Stress-induced NQO1 controls stability of C/EBPα against 20S proteasomal degradation to regulate p63 expression with implications in protection against chemical-induced skin cancer

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Abstract

Previously, we have shown a role of cytosolic NAD(P)H:quinone oxidoreductase 1 (NQO1) in stabilization of p63 against 20S proteasomal degradation resulting in thinning of epithelium and chemical-induced skin cancer [Oncogene (2011) 30,1098–1107]. Current studies demonstrate that NQO1 control of C/EBPα against20S proteasomal degradation also contributes to the up regulation of p63 expression and protection. Western and immunohistochemistry analysis revealed that disruption of NQO1 gene in mice and mouse keratinocytes led to degradation of C/EBPα and loss of p63 gene expression. p63 promoter mutagenesis, transfection and ChIP assays identified C/EBPα binding site between nucleotide position −185 to −174 that bound to C/EBPα and up regulated p63 gene expression. Coimmunoprecipitation and immunoblot analysis demonstrated that 20S proteasomes directly interacted and degraded C/EBPα. NQO1 direct interaction with C/EBPα led to stabilization of C/EBPα against 20S proteasomal degradation. NQO1 protection of C/EBPα required binding of NADH with NQO1. Exposure of skin and keratinocytes to chemical stress agent benzo(a)pyrene led to induction of NQO1 and stabilization of C/EBPα protein resulting in an increase in p63 RNA and protein in wild type but not in NQO1−/− mice. Collectively, the current data combined with previous suggest that stress-induction of NQO1 through both stabilization of C/EBPα and increase in p63 and direct stabilization of p63 controls keratinocyte differentiation leading to protection against chemical-induced skin carcinogenesis. The studies are significant since 2–4% human individuals are homozygous and 23% are heterozygous for NQO1P187S mutation and might be susceptible to stress-induced skin diseases.

Keywords

NQO1; C/EBPα; 20S proteasome; skin differentiation; p63
Introduction

Dicoumarol inhibitable cytosolic NAD(P)H:quinone oxidoreductase 1 (NQO1) competes with cytochrome P450 reductase and catalyzes two-electron reductive detoxification of quinones leading to protection against oxidative stress, mutagenicity and carcinogenicity (Ross, 2004; Vasiliou et al., 2006; Long and Jaiswal, 2000). The NQO1 gene expression is induced along with other phase II enzyme genes in response to xenobiotics and antioxidants (Kaspar et al., 2009). This provides protection for cells against oxidative stress and neoplasia. It is reported that diverse chemicals with the property to induce NQO1 and other enzymes block carcinogenesis (Zhang et al. 1994). NQO1−/− mice exhibited significantly increased sensitivity to skin carcinogenesis in response to benzo(a)pyrene (Long et al., 2000) and DMBA (Long et al., 2001). Studies also demonstrated that lower induction of p53 and decreased apoptosis contributed to chemical-induced skin carcinogenesis (Iskander et al., 2005). The only other member of NQO gene family NRH:quinone oxidoreductase 2 (NQO2) is also a cytosolic protein that metabolizes quinones and its derivatives (Long and Jaiswal, 2000). NQO2 is relatively less studied than NQO1 and its exact role in quinone metabolic detoxification and/or activation remain unknown. Human NQO1 gene is localized on chromosome 16q22 (Jaiswal et al. 1999). A C-->T polymorphism of human NQO1 gene produces a proline to serine (P187S) substitution that leads to degradation of enzyme (Traver et al., 1992; Siegel et al., 2001). Individuals carrying both mutated genomic alleles are completely lacking in NQO1 activity, whereas heterozygous individuals have intermediate NQO1 activity compared with wild type individuals (Siegel et al., 2001). Approximately 2–4% individuals are homozygous and 20–25% is heterozygous for this mutation (Ross, 2004).

The p53, p63 and p73, each having multiple isoforms belong to p53 family of transcription factors (Moll and Slade, 2004; Pietsch et al., 2008). These factors contribute various effects on downstream regulators of cell cycle progression and apoptosis. p63 is ubiquitously present. However, the role of p63 is most relevant in development of normal epithelium in skin (King and Weinberg, 2007). The p63-null mice die at or before birth, lacking skin and with profound limb underdevelopment, which can be partially rescued by returning p63 expression to the mice, suggesting the vital role(s) played by p63 in skin differentiation (Mills et al., 1999).

