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Release from quiescence stimulates the expression of human *NEIL3* under the control of the Ras dependent ERK–MAP kinase pathway

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ABSTRACT

Base excision repair (BER) is believed to be the predominant pathway for the repair of oxidative DNA damage. BER is initiated by lesion-specific DNA glycosylases that recognize and remove the damaged base. NEIL1, NEIL2 and NEIL3 are three mammalian members of the Fpg/Nei DNA glycosylase family with similar enzymatic properties. In this study we showed that both the transcription and protein levels of hNEIL3 fluctuated during the cell cycle. Based on predicted promoter elements of cell cycle-regulated genes and microarray data from various reports, we suggest that *hNEIL3* repression in quiescent cells might be mediated by the DREAM (DP1, RB p130, E2F4 and MuvB core complex) complex. Release from G0 by mitogenic stimulation showed an induction of *hNEIL3* in early S phase under the control of the Ras dependent ERK–MAP kinase pathway. In contrast, the total expression of *hNEIL1* was downregulated upon release from quiescence while the expression of *hNEIL2* was cell cycle independent. Notably, *hNEIL3* showed a similar regulation pattern as the replication protein hFEN1 supporting a function of hNEIL3 in replication associated repair. Thus, it appears that specialized functions of the NEILs are ensured by their expression patterns.

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1. Introduction

To preserve genetic information the DNA must be protected from damage generated spontaneously or induced by environmental agents [1]. To counteract DNA damage, different repair mechanisms have evolved for the many types of DNA lesions. DNA glycosylases in the base excision repair (BER) pathway recognizes and excise specific base lesions leaving an abasic site [2–5]. Repair synthesis is completed by several BER enzymes executing gap filling and ligation [2]. DNA glycosylases that recognize oxidized bases have been divided into two structural families, the Nth family and the Fpg/Nei family [6,7]. Three Fpg/Nei homologues designated hNEIL1, hNEIL2 and hNEIL3 in humans and mNeil1, mNeil2 and mNeil3 in mouse respectively, have been identified in mammals [8–12]. These are bifunctional glycosylases, also cleaving the exposed backbone at the abasic site. NEIL3 is the largest member of the family, consisting N-terminally of the characteristic Fpg/Nei motifs and C-terminally of a disordered extension with unique structural features containing a Ranbp-like zinc finger motif, a putative NLS and tandem repeated GRF zinc finger motifs.

NEIL1 and NEIL2 have been extensively studied biochemically. In the case of NEIL3, only the mouse ortholog, mNeil3, has been examined thoroughly [13]. In summary, the three homologues have broad and overlapping specificity for oxidative lesions and a preference for single stranded DNA, bubble and fork structures. Hydantions such as spiroimidodihydantoin (Sp) and guanidinohydantoin (Gh) are the preferred substrates for mNeil3. These lesions have garnered much attention due to their extremely high mutagenic potential in cells which is significantly greater than 8-oxoG [14]. Altogether, a function during replication or transcription when partly unwound regions of DNA are exposed has been suggested for the NEILs. All three proteins have been reported to be localized in the nucleus, while hNEIL1 has been shown to be found also in mitochondria [10,11,15,16]. Human *NEIL1* and *hNEIL2* are ubiquitously expressed although at different levels in distinct organs [9–11]. In contrast, *hNEIL3* is expressed in thymus and testis and in multiple forms of cancer, while *mNeil3* is expressed in hematopoietic tissue and testis, during embryonic development and in stem/progenitor rich regions in the brain [11,16–20]. Thus, hNEIL3/mNeil3 might function to remove lesions from the genome in proliferating cells. During the cell cycle, the expression of *hNEIL1* has been reported to be induced in S phase [9]. Functional interactions of hNEIL1 with WRN, PCNA, FEN1 and RPA have suggested that

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hNEIL1 is probably involved in repairing the replicating genome [21–25]. Unlike *hNEIL1*, *hNEIL2* expression is independent of the cell cycle stage and was recently shown to be involved in repairing oxidized bases in the transcribed genes of mammalian cells [10,26].

Transition through the mammalian cell cycle requires interplay of transcription factors that together induce or repress gene expression in a temporally defined manner. The E2Fs are a large family of transcription factors that bind target promoters and regulate their expression. There are eight known members of the E2F family in mammals, and they form active DNA-binding heterodimers with either DP1 or DP2 [27–29]. E2F activity is controlled in part by interactions with members of the pRB (pocket protein) family: the RB tumor suppressor, p107 and p130 [30]. The DREAM (DP1, RB p130, E2F4, and MuvB core complex) complex represses cell cycle-dependent genes during quiescence maintaining cells in G0 phase [31]. Upon re-entry into the cell cycle, the repressive complex is relieved by CDKs (cyclin-dependent kinases) that phosphorylate RB proteins in a cell cycle dependent manner [32,33]. The activation of CDKs involves the mitogenic stimulation of receptor tyrosine kinases (RTKs) and the downstream mitogen activated protein (MAP) kinase pathways [34–36]. Activation complexes as E2F1-NF-Y, E2F1-Sp1 can then be recruited to the promoter [37–39].

In the present study we have examined the cell cycle-dependent regulation of hNEIL3 at the transcriptional and protein level in comparison with other repair and replication associated genes and in cells from different origins. Furthermore, we have identified several putative cell cycle-regulated promoter elements and demonstrated the involvement of the Ras dependent ERK–MAP kinase pathway in the induction of *hNEIL3* expression.

2. Materials and methods

2.1. Cells

Human embryonic fibroblasts (HE) were obtained from the National Institute of Public Health (Folkehelsa, Oslo, Norway). MRC-5 (human fetal lung primary fibroblasts), HaCaT (human keratinocytes) and MCF-7 (human epithelial breast cancer cell line) were obtained from ATCC. HE cells were cultured in a 1:1 ratio minimal essential medium (MEM) + Dulbecco's modified Eagle's medium (DMEM) (Gibco, Life Technologies, Carlsbad, CA, USA) ratio supplemented with 10% fetal bovine serum (Standard quality FBS, PAA lab, GmbH, Austria), 1× GlutaMax (200 mM, Gibco, Life Technologies), and 1× penicillin–streptomycin (10,000 U/ml, Lonza, Basel, Switzerland). MRC-5, HaCaT and MCF-7 cells were cultured in DMEM supplemented with 10% FBS, 1× GlutaMax and 1× Pen-Strep.

