Molecular and Cellular Pathobiology

Metastasis Suppressor NM23-H1 Promotes Repair of UV-Induced DNA Damage and Suppresses UV-Induced Melanogenesis

Stuart G. Jarrett1, Marian Novak1, Sandrine Dabernat3, Jean-Yves Daniel3, Isabel Mellon2, Qingbei Zhang1, Nathan Harris1, Michael J. Ciesielski4, Robert A. Fenstermaker4, Diane Kovacic5, Andrzej Slominski5, and David M. Kaetzel1

Abstract

Reduced expression of the metastasis suppressor NM23-H1 is associated with aggressive forms of multiple cancers. Here, we establish that NM23-H1 (termed H1 isoform in human, M1 in mouse) and two of its attendant enzymatic activities, the 3′–5′ exonuclease and nucleoside diphosphate kinase, are novel participants in the cellular response to UV radiation (UVR)–induced DNA damage. NM23-H1 deficiency compromised the kinetics of repair for total DNA polymerase–blocking lesions and nucleotide excision repair of (6–4) photoproducts in vitro. Kinase activity of NM23-H1 was critical for rapid repair of both polychromatic UVB/UVA-induced (290–400 nm) and UVC-induced (254 nm) DNA damage, whereas its 3′–5′ exonuclease activity was dominant in the suppression of UVR-induced mutagenesis. Consistent with its role in DNA repair, NM23-H1 rapidly translocated to sites of UVR-induced (6–4) photoproduct DNA damage in the nucleus. In addition, transgenic mice hemizygous-null for nm23-m1 and nm23-m2 exhibited UVR-induced melanoma and follicular infundibular cyst formation, and tumor-associated melanocytes displayed invasion into adjacent dermis, consistent with loss of invasion-suppressing activity of NM23 in vivo. Taken together, our data show a critical role for NM23 isoforms in limiting mutagenesis and suppressing UVR-induced melanogenesis. Cancer Res; 72(1): 133–43. ©2011 AACR.

Introduction

Metastasis suppressor genes inhibit multiple steps of metastasis in human cancer (1, 2). The first metastasis suppressor gene to be described was nm23-m1, initially identified by its diminished expression in metastatic melanoma and breast carcinoma cell lines (3). Although metastasis suppressor function of nm23-h1 was validated in multiple settings (4, 5), its contribution to suppression of tumor initiation and progression is not well understood. Low NM23 expression in primary melanomas is correlated with poor clinical outcome, however, suggesting relevance of NM23 deficiency to initiation and/or progression in earlier stages of this tumor (6–8).

NM23-H1 possesses nucleoside diphosphate kinase (NDPK) activity, which maintains balance in intracellular nucleotide pools through transfer of γ-phosphates between nucleoside triphosphates and diphosphates (9). NM23-H1 also exhibits histidine protein kinase (hisK) activity (10), with the kinase suppressor of ras representing a relevant substrate (11). Nuclease activity was also described for the NM23-H2 isoform (12, 13), followed by our characterization of a 3′–5′ exonuclease function for the NM23-H1 isoform (14). 3′–5′ exucleases execute stepwise excision of damaged or mispaired nucleotides during DNA replication and repair (15), suggesting a possible role for NM23-H1 in these processes as well. We recently showed that ablation of the nm23 homolog in the yeast Saccharomyces cerevisiae, ynk1, results in compromised DNA repair and increased mutation rates in response to UV radiation (UVR) and etoposide treatment (16). More recently, we showed that mutations disrupting the 3′–5′ exonuclease activity of NM23-H1 result in reduced metastasis suppressor capacity in vivo (17). These observations suggest that loss of NM23-H1 expression may drive the genesis and progression of melanoma. Herein, 3′–5′ exonuclease and kinase activities of NM23-H1 are shown to be required for DNA repair and maintenance of genomic fidelity in human melanoma and mouse cells. In addition, transgenic mice harboring hemizygous deficiencies in the nm23-m1 and nm23-m2 genes are susceptible to UVR-induced melanogenesis, consistent...
with a key role for NM23 proteins in repair of UVR-induced DNA damage.

Materials and Methods

Cell culture, stable transfection, and UVR exposure

The human melanoma cell line WM793 is derived from a vertical growth phase (VGP) lesion (18, 19). WM793 and stably transfected variant lines were cultured in MCDB media (Sigma) supplemented with 2 mM CaCl2, 2.5 μg insulin and 2% fetal calf serum (Invitrogen). Procedures for stable transfection of melanoma cell lines have been described previously (17).

DNA short tandem repeat profiling (Genetica DNA Laboratories) of the WM793 cells used in this study confirmed identity at 9 genetic loci, including the sex identity locus amelogenin, with the WM793 profile established by the American Type Culture Collection.