Recent studies have shown a role of NQO1 in stabilization of p53 against 20S proteasome degradation (Asher et al., 2001; Gong et al., 2007). This stabilization of p53 is independent of ubiquitination (Asher et al., 2002). This function of NQO1 is in addition to its role in metabolism of quinone compounds as described above. NQO1 also stabilizes ornithine decarboxylase, tumor suppressor p33ING1b and AP1 factor c-Fos (Asher et al., 2005; Garate et al., 2008; Adler et al., 2010). Studies also showed that B[a]P induced NQO1 and NQO2 both stabilize p53 against 20S proteasomal degradation and protect against chemical-induced skin carcinogenesis (Gong et al., 2007). More recent studies have shown that NQO1 but not NQO2 stabilizes p63 against 20S proteasomal degradation resulting in protection against thinning of epithelium and chemical-induced skin cancer (Patrick and Jaiswal, 2011). These studies together suggest that NQO1 through stabilization of factors regulates biological processes leading to cell growth and differentiation. This property along with
property to detoxify quinones and derivatives led to protection against chemical-induced carcinogenesis.

In this report, we have extended studies to further understand NQO1 regulation of p63 which contributes to normal development of skin epithelium and protection against chemical-induced skin carcinogenesis. Disruption of NQO1 gene in mice led to degradation of C/EBPα and loss of p63 gene expression. p63 promoter mutagenesis and ChIP assays identified C/EBPα element that bound to C/EBPα and up regulated p63 gene expression. Coimmunoprecipitation analysis demonstrated that 20S and NQO1 both directly interacted with C/EBPα. 20S degraded C/EBPα while NQO1 protected C/EBPα against 20S proteasomal degradation. Exposure to environmental stressor benzo(a)pyrene, as well as antioxidant t-BHQ led to stabilization of C/EBPα protein resulting in increase in p63 RNA and protein in wild type but not in NQO1−/− mice. Collectively, the data suggest that stress-induction of NQO1 through both stabilization of C/EBPα and increase in p63 and direct stabilization of p63 controls keratinocyte differentiation leading to normal skin development and possibly contribute to protection against chemical-induced skin carcinogenesis.

RESULTS

As we discovered in previous studies (Patrick and Jaiswal, 2011), p63 levels in tumor-derived NQO1-null keratinocyte cell lines were significantly lower than those found in wild type cell lines (Fig. 1A). In an effort to determine correlation between C/EBPα and p63 expression we probed wild type (WT) and NQO1-null (1−/−) skin tissue and keratinocyte cell lines with antibodies against C/EBPα and discovered that similar to p63, C/EBPα expression was indeed lowered in NQO1-null keratinocytes, and also in skin tissue from NQO1-null mice. Immunohistochemistry studies of mouse skin tissue confirmed this downregulation of C/EBPα, showing substantially less staining in NQO1-null than wild type mouse epidermis (Fig. 1B). Since NQO1 is known to play a role in regulation of p63 expression, we investigated whether modulation of NQO1 is sufficient to regulate levels of C/EBPα protein as well. Overexpression of NQO1 construct led to substantial upregulation of C/EBPα in WT tumor-derived mouse keratinocytes, while knockdown of NQO1 via siRNA led to substantial downregulation of C/EBPα(Fig. 1C). Since NQO1 regulation of p63 is known to be partly due to protection of p63 protein from 20S proteasomal degradation, we investigated whether modulation of C/EBPα alone is sufficient to cause changes in p63 levels. Similar to NQO1 modulation, overexpression of C/EBPα constructs in keratinocytes led to upregulation of p63 levels, while specific knockdown of C/EBPα levels led to downregulation of p63 (Fig. 1D). To further elucidate the ability of NQO1 and C/EBPα to directly regulate p63 levels, we transfected WT keratinocytes with C/EBPα construct and tested whether knockdown of NQO1 would be sufficient to abnegate the effect on p63 upregulation. Overexpression caused upregulation of p63, which was downregulated in a dose-dependent manner when cells were simultaneously transfected with specific NQO1 siRNA (Fig. 1E).

