection for 6–8 d. Genomic DNA from G418-resistant clones was digested with BamHI and subjected to Southern blot analysis using a 0.6-kb XbaI–SacI fragment (3’ external to the construct) as a probe. Targeted clones were subsequently screened with a Neo DNA probe (Clal fragment) to confirm proper homologous recombination in the 5’ arm.

**Generation of the mHR23A−/− and mHR23A−/−/B−/− (DKO) mice and fibroblasts**

Cells from two independent targeted clones with 40 chromosomes were injected into 3.5-day-old blastocysts isolated from pregnant C57BL/6 females [Ng et al. 2002]. Male chimeraic mice were mated with C57BL/6 females to obtain heterozygous animals. Germ-line transmission was observed in the coat color of F1 offspring. Males and females heterozygous for mHR23A were interbred to generate mHR23A−/−, mHR23A+/−, and mHR23A+/+ mice. For the generation of double-mutant mHR23A/B mice, male and female animals heterozygous for both mHR23A and mHR23B [Ng et al. 2002] were interbred. Genotyping was performed by Southern blot or PCR analysis of genomic DNA from tail biopsies of 10–14-day-old pups.

Primary mHR23A MEFs (three independent lines per genotype) were isolated from E13.5 embryos obtained from matings between mHR23A−/− mice. Double-mutant mHR23A/B MEFs were isolated from E8.5 embryos derived from different crossings between mHR23A−/−/B−/− and mHR23A−/−/B−/− mice. Part of each embryo was used for genotyping, and the remaining tissue was minced and immersed in a thin layer of F10/DMEM culture medium (GIBCO-BRL) supplemented with 15% fetal tissue was minced and immersed in a thin layer of F10/DMEM buffer [25 mM Tris-HCl at pH 8.0, 1 mM EDTA, 10% glycerol, 0.01% Nonidet P-40, 1 mM dithiothreitol (DTT), 0.25 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor mix (chymostatin, leupeptin, antipain, and pepstatin A]) was added to a monolayer of MEFs. After 30 min on ice, the lysate was collected with a cell scraper and clarified by 2× centrifugation at 4°C. NP lysis buffer containing 0.3 M NaCl was added to the cell pellet and homogenized by sonication.

**RNA and protein analysis**

Total RNA was isolated from mHR23A MEFs using an RNeasy Mini Kit (QIAGEN). Twenty micrograms of total RNA was separated on a 0.9% agarose gel and transferred to Hybond-N+ membrane (Amersham Pharmacia Biotech). RNA blots were hybridized using full-length mHR23A and β-actin 32P-labeled cDNA probes.

Immunoblot analysis was performed on fibroblast extracts obtained by sonification (5 × 10⁶ cells in 300 µL of phosphate-buffered saline, PBS) or extraction. In the latter case, NP lysis buffer [25 mM Tris-HCl at pH 8.0, 1 mM EDTA, 10% glycerol, 0.01% Nonidet P-40 (NP-40), 1 mM dithiothreitol (DTT), 0.25 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor mix (chymostatin, leupeptin, antipain, and pepstatin A)] was added to a monolayer of MEFs. After 30 min on ice, the lystate was collected with a cell scraper and clarified by 2× centrifugation at 4°C. NP lysis buffer containing 0.3 M NaCl was added to the cell pellet and homogenized by sonication.

SDS–polyacrylamide gel electrophoresis was performed by loading 25–50 µg of total cellular protein per lane on 6%–8% gels. Proteins were blotted to nitrocellulose membranes [Schleicher & Schuell] and probed with polyclonal antibodies recognizing human HR23A or XPC, or with monoclonal antibodies recognizing the HA epitope (HA.11, Babco) or p62 subunit of TFIIH [3C9], kindly provided by J.M. Egly, Institut de Genetique et de Biologie Moleculaire et Cellulaire, Illkirch Cedex, France. Proteins were visualized using alkaline phosphatase-labeled proteins from the wild-type and targeted allele, respectively. Primer set 2: mHR23Bp1 (5’-GTTAGGGCATTGAAAAGAACAG-3’), mHR23Bp2 (5’-CTCAAGCTTGGTCTTACAGG-3’), and anti-sense pgk3 (5’-TAGGAGGAAGAGGATG-3’), giving 202- and 600-bp PCR fragments from the wild-type and targeted allele, respectively.