Mouse embryo fibroblasts (MEF) were isolated from C57BL/6 mouse embryos (13.5 day gestation), as well as syngeneic strains harboring either a single inactivating deletion in the nm23-m1 locus alone (M1 is the mouse homolog of the human H1 isoform; (20) or a tandem deletion of both the nm23-m1 and nm23-m2 loci (21)). MEFs were cultured in Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 10% fetal calf serum, 50 μg/mL penicillin, and 50 μg/mL streptomycin (Invitrogen). Melanocyte cultures were established from the epidermal skin layer from day 4 neonatal mice and cultivated as previously described (22). UVR was delivered to cell cultures with lamps (UVP) emitting a spectral output in the UVB/A (60% UVB, 290–320 nm; 40% UVA, 320–400 nm; <1% UVC, 250–290 nm) or UVC (254 nm) range.

DNA damage and repair assays

Cells were irradiated with either 15 J/m² UVB/A or 5 J/m² UVC following 24 hours in reduced serum medium (0.5%). Presence of UVR-induced DNA polymerase–blocking lesions was assessed using XL-PCR, which detects the total of a variety of polymerase-blocking lesions, such as base modifications, photoproducts, abasic sites, and strand breaks (23). Repair of a 10.4-kb fragment of the hprt gene was assessed by PCR as described (23). Efficiency of PCR amplification between damage and control samples was used to calculate repair at each time point as described (16). Removal of pyrimidine (6–4) pyrimidine photoproducts (6–4 photoproducts) and cyclobutane pyrimidine dimers (CPD) was measured by immunoslot blot assay (24). Equal loading of DNA was confirmed by 4,6-diamidino-2-phenylindole (DAPI; Invitrogen) staining (25).

Localized UVR exposure, in situ detergent extraction, and immunofluorescence

A clone of the WM793 melanoma cell line (793H1-FL8) was created to express NM23-H1 with a C-terminal 3X FLAG peptide sequence by stable transfection, as described (17). To achieve localized UV irradiation, cells were grown on plastic chamber slides (Lab-Tek), media was aspirated, and UVC (50 mJ/m²) applied through sterile UV-absorbing polycarbonate with 5-μm pores (Millipore; ref. 26). The membrane was removed and cells were either processed immediately, or medium was replaced and DNA repair allowed for the indicated periods. Cell extraction was carried out in situ by 2 washes of 0.1% Nonidet P-40 for 10 minutes on ice, followed by fixation in 4% paraformaldehyde for 10 minutes at 4°C. Antibodies directed to FLAG (anti-FLAG; Sigma), (6–4) photoproducts (anti-(6–4) photoproduct; Cosmo. Bio.), and secondary antibodies conjugated to DyLight Flours (DyLight 549 and 488; Jackson ImmunoResearch) were used for immunodetection. Fluorescence images were obtained using a Leica SP5 inverted confocal laser scanning microscope.

Mutation frequency in the hprt gene

Acquired resistance of cells to 6-thioguanine (6-TG) is conferred primarily by mutations within the hprt locus (27) and quantified as the number of 6-TG-resistant (6-TG r) colonies obtained after selection. Frequencies of spontaneous and UVR-induced hprt mutations in WM793-derived cell lines expressing wild-type and mutant variants of NM23-H1 were measured as described (27, 28). WM793-derived cell lines were seeded at 100 cells per well in a 6-well plate, with each line plated with 12 replicates, 6 of which were used for analysis of colony-forming efficiency and 6 for characterization of mutation spectra (Supplementary Methods). Cells were exposed to either UVB/A (5 J/m²) or sham treated, then grown in complete MCDB medium supplemented with 40 μmol/L 6-TG. Colonies were counted at 28 days following initial treatment, with colony-forming efficiency derived as the number of 6-TG-resistant colonies as a percentage of initial plating density.

UVB/A radiation of mice and melanoma surveillance

Protocols for use of mice were approved by the Institutional Animal Care and Use Committee at the University of Kentucky (Protocol 00801M2004). Parental C57BL/6 (n = 15) and the strain harboring hemizygotic deletion of the nm23-m1 and nm23-m2 loci (m1 / m2 ; n = 12) were subjected to an erythematous exposure of UVB/A radiation at postnatal day 5. A single dose of 4.000 J/m² was administered with lamps emitting a spectral output in the 290 to 400 nm range (UVB/A; UV). Irradiated mice displayed skin reddening and occasional superficial desquamation, but without morbidity or mortality. Tumor incidence and growth rate were monitored weekly, with caliper measurements (29) initiated at first appearance of a raised lesion. Mice were euthanatized 12 months post-UVR treatment and skin lesions analyzed by standard histopathology and immunohistochemical methods. Specimens were stained for either melanin using the Fontana–Masson procedure (American MasterTech) or dopachrome tautomerase (DCT) using the antibody anti-PEP8 (1:200; generously provided by Vince Hearing, National Cancer Institute) and developed with the Vectastain ABC kit with ImmPACT NovaRED (Vector Laboratories). Genotyping was done using published protocols (21).