To further investigate the mechanism of C/EBPα regulation of p63, we mapped the mouse p63 promoter using MATinspector and ProSite software, which yielded a number of putative C/EBPα sites upstream of the start ATG (Supplementary Fig. S1). We then cloned
the proximal 5′UTR of p63 and produced luciferase constructs which contained these putative sites to measure their regulating activity. Overexpression of C/EBPα in cells containing both the longest (2.3kb) and shortest (0.5kb) promoter sequences showed that C/EBPα is indeed capable of significantly inducing p63 transcriptional activity, and that most of the C/EBPα-specific induction is capable of occurring in the absence of the first two putative C/EBPα binding sites located at −384 and −271, respectively (Fig. 2A). To verify these results and more closely determine which putative sites were in fact active in the p63 promoter, we performed Chromatin Immunoprecipitation (ChIP) assay on WT keratinocytes using primers specific for a number of putative C/EBPα binding sites (Fig. 2B). Results indicated that only the −185 binding site showed activity. Additionally, we transfected WT keratinocytes with constructs containing wild type p63 promoter luciferase constructs as well as constructs which were mutated to abolish the specific C/EBPα binding sites and measured transcriptional activity after induction with NQO1 overexpression (white bars) as well as with C/EBPα overexpression (grey bars) (Fig. 2C). Results indicated that induction by NQO1 or by C/EBPα was only affected in the −185 binding site, indicating that only this site is active in keratinocytes. Further transcriptional studies found that co-expression of NQO1 and C/EBPα led to an additive effect on p63 transcription (Fig. 2D, left panels), suggesting that NQO1 upregulation of C/EBPα leads to enhanced C/EBPα effect on p63 promoter. Additionally, studies including a Y127/129A NADH binding mutant of NQO1, previously shown to have no upregulation effect on p63 (Patrick and Jaiswal 2011) compared to wild type NQO1 showed that abolition of NQO1 binding with NADH is sufficient to negate its ability to cause C/EBPα-mediated upregulation of p63 transcription (Fig. 2D, right panels). To determine the physiological effect on this system by known cell stressors, we performed studies using benzo[a]pyrene (BaP) and tert-butyl hydroquinone (t-BHQ), known inducers of the Nrf2-NQO1 stress response pathway (Fig. 2E). Results indicated that in both control WT keratinocytes (transfected with only empty vector) and in cells transfected with C/EBPα expression vector, stress-induced upregulation of NQO1 caused increase in p63 transcriptional activity, with an additive effect upon the exogenous induction caused by C/EBPα overexpression. Finally, we investigated whether induction or inhibition of NQO1 activity via known NQO1-modulation agents B[a]P and dicoumarol were sufficient to modulate C/EBPα binding to the −185 site on p63 promoter (Fig. 2F). We found a dose-dependent ability of B[a]P to induce C/EBPα binding to this promoter site, and conversely we found a dose-dependent ability of NQO1-inhibition by dicoumarol to downregulate C/EBPα binding to the −185 promoter site.