2.2. Cell cycle synchronization and analysis by flow cytometry

Synchronization of the cells in G0 phase was achieved by culturing cells as a confluent layer for 72 h followed by serum starvation (0.2% serum) for 72 h. The cells were G0 released by trypsination for 4 min at 37 °C (Trypsin-EDTA (200 mg/L), Lonza) and cultivated in standard growth medium at 25% confluence. Cells were harvested by trypsination at indicated time points, washed in ice-cold PBS and stored at −20 °C. Cells used for phase analysis were resuspended in PBS and fixed by addition of ice-cold 100% ethanol to a final concentration of 70%. The cells were stored at −20 °C. For FACS analysis the cells (about 10^6/ml) were stained with propidium iodide (50 μg/ml, Sigma–Aldrich, St. Louis, MO, USA) in 0.1 mg/ml RNaseA (Molzym GmbH & Co, Bremen, Germany)/0.1% Triton X-100 (Sigma–Aldrich)/4 mM Na-citrate buffer for 10 min at 37 °C and put on ice. Cells were subjected to flow cytometric analysis (BD LSRII flow cytometer (Becton Dickinson, San Jose, CA, USA), and the results were analyzed with the CellQuest software (Becton Dickinson).

Table 1
Primers used in Real-Time qRT-PCR.

Target	Sequence (5′ → 3′)	Acc. nr.
NEIL1	(+)-GCTGACCCTGAGCCAGAAGAT (−)-CCCCAACTGGACCACTTCCT	NM_024608.2
NEIL2	(+)-ACCTGTGACATCCTGTCTGAGAAGT (−)-TAATGATGTTCCCTAGCCCTGAGA	NM_145043.2
NEIL3	(+)-GGTCTCCACCCAGCTGTTAAAG (−)-CACGTATCATTTTCATGAGGTGATG	NM_018248.2
UNG2	(+)-GCCAGAAGACGCTCTACTCC (−)-GTGTCGCTTCCTGGCGGG	NM_080911
FEN1	(+)-AGGGAGAGCGAGCTTAGGAC (−)-GGCAACACAGAGGAGGGAT	NM_004111
GAPDH	(+)-CCACATCGCTCAGACACCAT (−)-GCGCCCAATACGACCAAAT	NM_002046.3

2.3. Kinase and ROS inhibitor treatment of HE cells

Synchronized cells were trypsinized and resuspended in growth medium without serum. The cells were preincubated with kinase inhibitor for 15 min and plated in complete growth medium with addition of inhibitor at 25% confluence. Inhibitors used: SB203580 (p38a,b,b2 inhibitor, 10 μM, Promega Corp, Madison, WI, USA), SP600125 (JNK inhibitor, 20 μM, Sigma–Aldrich), U0126 (MEK inhibitor, 20 μM, Promega) and N-acetyl cystein (NAC, ROS inhibitor, 10 mM, Sigma–Aldrich). Cells were harvested at indicated time points as described.

2.4. Total RNA isolation, cDNA synthesis and Real-Time qRT-PCR

Total RNA was extracted from cell pellets using the RNeasy kit (QIAGEN GmbH, Hilden, Germany) according to protocol. The RNA was treated with TurboDNase (Applied Biosystems, Foster City, CA, USA) and the purity controlled by absorbance (260/280 nm and 260/230 nm) measurements using Nanodrop spectrophotometer. cDNA was synthesized from 50 ng RNA in 20 μl reaction using the High-Capacity cDNA Reverse Transcription kit (Applied Biosytems). Real-Time qRT-PCR was performed in 20-μl reactions containing 2.5 ng of cDNA and 100 nM primers using the Power SYBR Green PCR master mix and the Step One Plus Real-Time PCR system (Applied Biosystems) according to the system and kit instructions. The following thermal cycle parameters were used: 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. All samples were run in triplicate. Melting point analyses were performed to confirm the specificity of the PCR. Relative quantitation (RQ) or fold change of gene expression was calculated using the comparative CT method described by the manufacture (Applied Biosystems) GAPDH was used as the reference gene for normalization, G0 as the reference sample for RQ calculation. Primers (Table 1) were designed using the Primer Express software version 2.0 (Applied Biosystems).

2.5. Immunoprecipitation and Western blotting

All procedures were performed at 4 °C. Cell pellets containing about 10^7 cells collected at given time points were suspended in 1 ml of lysis buffer (50 mM Tris–HCl pH 7.5, 500 mM NaCl, 1.0% NP-40, 1% Protease Inhibitor Cocktail (P8340, Sigma–Aldrich)), and disrupted by sonication. Cell lysates were centrifuged, and protein concentration was determined by the Bradford method using the DC Protein Assay Kit II (Bio-Rad, Hercules, CA, USA). 1 mg of extract was incubated with 10 μg of normal rabbit IgG (sc-2027,

Santa Cruz Biotechnology, Santa Cruz, CA, USA) and 20 μl Protein A/G PLUS Agarose (sc-2003, Santa Cruz Biotechnology). The pre-cleared extract was incubated with 1 μg of NEIL3 PolyClonal Antibody (11621-1-AP, ProteinTech Group, Chicago, IL, USA) and 20 μl Protein A/G PLUS Agarose (sc-2003, Santa Cruz Biotechnology). The beads were washed four times and after the final wash the sample was divided into two tubes. The contents of one tube were subjected to Western blot analysis and the other to DNA glycosylase activity.