Statistical analyses

Values are reported as means ± SEM. All analyses, including Fisher exact test, Student t test, and 2-way ANOVA, were conducted with SPSS software (SPSS Inc).
Results

NM23 expression promotes repair of UVR-induced DNA lesions in transformed and nontransformed cells

Impact of NM23-H1 expression on repair of UVR-induced DNA damage was measured initially in the NM23-deficient, VGP-derived melanoma cell line WM793 (19). Low NM23 expression was confirmed by comparison with multiple melanoma and breast cancer cell lines representing various stages of progression (Fig. 1A). The impact of nm23 gene ablation on DNA repair was also assessed in MEFs obtained from mice harboring either a single inactivating deletion in the nm23-m1 locus alone (m1 is the mouse homolog of the human h1 isoform) or a tandem deletion of both the nm23-m1 and nm23-m2 loci (Fig. 1B).

XL-PCR assay showed slow DNA repair after UVB/A (Fig. 1C) and UVC (Fig. 1D) exposure of vector-transfected WM793 cells (t1/2 ~ 1 hour), with forced expression of wild-type NM23-H1 accelerating repair (t1/2 ~ 25 minutes; P ≤ 0.05). MEFs bearing a homozygous deletion of the nm23-m1 locus exhibited delayed repair of lesions obtained from UVB/A (Fig. 1E) and UVC (Fig. 1F) exposure (t1/2 ~ 4.3 and 4.5 hours, respectively) compared with MEFs from parental C57BL/6 mice (t1/2 ~ 1.4 and 2.3 hours, respectively). Tandem deletion of nm23-m1 and nm23-m2 (21) resulted in an additional delay, up to approximately 5.2 hours post-UVC exposure (P ≤ 0.05). These data indicated an essential contribution for the H1/M1 isoform of NM23 in the early repair response to UVR-generated DNA damage, and probably, an additional contribution from the H2/M2 isoform.

NM23-H1 and the mouse homolog NM23-M1 promote nucleotide excision repair-mediated removal of (6–4) photoproducts

Nucleotide excision repair (NER) is the principal pathway for repair of UVR-generated (6–4) photoproducts and CPDs (30).

Figure 1. NM23 deficiency compromises UVR-induced DNA repair in WM793 melanoma cells and MEFs. A, relative NM23 protein expression was examined in multiple melanoma and breast cancer cell lines and B, MEFs from the parent C57BL/6, m1+/+ or m1−/−: m2−/− strains. C, UVB/A (15 J/m²) or D, UVC (5 J/m²)-induced DNA damage repair was assessed in WM793 variants without (vector) or with NM23-H1 overexpression (+H1). E, UVB/A-induced or F, UVC-induced DNA damage repair was determined in MEFs from the parent C57BL/6, m1+/+ or m1−/−: m2−/− strains. Values not sharing a common symbol are significantly different to control as determined by 2-way ANOVA (P < 0.05; n = 3–4). BrCa, breast cancer.
repair of these UVR-induced lesions, we measured removal of (6–4) photoproducts and CPDs by an immunoblot approach (24). (6–4) photoproduct repair was relatively slow in control-transfected WM793 cells (t1/2 ~ 4.7 hours; Table 1 and Fig. 2A), and significantly accelerated by forced NM23-H1 expression (t1/2 ~ 1.5 hours; P < 0.05). MEFs obtained from C57BL/6 mice (Table 1 and Fig. 2B) exhibited significantly faster repair (t1/2 ~1.7 hours; P < 0.05) than MEFs harboring either a single homozygous deletion of the nm23-m1 locus (Table 1; t1/2 ~4.3 hours; P < 0.05) or concurrent homozygous deletions of both the nm23-m1 and nm23-m2 loci (t1/2 ~4.8 hours; P < 0.05). Melanocytes isolated from mice with hemizygous deficiency at both the m1 and m2 loci (Table 1 and Fig. 2C) also exhibited reduced repair of (6–4) photoproducts compared with wild-type melanocytes (t1/2 ~0.9 hours vs. 4 hours; P < 0.05; Table 1 and Fig. 2C). CPD repair was much slower than that of (6–4) photoproducts in all cell types (Supplementary Fig. S1A–C), and NM23-H1 status had no impact on the rate of CPD removal.