To elucidate the role of the 20S proteasomal complex in C/EBPα regulation, we performed studies with known inhibitor of proteasomal activity MG-132 (Fig. 3A). We found that inhibition of 20S proteasomal activity was sufficient to inhibit C/EBPα degradation in both wild type and NQO1-null lines, suggesting that downregulation of C/EBPα protein in NQO1-null keratinocytes is at least in part due to degradation of protein by proteasomal pathways. Next we investigated whether the effect of proteasomal degradation was apparent when NQO1 was exogenously modulated. AfterNQO1 was inhibited via siRNA, protein levels of C/EBPα were downregulated, while this NQO1-mediated downregulation was mostly prevented after proteasomal inhibition with MG-132 (Fig. 3B). To determine that the 20S (ubiquitin-independent) pathway is responsible for C/EBPα depletion and not simply
the 26S (ubiquitin-dependent) pathway, we performed in vitro degradation studies using in vitro translated recombinant C/EBPα protein incubated for increasing periods with purified 20S proteasome (Sigma). We found that C/EBPα rapidly degrades in the presence of 20S proteasomal complex (Fig. 3C) and that therefore the ubiquitin-independent pathway is capable of regulating the degradation of C/EBPα protein. Further in vitro degradation studies using recombinant C/EBPα and NQO1 proteins incubated with purified 20S proteasomal complexes revealed that just as we previously discovered with p63 (Patrick and Jaiswal, 2011), while C/EBPα was efficiently degraded in presence of 20S proteasome, this 20S-mediated degradation was counteracted in the presence of NQO1 and NADH (Fig. 3D). This suggested that NQO1+NADH are sufficient to directly oppose 20S-mediated proteolysis of C/EBPα. Further studies showed that addition of NQO1 inhibitor dicoumarol was sufficient to abrogate NQO1-mediated protection of C/EBPα degradation by the proteasome (Fig. 3E). This also suggested that NADH bound NQO1 is essentially required for its inhibitory activity against proteolytic degradation of C/EBPα since dicoumarol is known to compete with NADH binding with NQO1 (Gong et al., 2007). To show in greater detail the necessity of NQO1+NADH in protection of C/EBPα, we performed degradation studies using NADH binding mutated forms of NQO1. We found that loss of NADH cofactor binding capacity (by mutation of tyrosines at 127 or 129) in NQO1 significantly impaired its ability to prevent 20S-mediated degradation of recombinant C/EBPα (Fig. 3F, Supplementary Fig. S2). In previous investigations we noted that NQO1 containing the well-known C>T polymorphism (which causes NQO1 protein instability) lost its ability to prevent p63 degradation; the same effect is apparent in the case of C/EBPα where degradation proceeds mostly unimpeded (Fig. 3F). It is noteworthy that addition of V5-tag to C-terminus of NQO1P187S led to stabilization of otherwise unstable mutant protein that protected C/EBPα against 20S degradation (Supplementary Fig. S2). Therefore, it is loss of mutant NQO1P187S protein and not the mutation itself that led to the inability of NQO1 to protect C/EBPα against 20S.

In an effort to determine whether the ability of NQO1 to directly oppose 20S-mediated degradation of C/EBPα is due to a direct interaction with either 20S proteasomal complex or C/EBPα, we performed several immunoprecipitation studies using both endogenously expressed and overexpressed proteins. We found that, similar to p63 and p53, C/EBPα is capable of interacting directly with both NQO1 (Fig. 4A) and with the 20S proteasome (Fig. 4B). NQO1 is, as was reported previously, not found to interact directly with the 20S proteasome (Fig. 4C). These results suggest that NQO1 protection of C/EBPα may be similar in nature to its protection of p53 and p63.

We next investigated the effects of known environmental stressor/carcinogen benzo[a]pyrene (B[a]P) (Fig. 5) and NQO1 inducing agent tert-butylhydroquinone (t-BHQ) (Supplementary Fig. S3) to determine their effects on NQO1-mediated induction of C/EBPα and its role in p63 gene expression. Studies were performed in both in vivo and in vitro systems using adult wild type and NQO1-null mice and tumor-derived keratinocyte cell lines from these mouse lines, respectively. We discovered that B[a]P induction of NQO1 was accompanied by induction of C/EBPα and p63 protein levels (Fig. 5). Results of these studies confirmed that environmental and chemical agents may lead to C/EBPα...
overexpression downstream to NQO1 induction, and prompted our investigation of other NQO1-inducing agents. We found that antioxidant t-BHQ in a dose- and time-dependent manner induced NQO1 expression that led to stabilization of C/EBPα protein and induction of C/EBPα target gene p63 expression (supplementary Fig. S3). After having established C/EBPα transcriptional activation of p63, we used p63 induction as a marker of C/EBPα activity and assayed both p63 transcription product (via quantitative PCR) and p63 translation product (via Western blot). We found that induction of NQO1 led to increased C/EBPα transcriptional activity (presumably by NQO1 rescue of C/EBPα from proteasomal degradation) and this increase was apparent in the later increase of p63 transcript and subsequent increase of p63 protein.