Proteins were separated on 10% denaturing SDS-polyacrylamide gel (NuPage, Invitrogen Life Technologies, Carlsbad, CA, USA) and transferred to Hybond-P PVDF Membrane (Amersham Biosciences, Piscataway, NJ, USA). The membrane was incubated with the primary antibody NEIL3 PolyClonal Antibody (11621-1-AP, ProteinTech Group) at 4 °C overnight followed by incubation with peroxidase-labeled goat polyclonal secondary antibody to rabbit IgG-H and L(HRP) (ab6721, Abcam, Cambridge, MA, USA) for 1 h at room temperature. The membrane was treated with chemiluminescence reagent Immun-Star™ WesternC™ Chemiluminescence Kit (170-5070, Bio-Rad) and bands were visualized by ChemiDoc XRS system (Bio-Rad).

2.6. Gh assay for enzymatic cleavage of DNA.

The guanindinohydantoin (Gh) containing oligonucleotide substrate was a kind gift from Prof. Cynthia J. Burrows, Department of Chemistry, University of Utah. The oligonucleotide (5′ tgttcatcatgcgtc Ghtcggtatatcccat 3′) containing a single Gh was ^{32}P-endlabeled by T4 polynucleotide kinase (PNK) (New England Biolabs, Ipswich, MA, USA) and [γ-^{32}P]adenosine triphosphate (NEG502A, PerkinElmer, Waltham, MA, USA) by incubation for 30 min at 37 °C. PNK was deactivated at 80 °C. The labeled oligonucleotide was purified by 20% native polyacrylamide gel electrophoresis and visualized by storage phosphor autoradiography (Typhoon 9410 (Molecular Dynamics, Sunnyvale, CA, USA)). The radiolabeled substrate was isolated from the gel, eluted in dH_2O and stored at 4 °C. The incision assay was performed as previously reported [11] with modifications. To immunoprecipitate samples, 10 fmol DNA substrate (^{32}P-labeled oligomer), 100 ng ss competitor DNA (35-nucleotide random sequence), were mixed in reaction buffer (50 mM MOPS pH 7.5, 1 mM EDTA, 5% glycerol and1 mM DTT) in a total volume of 10 μl and incubated for 60 min at 37 °C. 10 μl of formamide loading dye was added, and the samples were denatured at 80 °C for 10 min. The reaction products were resolved in 20% denaturing polyacrylamide gels and visualized by autoradiography as for the substrates. Purified core hNEIL3 enzyme was purified as previously described and used as positive control [40].

2.7. Total RNA isolation and Northern blot analysis

Total RNA was isolated from HaCaT cells using the TRIzol Reagent (Ambion, Applied Biosystems) according to manufacture's instructions. mRNA was isolated from total RNA using the MicroPoly(A)Purist Kit (Ambion) and 8 μg/lane was subjected to 1% (w/v) denaturing agarose gel electrophoresis at 5 V/cm. RNA was transferred to an Ambion BrightStar-Plus membrane by downward transfer from gel and crosslinked (at Preset energy setting) to the membrane in a CL-1000 UV-Crosslinker (UVP, Upland, CA). The Northern Max kit (Ambion) was used in the blotting, prehybridization/hybridization and washing steps of the membrane as described by the manufacture. The probes were labeled using Amersham Rediprime II Random Prime labeling system (Amersham Biosciences) and *e*asytide dCTP (α-^{32}P) (NEG513H, PerkinElmer). The filter was exposed to a storage phosphor screen (GE Healthcare) and the hybridization signals were detected and quantified using the Typhoon 9410 and ImageQuant software (Molecular Dynamics).

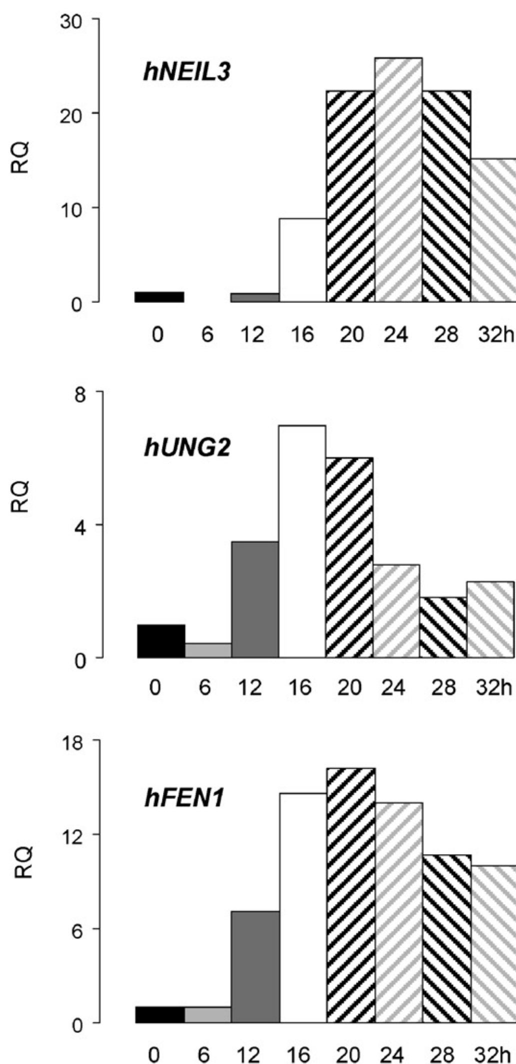
3. Results

3.1. Release from quiescence stimulates the expression of hNEIL3

As *hNEIL3* expression is confined to proliferative tissues and cells, we examined the transcription level of *hNEIL3* during cell cycle progression by taking advantage of the Real-Time qRT-PCR technique. Cells cultured in vitro can be arrested in the G0 phase of the cell cycle, either by serum starvation or by contact inhibition repressing pRB-phosphorylation by distinct mechanisms. Serum deprivation results in a decrease of the positive regulators cyclin D1 and cyclin D3, whereas contact inhibition acts via regulation of the inhibitory proteins p16 and p27 [41]. We combined these conditions as this has been shown to synchronize human embryonal lung fibroblasts with highest efficiency [41]. Release from the G0 phase and cell cycle entry was achieved by culturing the cells at subconfluence in complete serum containing medium. We have compared *hNEIL3* transcription in human embryonic (HE) fibroblasts with *hUNG2* and *hFEN1*, two known replication associated enzymes [42–48]. As seen in Fig. 1, *hNEIL3* transcription was upregulated during cell cycle progression. Human *NEIL3* transcription was induced in early S phase, *hUNG2* and *hFEN1* in late G1 phase. Human *NEIL3* and *hFEN1* transcription was sustained through G2/M phase while *hUNG2* transcription declined in late S phase. Moreover, *hNEIL3* was the most highly induced gene, reflecting the low basal level in quiescent cells.