3′–5′ exonuclease and kinase activities of NM23-H1 are required for prompt repair of UVB/A-induced DNA damage

To measure relative contributions of the 3′–5′ exonuclease and kinase activities of NM23-H1 to repair of DNA lesions induced by UVB/A, a panel of WM793-derived cell lines stably expressing enzymatically defective variants of NM23-H1 was employed (Supplementary Fig. S2). These cell lines consisted of mixed transfant populations (10–100 independent colonies per cell line) to minimize effects of clonal phenotypic variation (14, 17). The NM23-H1 mutants consisted of E5A, which is deficient in 3′–5′ exonuclease activity but exhibits normal NDPK and hisK function (EXO-, kinase+); K12Q, which is defective in both activities (EXO-, kinase-); P96S, which is partially kinase deficient (EXO-/-, kinase-/-); and H118F, which exhibits total loss in kinase activity but normal 3′–5′ exonuclease activity (EXO+, kinase-/-).

As determined using the XL-PCR assay, each of the mutants exhibited some degree of delayed repair of UVB/A-induced damage relative to wild-type NM23-H1 (Table 1). The 3′–5′ exonuclease–deficient mutants E5A (t1/2 ~ 51 minutes; Supplementary Fig. S3A) and K12Q (t1/2 ~ 62 minutes; Supplementary Fig. S3B) displayed delayed repair relative to wild-type NM23-H1 over the first 3 hours of repair. The kinase-deficient mutants P96S (t1/2 ~ 33 minutes; Supplementary Fig. S3C) and H118F (t1/2 ~ 49 minutes; Supplementary Fig. S3D) exhibited delayed repair that was less marked than with E5A and K12Q, but significantly different from wild-type. These data strongly suggest contributions of both the 3′–5′ exonuclease and kinase functions of NM23-H1 to repair of UVB/A-induced lesions.

<table>
<thead>
<tr>
<th>Table 1. NM23 deficiency compromises UVR-induced DNA repair</th>
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<tbody>
<tr>
<td><strong>Polymerase-blocking lesions</strong></td>
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<tr>
<td></td>
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<tr>
<td>Repair half-time, minutes</td>
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<tr>
<td>Melanocytes</td>
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<tr>
<td>C57BL/6</td>
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<td>M1+/-:M2+/-</td>
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<tr>
<td>MEFs</td>
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<td>C57BL/6</td>
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<tr>
<td>M1+/-:M2+/-</td>
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<tr>
<td>M1+/-:M2-/-</td>
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<tr>
<td>WM793 melanoma</td>
</tr>
<tr>
<td>Vector</td>
</tr>
<tr>
<td>Wild-type H1</td>
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<tr>
<td>E5A (kin-:exo-)</td>
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<tr>
<td>K12Q (kin-:exo-)</td>
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<tr>
<td>P96S (kin-:exo-)</td>
</tr>
<tr>
<td>H118F (kin-:exo-)</td>
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</table>

*Repair efficiencies are expressed as time taken in minutes to repair 50% of initial damage and expressed as mean ± SEM from at least 3 replicate measurements.

b, n.d. not determined.

*Means within a cell type not bearing a common superscript are significantly different (P < 0.05), as determined by 2-way ANOVA and mean separation.
repair in the kinase-deficient mutants K12Q (t1/2 ~ 72 minutes; Supplementary Fig. S4B), P96S (t1/2 ~ 57 minutes; Supplementary Fig. S4C), and H118F (t1/2 ~ 49 minutes; Supplementary Fig. S4D) relative to wild-type NM23-H1—transfected cells were observed (Table 1), indicating involvement of the kinase activity in NER proficiency. Repair activity of the E5A variant (t1/2 ~ 38 minutes) was not different from that of wild-type NM23-H1, however, suggesting lack of involvement of the 3’–5’ exonuclease function. Direct quantification of UVC-induced (6–4) photoproducts by immunoslot blot analysis again revealed the 3’–5’ exonuclease–deficient mutant, E5A was fully active in DNA repair (Supplementary Fig. S5A), whereas the kinase-deficient mutants, K12Q (Supplementary Fig. S5B), P96S (Supplementary Fig. S5C), and H118F (Supplementary Fig. S5D) exhibited delayed repair relative to wild-type NM23-H1 during the early time points of the DNA repair response. NM23 expression did not regulate expression of a spectrum of NER-relevant proteins in the WM793-derived lines (Supplementary Fig. S6). Taken together, these results indicated that the kinase activity of NM23-H1, but not the 3’–5’ exonuclease, contributes significantly to NER-mediated removal of UVR-induced (6–4) photoproducts, and strongly suggest that the effect is not mediated indirectly by regulating expression of participants in the NER response.