**DISCUSSION**

In previous studies we have investigated the role of NQO1 in protection of p53 and p63 against proteasomal degradation by ubiquitin-independent mechanisms (Gong et al., 2007; Patrick and Jaiswal, 2011). The role of NQO1 in protection of these factors, as well as protection of others such as ODC and c-Fos, has established NQO1 as a physiologically-relevant agent in transcription factor biology. In these studies we expand the role of NQO1 modulation of transcription factor p63 to include a previously-uninvestigated pathway by which NQO1 can exert regulation of p63 and therefore p63-dependent processes such as epithelial development.

After continuing studies of NQO1 regulation of p63 expression we found that p63 transcription was decreased in NQO1-null relative to wild type keratinocytes (Patrick and Jaiswal, 2011) and subsequently we expanded our line of research to investigate the cause of this downregulation further. We noted that C/EBPα expression corresponded well to p63 expression in both tissues and cell lines and that manipulation of C/EBPα led to changes in p63 expression which mirrored changes caused by manipulation of NQO1. Further investigation of the p63 promoter revealed putative C/EBPα binding sites which were investigated for transcriptional activity, leading to the discovery that similar to results found in human p63 (Higashikawa et al., 2007), there is a physiologically-active C/EBPα binding site at the mouse p63 promoter. Identification of a putative site allowed us to characterize its inducibility with wild type but not null NQO1, and show that its binding to p63 promoter is proportionate to NQO1 expression. These results help to establish a role of NQO1 in positive regulation of p63 transcription via upregulation of C/EBPα which can itself directly drive p63 promoter activity.

Our previous investigations of regulatory activities of NQO1 led to 20S (ubiquitin-independent) proteasomal degradation as a mediating factor in transcription factor degradation. We therefore looked to this same mechanism to explain how NQO1 may be protecting C/EBPα from depletion in wild type systems. Our numerous in vitro degradation studies showed that in fact C/EBPα is strongly degraded by 20S proteasomal complexes and that this degradation can be thwarted by presence of NQO1. Furthermore we discovered that like p53 and p63, NADH was an important parameter in ability of NQO1 to exert this protective effect upon C/EBPα against proteolysis due to 20S and that compromising the stability of NQO1 had consequences on C/EBPα stability against 20S-specific degradation.
Furthermore we investigated whether the characterized ability of NQO1 to bind directly to its target protein held true for C/EBPα. Studies involving both tagged NQO1 and C/EBPα, as well as endogenous-protein studies in both wild type and NQO1-null keratinocyte cell lines provided data which support the hypothesis that NQO1 does in fact bind directly to C/EBPα and thereby directly prevent its depletion from cells by proteasomal degradation. This is important to our understanding of NQO1 in that it further elucidates its ability and tendency to directly interact with transcription factor proteins to facilitate their protection from 20S-mediated proteolysis. This, in addition to the necessity of NADH cofactor and wild type genotype at P187 for protective activity, strongly indicate that NQO1 likely employs the same mechanism in protection of the ever-expanding collection of transcription factors from degradation.

To determine the physiological relevance of C/EBPα in the NQO1-p63 axis we investigated the effect of known NQO1-inducing agents, both environmental stressors such as benzo[a]pyrene and chemical inducers of NQO1 such as tert-butylhydroquinone, on C/EBPα induction and transcriptional activity. In all cases of NQO1 induction we found a dose- and time-dependent induction of C/EBPα as well as a later induction of C/EBPα transcriptional activity at the p63 promoter, leading to induction of p63 protein levels. These studies further suggest that C/EBPα is an efficient mediator of NQO1 modulation of p63 expression in keratinocytes and that C/EBPα-mediated activation of p63 promoter and subsequent increase of p63 mRNA in keratinocytes is a common physiological response following NQO1 induction by multiple means and preceding the direct protection of p63 protein levels by NQO1.

While it still appears that the primary mechanism for NQO1 modulation of p63 is via direct interaction and protection from ubiquitin-independent proteasomal degradation, there exists a secondary but significant pathway by which NQO1 and NADH mediate p63 expression in epithelium. In our hypothetical model (Fig. 6), in wild type systems, NQO1 protection of C/EBPα leads to buildup in tissues, which ultimately leads to increased C/EBPα transcriptional activity at established transcription factor binding sites on the p63 promoter and subsequent increase in p63 transcription, leading to increase in p63 levels, which can either be degraded by default by 20S proteosome or be protected by NQO1 interaction, leading to p63 buildup and subsequent increase in p63-dependent pathways such as keratinocyte differentiation. In NQO1-deficient systems, lack of NQO1 expression leads to a shift in the equilibrium between C/EBPα default degradation by 20S proteosome and protection, leading to increased C/EBPα proteolysis and subsequently decreased protein levels. The decreased C/EBPα protein levels leads to decreased transcriptional activity at the p63 promoter and thereby less p63 transcription. This decreased p63 transcription coupled with lack of NQO1-mediated rescue of p63 protein from 20S proteasomal degradation lead to stark decrease in overall p63 levels and thereby decreases in p63-dependent pathways.