To address the question whether the transcriptional regulation of *hNEIL3* in HE cells was cell lineage specific or a more general regulation pattern in proliferating cells, we investigated the *hNEIL3* level in primary human fetal lung fibroblasts (MRC-5), human keratinocytes (HaCaT) and human breast tumor epithelial cells (MCF-7). Confluent and serum starved cells were released and followed for 4 days until they once more reached confluence. As seen in Fig. 2, *hNEIL3* transcription was induced in all the cell cultures, albeit variations in the time of induction and level of expression were observed in the different cell lines. These variations most probably reflect the efficiency of synchronization, cell phase progression and the capacity to be contact inhibited. HE cells were relatively synchronized for 32 h after G0 release, at later time points they were more randomly distributed showing a reduced level at day 2. The MCF-7 cells tended to grow as a two-layer which confirm that they are unable to be contact inhibited and may reflect the relatively high level of *hNEIL3* at day 0. Overall, *hNEIL3* was highly expressed in cells that were in a proliferative state, at days 3–4 when reaching confluence and cell cycle arrest the *hNEIL3* level dropped.

These findings prompted us to examine whether the amount of hNEIL3 protein reflected changes in transcriptional level. We could not detect hNEIL3 protein in Western blot experiments of total protein extracts from unsynchronized HE or HaCaT cells (data not show). However, a prominent band of around 70 kDa was detected when total protein extracts from unsynchronized HE or HaCaT were immunoprecipitated with NEIL3 antibody. This band was absent in the control experiments that did not include NEIL3 antibody in the immunoprecipitation sample (Fig. 3A). The 70 kDa band was cut from the gel and subjected to MS-based sequencing. Altogether, 4 different peptides identified corresponded to hNEIL3. We next subjected the immunoprecipitated samples containing or lacking hNEIL3 antibody to a standard DNA glycosylase activity assay toward ssGH. As seen in Fig. 3B, activity toward ssGh was detected in the lane in which hNEIL3 was immunoprecipitated and absent in the sample lacking NEIL3 antibody. Next, Western blot



Hours / %	0	6	12	16	20	24	28	32
G0/G1	83	72	75	75	45	21	17	41
S	2,1	1,3	1,6	3,2	28	21	6,3	5
G2/M	10	14	13	12	14	34	51	34

Fig. 1. Upregulation of *hNEIL3* in quiescent HE fibroblasts stimulated to proliferate. HE fibroblasts were serum starved at confluence and G0 released by replating 1:4 in serum containing culture medium. Normalized *hNEIL3, hUNG2* and *hFEN1* relative transcript levels (RQ) were measured during cell cycle progression upon G0 release at indicated time points after release by qPCR. G0 cells used as the reference sample for RQ calculation. Cell cycle distribution was monitored using propidium iodide staining followed by flow cytometry after release from the block. The percentage of cells in each cell cycle phase is presented in the table. Experiment was repeated twice, one representative experiment is displayed.

experiments, as shown in Fig. 3C revealed that hNEIL3 levels, virtually undetected in arrested cells, showed a similar increase in HaCaT cells during cell cycle progression. Finally, to measure whether NEIL3 activity followed the amount of hNEIL3 protein, DNA glycosylase assays were carried out using immunoprecipitated hNEIL3 from various time points after release from quiescence. As seen in Fig. 3D, DNA glycosylase activity mirrored hNEIL3 protein levels.

3.2. Opposite pattern of hNEIL1 and hNEIL3 gene expression

Next, we compared the relative transcript level of the three *hNEIL* homologues in contact inhibited and serum starved synchronized HE and HaCaT cells. Interestingly, opposite patterns of regulation of *hNEIL1* and *hNEIL3* in quiescent cells were observed (Fig. 4A and B). In contrast, for *hNEIL2* transcription, phase independent expression was observed (Fig. 4A and B). Intriguingly, our results showed a strong repression of *hNEIL1* transcription upon G0 release. The repression was also observed for the serum starved, contact inhibited MRC-5 cells (data not shown), this is in contrast to the expression pattern in serum starved MRC-5 cells reported by Mitra et al. [9]. Several *hNEIL1* transcripts have previously been detected in Northern blots [9,11,19]. As these cannot be distinguished by the primer pair used in the Real-Time qRT-PCR reactions we performed Northern blotting of mRNA from HaCaT cells upon G0 release to identify the different transcripts (Fig. 4C). In our experiments, the 4 kb and the 1.8 kb *hNEIL1* transcripts along with the smaller RNA specie observed by Mitra et al. were identified and normalized to the β-*ACTIN* levels (Fig. 4C). The 4 kb band is the main transcript detected in human tissues, while the 1.8 kb band is the so called completely processed product. At G0, an intense 4 kb *hNEIL1* transcript was observed which decreased 50 times 27 h after release and was absent in unsynchronized cells. Both the 1.8 kb and the smaller transcript also decreased significantly (3–6 times) upon release. In contrast, *hNEIL3* transcription was induced in cycling cells. The more prominent band in the unsynchronized cells could reflect that the cells are in a higher proliferative state than cells which are released from G0.