**DNA repair assays and microneedle injection**

UV sensitivity was determined as described [Ng et al. 2002]. MEF cultures were exposed to different doses of UV-C light (254 nm, Philips TUV lamp) and allowed to grow for another 3–5 d, before reaching confluence. The number of proliferating cells was estimated by scintillation counting of the radioactivity incorporated during a 3-h pulse with [3H]thymidine [5 µCi/mL, specific activity, s.a.: 50 Ci/mmol, Amersham]. Cell survival was expressed as the ratio of [3H]incorporation in irradiated and unirradiated cells.

UV-induced global genome repair was assayed using the UDS method as described [Vermeulen et al. 1994]. Cells were exposed to 16 J/m² of 254-nm UV light, allowed to recover for 16 h, labeled with [5,6-3H]uridine [10 µCi/mL, s.a.: 50 Ci/mmol], and processed for autoradiography. The relative rate of RNA synthesis was expressed as the number of autoradiographic grains over the UV-exposed nuclei divided by the number of grains over the nuclei of unirradiated cells on parallel slides.

Microneedle injection of control cells (CSRO) was performed as described previously [Vermeulen et al. 1994]. After injection of at least 50 homopolykaryons, cells were cultured for the desired time in normal culture medium before they were assayed for their repair capacity by means of UV-induced UDS.
goat anti-rabbit or peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies.

**Immunoprecipitation**

Immunoprecipitations (IP) were performed on whole-cell extracts (WCE). Cells were harvested in lysis buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM DTT, 0.25 mM PMSE, and complete protease inhibitors, Roche) containing 10 mM iodoacetamine [Sigma]. WCEs were kept on ice for 30 min and centrifuged at 4°C prior to each precipitation. Supernatants of WCE were transferred to a fresh microtub, mouse monoclonal anti-GFP antibodies [7.1 + 13.1; Roche] were added, and the mixture was incubated at 4°C for 3–4 h with mild rotation. Subsequently, 50 µL of protein-G sepharose beads (4 Fast Flow, Amersham), equilibrated with lysis buffer, were added and incubated at 4°C overnight with mild rotation. Beads were collected (5000 rpm, 1 min) and washed three times with lysis buffer at 4°C for 10 min and once with wash buffer (50 mM Tris-HCl at pH 7.5, 0.25 mM NaCl, 0.1% NP-40, 0.05% sodium deoxycholate). SDS sample buffer was added to the beads, and samples were boiled for 10 min. Immunoprecipitated samples were separated on 6% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Immunodetection was performed with rabbit polyclonal anti-human XPC or mouse monoclonal anti-GFP (7.1 + 13.1; Roche).

**Immunofluorescence labeling**

Cells were grown on glass coverslips at 60%–80% confluency. After washing twice with PBS, cells were fixed with 2% paraformaldehyde in PBS at room temperature (RT) for 10 min and permeabilized with 0.1% Triton X-100 in PBS at RT twice for 10 min. After extensive washing (three times of 5 min each) with PBS+ (PBS supplemented with 0.15% glycine and 0.5% BSA), cells were incubated with affinity-purified primary antibodies in PBS+ in a moist chamber at RT for 90 min. Immediately after exposure, the filter was removed, medium was added back to the cells, and culturing was continued. After various time periods (as indicated in the text), cells were processed for immunolabeling. To identify cells in mixtures of control and mutant fibroblasts, cells were labeled with latex beads (diameter 0.79 µm; Polybead Carboxylate Microspheres, Polysciences) added to fibroblast cultures 2 d prior to mixing of the cells. Cells were thoroughly washed in PBS (three times) before trypsinization to remove the unincorporated beads and seeded in a 1:1 ratio on coverslips and cultured for 2 d.