**UVR-induced nuclear translocation and recruitment of NM23-H1 to local sites of (6–4) photoproducts**

Immunolocalization studies were conducted to verify that NM23-H1 was recruited directly to (6–4) photoproduct lesions, coincident with its enhancement of (6–4) photoproduct removal (Fig. 3). To induce highly localized production of (6–4) photoproducts, UVC-irradiation was applied to 793H1-FL8 cells through UV-blocking Millipore filters containing 5 μm pores. After removing soluble NM23-H1 proteins by *in situ* detergent extraction, sequential application of anti-FLAG and anti-(6–4) photoproduct antibodies revealed rapid colocalization of NM23-H1 with sites of nuclear (6–4) photoproduct enrichment. Colocalization of FLAG-H1 and (6–4) photoproducts was apparent at 5 minutes of the UVC treatment, reached a maximum at 1 hour, and was dissipated by 12 hours. This
time course closely paralleled that of NM23-H1–assisted disappearance of (6–4) photoproducts in the immunoslot blot assay. These results showed that NM23-H1 is rapidly recruited to sites of UVR-induced DNA damage and strongly suggested its direct participation in the early DNA damage repair response.

**NM23-H1 suppresses spontaneous and UVB/A-induced mutations**

Having shown both the necessity of NM23-H1 expression for prompt repair of UVR-induced lesions and its rapid recruitment to sites of DNA damage, studies were next conducted to determine the extent to which the NM23 protein suppresses mutagenesis. Rates of spontaneous and UVR-induced mutagenesis within the *hprt* locus were quantified for the WM793 cell line panel and MEFs harboring a homozygous deletion of the *nm23-m1* locus, using the 6-thioguanine-resistance (6-TG) colony formation assay (27, 31). In the absence of UVR exposure, colony formation was reduced 10-fold by forced NM23-H1 expression (*P* ≤ 0.05; Fig. 4A). The 3′–5′ exonuclease–deficient mutants E5A and K12Q were also less effective than the wild-type protein in suppressing *hprt* mutations, with a 5-fold elevation in colony formation (*P* ≤ 0.05), whereas P96S and H114F did not differ from wild type. In a similar fashion, MEFs expressing *nm23-m1* had a reduction colony formation compared with lines harboring an *nm23-m1* deletion (3.5-fold; *P* ≤ 0.05; Fig. 4B). Exposure to UVB/A resulted in increased colony formation in all cell lines relative to that seen in basal conditions. Forced NM23-H1 expression suppressed the UVR-induced increase in colony formation compared with the vector-transfected control (4-fold; *P* ≤ 0.05; Fig. 4C). E5A and K12Q both exhibited reduced suppressor activity, with colony formation not significantly differing from NM23-deficient control cells. In contrast, colony formation in cells expressing P96S and H114F equaled that seen with wild-type NM23-H1. As predicted, higher efficiencies of colony formation were seen in MEFs harboring an *nm23-m1* deletion (4-fold; *P* ≤ 0.05; Fig. 4D). These data indicated that NM23-H1 deficiency confers increased mutagenic potential under basal conditions and following UVR exposure and indicated that the 3′–5′ exonuclease activity of NM23-H1 is the predominant function required to suppress UVB/A-generated mutations.

To obtain mutational spectra within the impacted *hprt* locus (Table 2), 15 individual 6-TG<sup>+</sup> colonies were cloned from control- and NM23-H1–derived cell lines (spontaneous and UV-induced), followed by extraction of chromosomal DNA and sequencing between nucleotide positions 296 and 1,051 (exons 2–9). Forced expression of NM23-H1 resulted in a 46% reduction in total spontaneous mutations and a 55% reduction in UVR-induced mutations. Single-base substitutions predominated in both cell lines, with higher rates of spontaneous and UVR-induced transitions and transversions in colonies derived from the vector-transfected control. Interestingly, C>T and T>C substitutions characteristic of UVR-induced damage occurred at higher frequencies in those clones.

**NM23-H1 inhibits progression of WM793 melanoma cells to growth factor independence**

WM793 is a relatively invasive cell line derived from a VGP melanoma but exhibits little metastatic potential when explanted in immunocompromised mice (17). Serial passaging in growth factor–free culture medium, however, eventually gives rise to colonies capable of autonomous proliferation (32), a well-recognized phenotype of metastatic cells. As this process requires multiple mitotic events and is likely to be driven by genomic instability, it was adapted as a novel assay for malignant progression.