3.3. Identification of E2F elements in the promoter region suggest Rb dependent regulation of the hNEIL3 gene

The majority of promoters of cell cycle-regulated genes are GC-rich and TATA-less and with a high frequency of E2F binding sites [37,49]. We have used the MatInspector program to search for characteristic promoter elements of cell cycle-regulated genes. Several E2F, Sp1, CREB, NRF-1, NF-Y transcription factors and cell cycle-dependent element (CDE)/cell cycle gene homology region (CHR) cis-regulatory elements were predicted (Fig. 5). Several of the E2F, Sp1 and the CDE/CHR binding elements were in close proximity to the transcriptional initiation site.

3.4. Human NEIL3 induction upon cell cycle entry is mediated through the MAP kinase signaling cascade

We have demonstrated a cell cycle-dependent regulation of *hNEIL3* and the putative E2F elements in the 5′UTR suggested an Rb dependent regulation. The transition of cells from G0/G1 to S is regulated by cyclin D1/E and their cognate CDKs which phosphorylates and inactivates the retinoblastoma protein family [32,33,50]. The cyclin D1/E level is induced by mitogenic stimulation, involving RTKs, MAP kinases and activation of several families of transcription factors [34–36]. In order to further elucidate the regulation of *hNEIL3* expression we examined the dependence on one or several of the MAP kinase pathways upon mitogenic stimulation and G1/S transition of quiescent cells. Four MAP kinase pathways have been identified [51,52]. These are the extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-Terminal kinase (JNK), p38, and ERK5 cascades. Specific chemical inhibitors of the four MAP kinase cascades have been widely used in cell culture systems to study the different pathways. We treated contact inhibited and serum starved HE cells with the kinase inhibitors SB203580 (p38a,b,b2 inhibitor), SP600125 (JNK inhibitor), U0126 (MEK ERK1/2 inhibitor) and N-acetyl cystein (NAC, a ROS inhibitor) followed by G0 release. The antioxidant NAC is frequently used as an experimental tool to

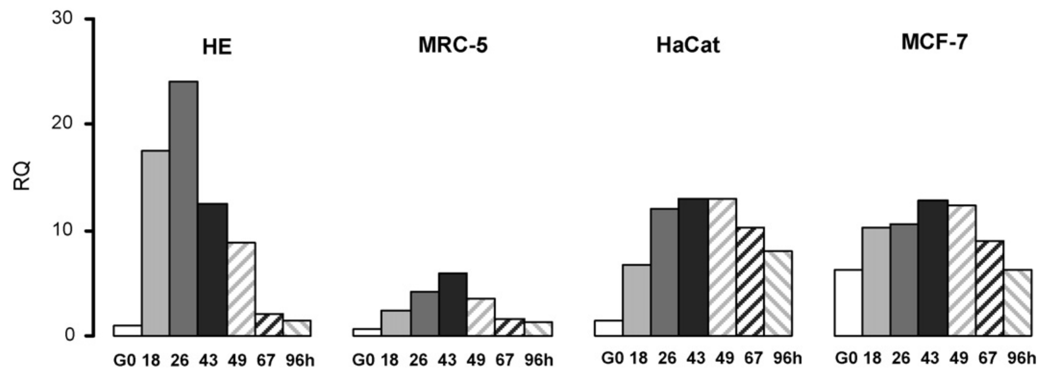


Fig. 2. Human *NEIL3* was highly expressed in cells that were in a proliferative state. HE, MRC-5, HaCaT and MCF-7 cells were serum starved at confluence and G0 released by replating 1:4 in serum containing culture. Normalized *hNEIL3* relative transcript level (RQ) was measured at indicated time points after release by qPCR, using G0 HE cells as the reference sample for RQ calculation Experiment was repeated twice, one representative experiment is presented.