**Light microscopy and image analysis**

Immunofluorescent microscopy images were obtained with either a Leitz Aristoplan microscope equipped with epifluorescence optics and a PlanApo 63×/1.40 oil immersion lens or a Leica DMRBE microscope equipped with epifluorescence optics and a PL Fluotar 100×/1.30 oil immersion lens. For the detection of GFP-tagged proteins in the living cells we used a Olympus IX70 microscope equipped with epifluorescence optics and an Olympus PlanApo 60×/1.40 oil immersion lens. GFP images were obtained after excitation with 455–490 and long pass emission filter (>510 nm). Cy-3 images were obtained after excitation with 515–560 and long pass emission filter (>580 nm).

**Generation of XPC-GFP fusion cDNA construct and cotransfection studies**

Full-length human XPC cDNA (ScaI-Asp7181 fragment) was cloned in an EcoRI–Asp7181-digested eukaryotic expression vector pEGFP-N3 (Clontech) containing a 3’-histidine-hemagglutinin tag (generated by insertion of a double-stranded oligonucleotide in SspI–NotI-digested pEGFP-N3; kindly provided by D. Hoogstraten, Erasmus Medical Center, Department of Cell Biology and Genetics, Medical Genetics Center, Rotterdam, The Netherlands). For simplicity, the resulting tagged cDNA construct hXPC-EGFP–His6-HA–N3 is referred to as hXPC-GFP.

Full-length cDNAs of the hHR23B (in a pSLM vector; Pharmacia Biotech) and hXPC-GFP were cotransfected into DKO MEFs using puromycin as a selectable marker. The transfection was performed using SuperFect Transfection Reagent (QIAGEN); puromycin was added 24 h after transfection to a final concentration of 1 μg/mL, and the cells were maintained under selection for 20–40 d. Stable puromycin-resistant clones were isolated, and integration of the cDNA construct was confirmed by DNA blotting (data not shown).

**Exposure of cells to DNA-damaging agents**

Cells stably expressing hXPC-GFP/hHR23B were rinsed with PBS, exposed to UV-C light (254 nm; Philips TUV lamp, dose as indicated in the text), and subsequently cultured at 37°C for various time periods (as indicated in the text). XPC was detected either by immunoblot analysis or by visualization in living cells using fluorescence microscopy. A similar approach was used to study the effect of N-acetoxy-2-acetylaminofluorene (NA-AAF, final concentration 50 or 100 µM), mitomycin C (MMC; Sigma, final concentration 1.2 or 2.4 µg/mL), ionizing radiation (γ-rays from a 137Cs source, single dose of 6 and 10 Gy), the proteasome inhibitor N-CBZ-LEU-LEU-LEU-AL (CRZ-LLL; Sigma, final concentration 5 or 10 µM), the transcription inhibitors 5,6-dichloro-1β-D-ribofuransyl-benzimidazole (DBR; Sigma, final concentration of 100 µM, 2–3 h) and α-amanitin [Sigma, final concentration 10 µg/mL, 1–3 h], the translation inhibitor cycloheximide (CHX; Boehringer, final concentration 30, 50, and 100 µg/mL, 1–3 h), and heat shock (39.5°C and 41°C, for 2–12 h).

Local UV irradiation was obtained by covering cells grown on glass coverslips with an isopore polycarbonate filter with pores of 5.0 µm diameter (Millipore, TMTP) during UV irradiation (4 × 16 J/m2 of UV-C). Immediately after exposure, the filter was removed, medium was added back to the cells, and culturing was continued. After various time periods (as indicated in the text), cells were processed for immunolabeling.

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A novel regulation mechanism of DNA repair by damage-induced and RAD23-dependent stabilization of xeroderma pigmentosum group C protein

Jessica M.Y. Ng, Wim Vermeulen, Gijsbertus T.J. van der Horst, et al.

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