All lines displayed similar growth rates during the adaptation cycle (data not shown) and after the first growth period in protein-free medium (Supplementary Fig. S7A; passage 1). After a total of 18 weeks of selection, colony formation and growth were accelerated significantly in parent WM793 cells, consistent with progression to growth factor–independent survival and proliferation. Forced expression of NM23-H1 significantly retarded colony development, strongly suggesting suppression of mutations required for growth factor independence. The mutant K12Q deficient in both 3′–5′ exonuclease and NDPK activity failed to suppress colony development (Supplementary Fig. S7A; passage 2), whereas the E5A, P96S,
and H118F mutants displayed intermediate phenotypes (Supplementary Fig. S7B; passage 2). Immunoblot analyses verified persistent forced NM23-H1 expression in the selected cell lines, with the exception of the wild-type protein that reverted to reduced levels, suggesting negative selection against it (Supplementary Fig. S7C). Failures of the 3′–5′

Figure 4. The 3′–5′ exonuclease activity and NDPK activities of NM23-H1 are necessary for preventing both spontaneous and UVR/A-induced mutations. Cell lines were either mock-treated or exposed to UVR/A (5 J/m²), with colony-forming efficiency determined at 28 days post-6-TG treatment as described in Materials and Methods. A, spontaneous mutations in NM23-H1-deficient human melanoma-derived cell line (WM793) and a panel overexpressing either wild-type NM23-H1 or enzymatic mutant variants (E5A, K12Q, P96S, and H118F) and B, MEFs from the parent C57BL/6 or m1/C0/C0 strains. C, UVR-induced mutations in NM23-H1-deficient human melanoma-derived cell line (WM793) and a panel overexpressing either wild-type NM23-H1 or enzymatic mutant variants (E5A, K12Q, P96S, and H118F) and D, MEFs from the parent C57BL/6 or m1/C0/C0 strains. Values not sharing a common superscript are significantly different as determined by Fisher exact test (P < 0.05; n = 5).

Table 2. Mutation spectra of spontaneously and UVR-induced 6-TG clones

<table>
<thead>
<tr>
<th>Class of mutation</th>
<th>Spontaneous</th>
<th>UVR</th>
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<tr>
<td></td>
<td>Vector</td>
<td>+ H1</td>
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<tr>
<td>Base substitutions</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>Transitions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C&gt;T</td>
<td>3.2 ± 1</td>
<td>0.9 ± 0.5</td>
</tr>
<tr>
<td>A&gt;T</td>
<td>1.6 ± 0.3</td>
<td>0.2 ± 0.3</td>
</tr>
<tr>
<td>G&gt;C</td>
<td>1.6 ± 0.3</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>T&gt;C</td>
<td>0.9 ± 0.6</td>
<td>0.7 ± 0.4</td>
</tr>
<tr>
<td>Transversions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T&gt;G</td>
<td>4.2 ± 0.7</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>C&gt;A</td>
<td>3.9 ± 1</td>
<td>0.6 ± 0.8</td>
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<tr>
<td>G&gt;A</td>
<td>1.3 ± 0.2</td>
<td>0.4 ± 0.4</td>
</tr>
<tr>
<td>Tandem</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC&gt;TT</td>
<td>4.2 ± 2</td>
<td>3.3 ± 5</td>
</tr>
<tr>
<td>Frameshifts</td>
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<tr>
<td>(1-2 bp)</td>
<td>10.4 ± 4</td>
<td>8.5 ± 6</td>
</tr>
<tr>
<td>Others</td>
<td>3.9 ± 4</td>
<td>3.0 ± 5</td>
</tr>
<tr>
<td>Total</td>
<td>51 ± 5</td>
<td>27.7 ± 5</td>
</tr>
</tbody>
</table>

*Average number of mutations to occur per clone between positions 296–1,051 bp of the hprt gene (n = 15 per group).
exonuclease- and NDPK-deficient mutants to resist progression to growth factor independence are consistent with antimutator functions of these enzymatic activities of NM23-H1. Colonies obtained prior to growth factor independence, had elevated levels of endogenously generated 8-OHdG, were observed in NM23-H1–deficient cells and the K12Q variant which lacks both the 3′–5′ exonuclease and kinase functions. After acquisition of growth factor independence, all mutant variants of NM23-H1 (E5A, K12Q, P96S, and H118F) showed elevated 8-OHdG content relative to the NM23-H1–expressing cells (Supplementary Fig. S8A and B). No evidence of NM23-H1–dependent alterations in gross chromosomal structure (Supplementary Fig. S9A–D) or microsatellite instability (Supplementary Fig. S10A–D) was obtained. Overall, these data further supported an essential role for the 3′–5′ exonuclease and kinase functions of NM23-H1 in reducing accumulation of DNA damage contributing to malignant progression.