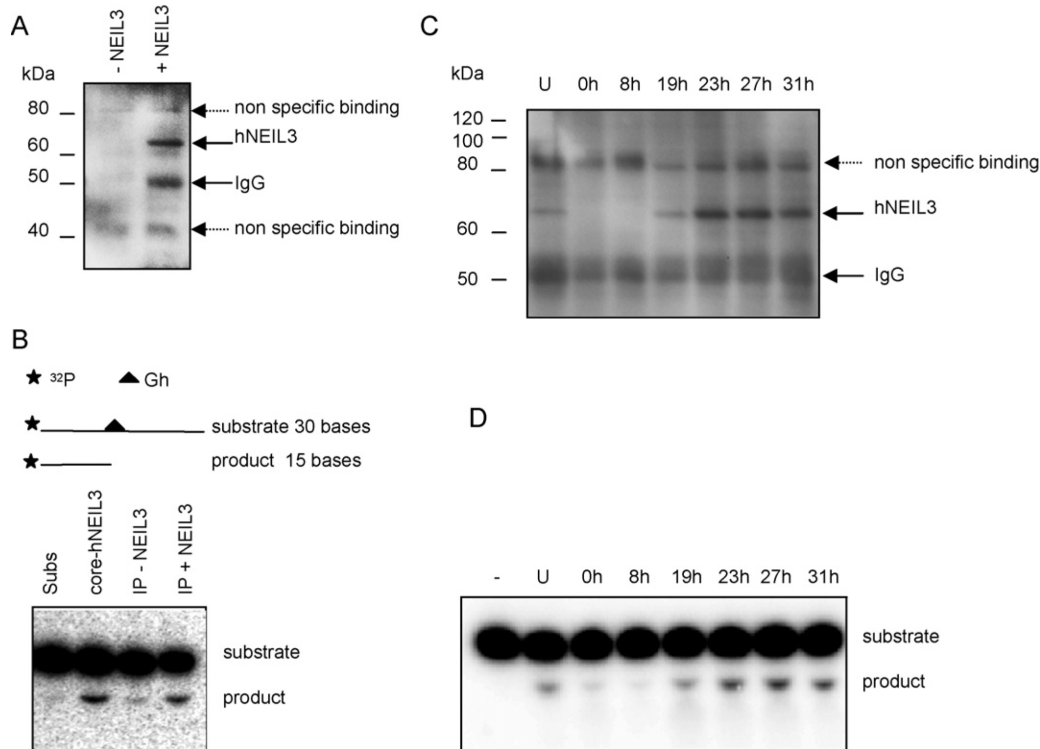
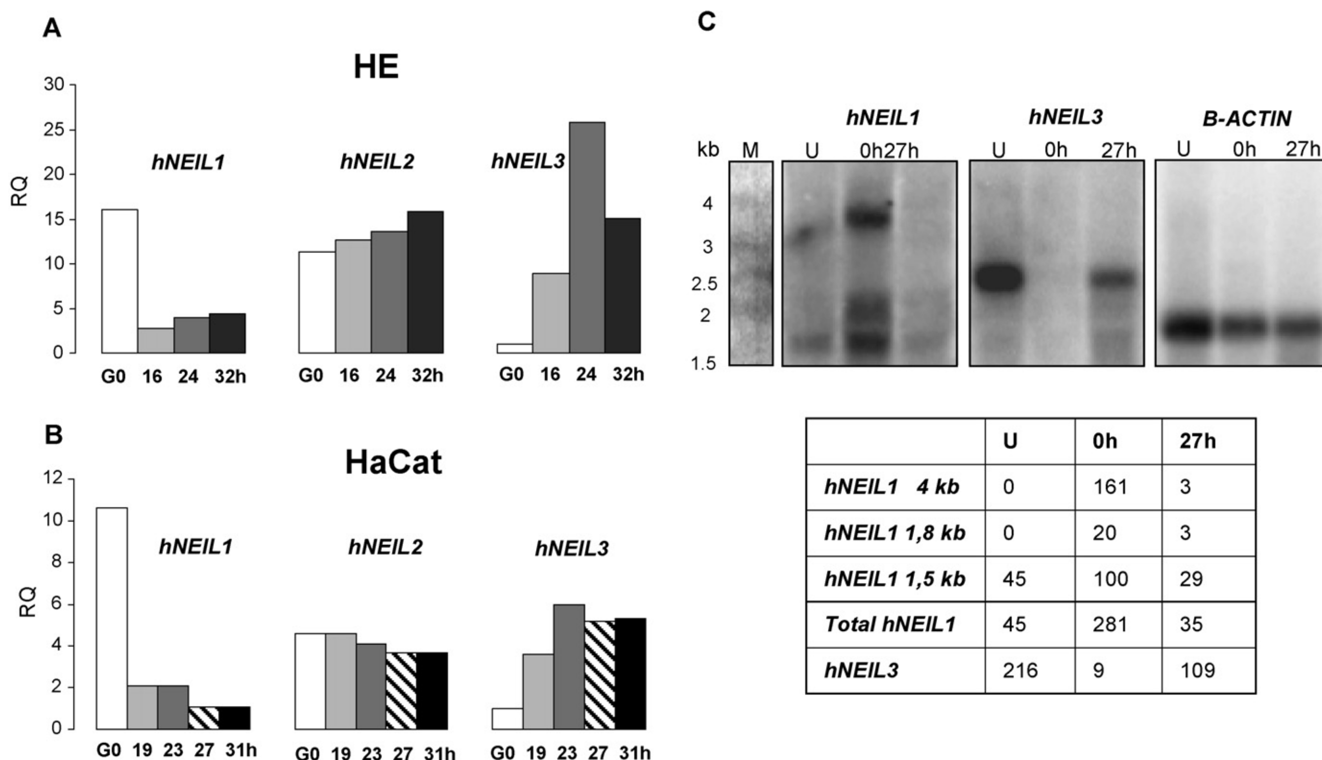


Fig. 3. Human NEIL3 protein levels and DNA glycosylase activity mirrored *hNEIL3* expression profile. Unsynchronized HaCaT cells were immunoprecipitated by NEIL3 antibody. (A) Western blot analysis of unsynchronized HaCaT cells that were immunoprecipitated with NEIL3 (+ NEIL3) or a control lacking NEIL3 antibody (− NEIL3). Arrows indicate the 70 kDa band sequenced, The IgG heavy chain and non-specific binding proteins. (B) Immunoprecipitated samples were incubated with a ^{32}P-labeled single stranded Gh oligonucleotide in a standard DNA glycosylase activity assay. Substrate alone (Subs), purified core hNEIL3 (core-hNEIL3), immunoprecipitate without NEIL3 antibody (IP − NEIL3) and immunprecipitate with NEIL3 antibody (IP + NEIL3). HaCaT cells were serum starved at confluence and G0 released by replating 1:4 in serum containing culture. (C) Western blot analysis detecting endogenous immunoprecipitated hNEIL3, and (D) DNA glycosylase activity on an ssGh substrate were measured during cell cycle progression upon G0 release at indicated time points after release. U: unsynchronized cells.

assess the involvement of ROS in various signal transduction pathways [53–56]. All inhibitors at the given concentrations, except for the p38 inhibitor, inhibited proliferation totally. As seen in Fig. 6 *hNEIL3* induction was dependent on activated kinases, mostly by the Ras dependent ERK–MAP kinase pathway. At 17 h after stimulation the transcription was inhibited totally, and at 23 h there was a 50–60% inhibition. The JNK inhibitor had a minor effect on *hNEIL3* transcription while no significant effect was observed by p38 or ROS inhibition. Human *FEN1* showed a similar regulation pattern as *hNEIL3*. In contrast, the p38 and JNK inhibitors mediated an upregulation of the *hUNG2* transcript level, while the ERK inhibitor appeared to delay the transcription. NAC had no significant effect on *hUNG2* transcription. The repression of *hNEIL1* transcription at G1/S transition was not affected by any of the kinases or ROS inhibitors (data not shown).