**NM23 deficiency sensitizes mice to UVB/A-induced melanomagenesis**

Our cell culture–based studies indicated that NM23 proteins contribute to repair of UVR-induced DNA damage and suppression of both spontaneous and UVR-induced mutations. To determine the extent to which NM23 deficiency in vivo could confer increased susceptibility to UVR-induced DNA damage and tumorigenesis, the mouse strain harboring a tandem, hemizygous deletion of the nm23-m1 and nm23-m2 loci (m1/C0/++; m2/C0/++) was subjected to an erythematous exposure to UVB/A irradiation at postnatal day 5. The homozygous-null condition is lethal in late embryonic and early neonatal development and could not be analyzed. UVB/A-irradiation resulted in appearance of melanocytic papules on the tails of almost all (11/12) m1/C0/++; m2/C0/++ mice (Fig. 5A). Tumors appeared at a median age of approximately 5 months of age (Fig. 5B), reaching maximum sizes of 10 to 90 mm3 (1–3 per mouse) by the 12-month termination point of the study. No lesions or

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**Figure 5.** NM23 expression suppresses UVB/A-induced melanomagenesis in vivo. The parental C57BL/6 and hemizygous-null m1/C0/++; m2/C0/++ mouse strains (15 and 12 mice per strain, respectively) were exposed to a single neonatal erythematous dose of UVB/A (4,000 J/m2) at postnatal age day 4, as described in Materials and Methods.

A, representative gross view of a raised pigmented tail lesion in a m1/C0/++; m2/C0/++ mouse at 12 months following UVR exposure.

B, incidence and time course of UVR-induced tail lesions in C57BL/6 and m1/C0/++; m2/C0/++ mice. C, representative H&E section from an m1/C0/++; m2/C0/++ mouse containing a grossly identified tail tumor. At right is a higher magnification of the boxed area. Evident is a follicular infundibular inclusion cyst (fc) with proliferation of melanocytes (mh, melanocytic hyperplasia; thin arrows) along its wall and in the keratinous content (thick arrows). bm, basement membrane; d, dermis; e, epidermis. D, confirmation of melanin pigmention within the keratin flakes (thin arrows) was obtained by positive Fontana-Masson (FM) staining. E, melanocyte identity (arrows) was confirmed by staining of DCT.
hypermelanotic lesions were observed in the 12-month study in nonirradiated mice of the parental C57BL/6 or m1−/−; m2−/− strain, nor in 15 C57BL/6 mice irradiated with UVB/A (data not shown).

To characterize UVR-induced melanotic tumors obtained in m1−/−; m2−/− mice, histologic examination was conducted on each of the 11 tail tumors, as well as 2 lesion-free tail samples from the parental C57BL/6 strain. In m1−/−; m2−/− tumors, formation of follicular infundibular cysts (epidermoid type) was observed, with marked proliferation of dendritic and heavily pigmented melanocytes along the basement membrane of the cyst, consistent with in situ melanoma (Fig. 5C). Local invasion of these melanocytes was seen in adjacent dermis (Fig. 5C). High melanogenic activity of the melanocytes is illustrated by transfer of melanin to keratinocytes within the cyst wall and keratin flaps within the cyst interior, as confirmed by Fontana–Masson staining (Fig. 5D) and negative iron staining for hemosiderin (not shown). Melanocyte identity was confirmed by staining with antibody (PEP8) directed to DCT (Fig. 5E). Mole-like lesions were also obtained in m1−/−; m2−/− mice which exhibited accumulation of melanized dendritic melanocytes within the dermis, a feature not seen in tails of parental C57BL/6 mice. Of note is the similarity in morphologic and cytologic features of these tumors with UVR-induced melanomas obtained in a well-characterized mouse strain engineered for overexpression of hepatocyte growth factor (HGF; ref. 33). In that strain, melanomas are characterized by heavily pigmented epithelioid and dendritic melanocytes of follicular origin, which is typical of spontaneous and induced melanomas in rodents.

Discussion

These studies show a previously unrecognized role for the human NM23-H1 and mouse NM23-M1 isoforms in promoting the early repair response to UVR-initiated DNA damage, as well as suppression of UVR-initiated skin tumorigenesis. Such a function was initially suggested by demonstrations of anti-mutator activity for NM23 homologues in bacteria (34, 35) and yeast (16), as well as DNA cleaving activities in vitro for recombinant NM23-H2 (13) and NM23-H1 (14). This study seems to explicitly define for the first time a DNA repair function for NM23-H1 (and probably NM23-H2) in the human and mouse, and specifically as a promoter of the NER pathway for repair of UVR-induced (6–4) photoproducts.

Analysis of catalytically defective mutants of NM23-H1 revealed contributions of its kinase and 3′–5′ exonuclease functions in the early response to UVR-induced DNA damage against polymerase-blocking lesions and (6–4) photoproducts. Whereas 3 variants used in this study (K61Q, P80S, and H113F) exhibit moderate to severe deficiencies in NDPK and hisK activities, none are selective in their targeting of the two kinases (17). The NDPK activity seems a likely participant in light of its prior implication in antimitator functions in E. coli (35). All of the kinase-inactivating and/or 3′–5′ exonuclease–inactivating mutations were inhibitory to repair of overall UVR-induced DNA polymerase–blocking lesions in the hprt locus, strongly suggesting involvement of both activities across a spectrum of DNA damage. Mutational analysis showed that only the kinase-defective mutants exhibited a deficit in promoting NER removal of (6–4) photoproducts, whereas the 3′–5′ exonuclease–defective mutants were fully active.

The apparent lack of contribution from the 3′–5′ exonuclease to repair of (6–4) photoproducts was anticipated in light of currently accepted molecular mechanisms of NER, which do not invoke 3′–5′ exonuclease activity (36). However, the 3′–5′ exonuclease of NM23-H1 clearly promotes repair of total UVR-induced (i.e., DNA polymerase blocking) lesions and suppresses spontaneous and UVR-induced mutations, suggesting this activity may contribute to pathways other than NER, such as base excision repair, double strand-break repair, and translesion synthesis, in which 3′–5′ exo-exonucleases have been implicated (37, 38).

The rapid colocalization of NM23-H1 with (6–4) photoprod-uct lesions raises the possibility that enzymes involved in dNTP synthesis are functionally coupled with DNA repair components at sites of DNA damage (39–41). Indeed, recent studies have implicated deoxyribonucleotide metabolizing enzymes in the DNA repair response, such as ribonucleotide reductase, p53R2 and thymidine kinase (42–45). The possibility remains open that the NDPK activity may deliver nucleotides directly to the DNA machinery during DNA repair processes, as hypothesized by multiple investigators (39–41).

NM23 expression did not promote repair of CPDs, suggesting it contributes to the early/acute phase of the DNA damage response [e.g., (6–4) photoprod-repair] and not the longer term regulation involved in repair of CPD repair. The selective enhancement of only (6–4) photoprod-repair by NM23 isorms is not surprising, as a number of studies have reported important differences in repair of (6–4) photoprod-ucts and CPDs (46, 47).

The strong penetrance of UVR-induced melanomagenesis and cyst formation in mice deficient in nm23-m1 and nm23-m2 provides important in vivo validation of the DNA repair-promoting function of NM23 proteins. These phenotypes strongly suggest specific contributions to the NER pathway, as knock-out lesions in such NER participants as XPC and XPA results in a similar spectrum of UVR-induced skin tumors (48, 49). nm23-h1 remains the prototypical metastasis suppressor gene, with a substantial body of literature affirming its ability to suppress tumor cell motility, invasive potential, and the overall metas-tatic process in the absence of short-term effects on cell proliferation or primary tumor–forming potential. Our find-ings also establish the nm23 genes and their cognate proteins as suppressors of UVR-induced initiation and progression of melanoma in mice.

Tumor suppressor activity of the nm23-m1 and/or nm23-m2 genes is robust, having been shown in the hemizygous-null condition. This high penetrance may be due to participation of NM23-M1 in multiple pathways for repair of UVR-induced DNA damage and/or cooperation with loss in NM23-M2 expression. The ability of NM23-H1/M1 expression alone to promote the initial phase of the DNA repair response and suppress mutagenesis strongly suggests deficiency of this isoform is a major factor in the UVR-induced melanoma phenotype. Additional contributions of the M2 isoform are also suggested by the additional deficit seen in repair of...
UVR-induced damage in MEFs derived from ml−/−; m2−/− mice (Fig. 1). In a previous study, nullizygosity in the nm23-m1 locus failed to render susceptibility to diethylnitosamine-induced liver carcinogenesis, although progression to metastasis was enhanced (50). This discordance with our current study may be related to differences in the genotoxic stressors used (UV vs. chemical), the organs targeted for mutagenesis (skin vs. liver), and/or the genetic background of the mouse strains employed (129Sv/C57BL/6 vs. C57BL/6).

Loss of NM23 expression may represent a “double-hit” in which melanoma cells are not only more motile and invasive but also genetically unstable and prone to progression- and metastasis-driving mutations. In support of this model, low NM23-H1 expression in primary melanomas has been associated with poor outcome (6–8). The possibility is suggested that NM23-deficient metastases in melanoma and breast carcinoma may arise from an NM23-deficient and, thus, genetically unstable subpopulation within the primary tumor. Our findings also raise the question of whether NM23 deficiency in the mouse results in mutations characteristic of human melanoma progression (e.g., b-raf/raf, pten, akt, and others), and if so, whether they arise by a stochastic or directed process. Mouse models of melanoma capable of progression to NM23 deficiency–dependent metastasis should provide insights into this important issue in cancer biology.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Stuart G. Jarrett, Marian Novak, Sandrine Dabernat, et al.


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