4. Discussion

Under our experimental conditions, our data established that the three members of the Nei/Fpg family of DNA glycosylases, *hNEIL1*, *hNEIL2* and *hNEIL3* were distinctly regulated during cell cycle progression. Human *NEIL3* was highly upregulated upon release from quiescence. In contrast, *hNEIL1* was repressed while the expression of *hNEIL2* was unaffected. Synchronous cell populations can be obtained by several methods in order to study events



	U	0h	27h
hNEIL1 4 kb	0	161	3
hNEIL1 1,8 kb	0	20	3
hNEIL1 1,5 kb	45	100	29
Total hNEIL1	45	281	35
hNEIL3	216	9	109

Fig. 4. Human *NEIL1* and *NEIL3* were down- and up-regulated respectively upon release from quiescence. (A) HE fibroblasts, and (B) HaCaT cells were serum starved at confluence and G0 released by replating 1:4 in serum containing culture medium. Normalized *hNEIL1*, *hNEIL2* and *hNEIL3* relative transcript level (RQ) were measured at indicated time points after release by qPCR, using G0 cells as the reference sample for RQ calculation Experiment was repeated twice, one representative experiment is presented in the figure. (C) Northern blot analysis of *hNEIL1* and *hNEIL3* mRNA using *B-ACTIN* for normalization at the indicated time points. U: unsynchronized cells. 8 μg mRNA was loaded on the gel and the Ambion RNA Millennium Marker was used as a RNA size standard.

occurring through the cell cycle. Several methods, including contact inhibition, serum deprivation, thymidine blocks or drugs are widely used to synchronize cells [41,57]. In this study we chose to synchronize cells by both contact inhibition and serum deprivation as these conditions in combination have shown to give additive anti-proliferative effect and in addition contact inhibition play the fundamental role in proliferation control in vivo (i.e. wound-healing [41]).

The expression of *hNEIL3* was regulated both at the transcriptional and protein level as the cells entered the cell cycle from quiescence, showing an induction in early S phase which prolonged through G2/M as for *hFEN1*. The onset of induction was lagging approximately 4 h compared to *hUNG2* and *hFEN1* but the induction level was significantly higher than for *hUNG2* in particular, reflecting a very low basal level of *hNEIL3* in quiescent cells and non-proliferating tissue. Moreover, *hNEIL3* has been picked in several genome wide screens that identify genes that are cell cycle-regulated, with highest expression in the G2 phase [58,59]. The *hNEIL3* expression seems not to be confined to any cell lineage, but the onset and level of induction upon G0 release varies. Efficiency of synchronization, cell phase progression or the capacity to be contact inhibited may affect the expression pattern of *hNEIL3* in the different lineages. Loss of regulation at the G1/S transition appears to be a common event among virtually all types of human tumors [60,61]. This was observed with the MCF-7 cells from breast tumor which tended to grow as a two-layer not being contact inhibited. In addition, lineage specific expression of distinct transcription factors could also have an influence on the transcript level of *hNEIL3* in the various lineages.

Human *NEIL3* being a cell cycle-regulated gene was supported by the analysis of its 5′ upstream promoter region which revealed several features that characterize such genes. Of particular interest are the E2F binding sequences, the cell cycle-dependent element (CDE) and the cell cycle genes homology region (CHR) located proximal to the transcription initiation site. Interestingly, in genome wide studies using chip-on-chip to identify E2F4 and p130 (Rb

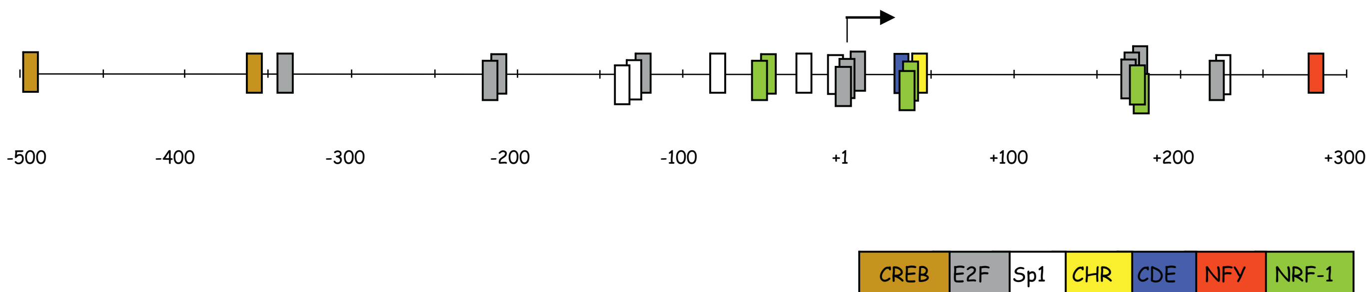


Fig. 5. Analysis of the −500 to +300 base pair region of human *NEIL3* revealed the presence of several putative E2F sites. The MatInspector program was used to search for promoter elements that are involved in cell cycle regulation. The transcription initiation site is shown by an arrow.

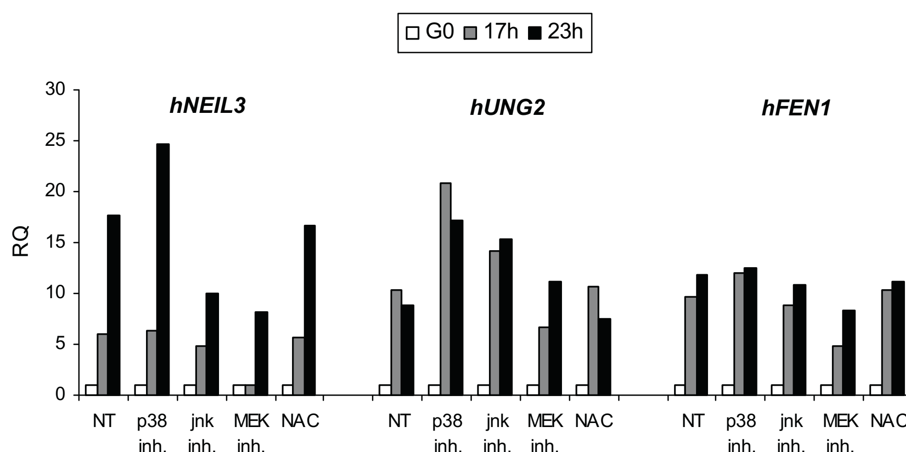


Fig. 6. The effect of kinase and ROS inhibitors on transcription of human *NEIL3, UNG2* and *FEN1*. HE fibroblasts were serum starved at confluence and the cells were pre-incubated with kinase and ROS inhibitors for 15 min. Cells were G0 released by replating 1:4 in serum containing culture medium with inhibitors. Inhibitors used: SB203580 (p38a,b,b2 inhibitor, 10 μM), SP600125 (JNK inhibitor, 20 μM), U0126 (MEK inhibitor, 20 μM) and N-acetyl cystein (NAC, ROS inhibitor, 10 mM). NT: non-treated cells. Normalized *hNEIL3, hUNG2* and *hFEN1* relative transcript level (RQ) were measured at indicated time points after release by qPCR, using G0 cells as the reference sample for RQ calculation. Experiment was repeated twice, one representative experiment is presented.

pocket protein) targets for transcriptional regulation of growth arrest the *hNEIL3* and *hFEN1* genes were picked up [31,62,63]. Moreover, in the study by DeCaprio and co-workers [31], *hNEIL3* and *hFEN1* were identified as genes whose promoter was strongly bound by the DREAM complex in quiescent cells, while *hUNG2* was bound by a different complex.

Rb pocket protein inactivation by phosphorylation at the G1/S transition of cells is a consequence of a cascade of phosphorylation events upon mitogenic stimulation involving RTKs [34–36,51,52]. We have demonstrated that the induction of *hNEIL3* transcription when cells entered the cell cycle was to a certain extent dependent on the activation of the Ras dependent ERK–MAP kinase pathway in particular. Human *FEN1* and *hUNG2* transcription were also affected by kinase inhibitors in which the ERK–MAP kinase pathway was the most important for induction. In the case of *hUNG2*, the activation of p38 and JNK kinases seemed to mediate repression. To get a more detailed picture of the signaling pathways involved, several other inhibitors and combination of inhibitors could have been used. Moreover, the use of specific mitogenic factors as a substitute for serum stimulation could also elucidate this question. Regardless, we present strong evidence supporting an Rb associated regulation of *hNEIL3* expression in mitogen stimulated cells.

In contrast to *hNEIL3, hNEIL1* transcription was strongly repressed upon G0 release in contact inhibited and serum starved conditions. Four *hNEIL1* transcripts have been described in Northern blot experiments: a weak 1.8 kb transcript corresponding to the fully processed *hNEIL1* together with two highly expressed larger transcripts, claimed to be unprocessed *hNEIL1*, of about 4 and 8 kb [9,11,19]. The fourth transcript is a smaller transcript identified in the MRC-5 cell line. Northern blot analysis revealed that the repression of *hNEIL1* in HaCaT cells upon release from quiescence was mainly due to downregulation of the 4 kb transcript but also a significant repression of the smaller transcripts. Interestingly, the 4 kb *hNEIL1* transcript was not detected in subconfluent serum starved MRC-5 cells [9]. We ascribe the different results to the conditions used in the experiments. Mitra and colleagues synchronized the cells by serum starvation at subconfluence and by using identical conditions with MRC5 cells we did not see the same high level of *hNEIL1* transcription at G0 nor did it repress *hNEIL3* and *hUNG2* transcription as efficiently as in combination with contact inhibition (data not shown). Thus, this 4 kb *hNEIL1* transcript appears to be specific in contact inhibited and contact inhibited/serum deprived conditions. It is reported that both serum deprivation and contact inhibition lead to significant and distinct changes in gene expression and apparently *hNEIL1* transcription was differently regulated when cells were interacting [64]. The drastic repression of *hNEIL1* transcription upon cell cycle entry was not dependent on the mitogen activated kinase pathways (data not shown).

Since all NEIL enzymes process DNA base lesions in single stranded DNA, a function during replication or transcription when partly unwound regions of DNA are exposed has been suggested. *hNEIL2* expression is independent of the cell cycle stage and recently a role for hNEIL2 in repairing oxidized bases in the transcribed genes of mammalian cells has been published [10,65]. The results showing high expression of all the *hNEIL1* transcripts in G0 arrested cells imply that the enzyme could have a function in quiescent contact inhibited cells and organs in addition to the proposed replication associated repair of oxidative lesions [21–25]. We could not observe the S-phase specific expression of *hNEIL1* using the Real-Time qRT-PCR technique as the fully processed 1.8 kb transcript cannot be distinguished from the other larger transcripts. Hence, we are not questioning the replication associated repair of hNEIL1. Still, an intriguing question is why the most abundant mRNA transcript in contact inhibited and in human tissue in general is the 4 kb specie referred to as unprocessed *hNEIL1*. A more in depth study of hNEIL1 regulation at the transcriptional and protein level in cells and organs would be elucidatory. Pertinent to this, RNA editing of *hNEIL1* was recently demonstrated to change the substrate specificity of the enzyme [66].

Finally, NEIL3 has been the elusive enzyme of the three NEIL homologues. In this paper we have shown for the first time that endogenous hNEIL3 has DNA glycosylase activity. As it has been very difficult to purify the full length recombinant enzyme, we have shown that by synchronizing cells it is possible to immunoprecipitate enzymatically active hNEIL3 which can be used to confirm results obtained from recombinant NEIL3 enzymes. The expression pattern of *hNEIL3* could indicate that hNEIL3 is mainly involved in replication associated repair. A predicted non-canonical PCNA motif [67] and our previously reported co-localization of hNEIL3 with RPA [11] support this hypothesis.

In conclusion, the hNEIL3 glycosylase expressed in highly proliferative cells was found to be cell cycle-dependent and induced when cells enter the S phase by mitogenic stimulation. The induction was to a certain extent under the control of the Ras dependent ERK–MAP kinase pathway. We suggest that the *hNEIL3* repression in G0 arrested cells could be mediated by E2F4 binding through

the DREAM complex to one or several of the putative E2F sites or CDE/CHR sites.

Conflict of interest statement

There are no conflicts of interest.

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