In summary, we demonstrated that NQO1 along with its cofactor NADH stabilizes C/EBPα against 20S proteasomal degradation. This resulted in transcriptional activation of p63. NQO1 stabilization of C/EBPα required NQO1 cofactor NADH and a direct interaction with C/EBPα. Chemical stress induced NQO1 leading to stabilization of C/EBPα and increased transcription of p63. NQO1/C/EBPα-mediated transcriptional activation of p63 (current
report) along with NQO1/NADH-mediated stabilization of p63 (Patrick and Jaiswal, 2011) contributed to normal skin epithelium development and protection against chemical induced skin cancer.

**MATERIALS AND METHODS**

**Animals, cell culture, inhibitors, mutants and treatments**

C57BL/6 NQO1-null mice were derived by us as was previously reported (Radjendirane et al., 1998). Keratinocytes from these mice were derived from tumors induced by application of DMBA to skin as was described previously (Ahn et al., 2006) and subsequently cultured in DMEM/10%FBS/1% antibiotics and subcultured 2–3x each week. WT mouse keratinocytes were transfected using Amaxa Nucleofector machine with cell line-specific reagents according to manufacturer recommendations (Lonza, Basel, Switzerland). 20S proteasome complex inhibitor MG132 was obtained from Sigma (St. Louis, MO) and diluted in DMSO to produce stocks. DNA transfections were performed using Effectene reagent (Qiagen, Valencia, CA) and siRNA transfections were performed using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA). Cell lines which were not lab-derived were obtained from ATCC (Mannassas, VA). Mutants of human NQO1 were produced using GeneTailor site-directed mutagenesis kit (Invitrogen) as previously reported (Patrick and Jaiswal, 2011) according to manufacturer suggestions, using sequence provided by Pubmed (NM_000903) to determine relevant residues from previously-cloned HuNQO1 vector, and all clones were subcloned into pCDNA 3.1 vector for use.

**Histology**

Seven to nine week old male wild type and NQO1−/− mice were euthanized via isofluorane according to IACUC-approved protocols, and dorsal skin areas were shaved with clippers and removed by surgery. The skin was fixed in a solution of formalin overnight at room temperature with gentle stirring, and brought to University of Maryland Dermatopathology laboratory for embedding in paraffin blocks and microtomy, as well as mounting to charged slides and subsequent H&E staining.

**Western blot analysis**

Cell lysates and tissue homogenates were homogenized/lysed in RIPA buffer (20mM Tris-HCl, pH 7.4, 150mM NaCl, 1mM EDTA, 0.5% Deoxycholate, 1% NP-40, 1% Triton X-100, 1mM PMSF, and protease inhibitor cocktail (Roche, Konzern-Hauptsitz, Switzerland)) chilled on ice and clarified by centrifugation at ~10000xg. Protein concentration was estimated by Bradford method with diluted Bio-Rad reagent (Bio-Rad, Hercules, CA). SDS-PAGE analysis was performed as previously described (Gong et al., 2007). Antibodies against NQO1 were derived in our lab as described previously (Radjendirane et al., 1998); antibodies against Δ Np63α (4A4 with quantitation of the prominent band at 51–55 kDa), C/EBPα (14AA), and glyceraldehyde phosphate dehydrogenase (GAPDH), and protein A/G agarose bead slurry were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced Chemiluminescent reagent was purchased from GE Healthcare (Piscataway, NJ) and Pierce Biotechnology (Thermo Fisher Scientific, Rockford, IL).
**Immunoprecipitation**

Cells were plated into 150mm cell culture dishes and grown until 75% confluence before transfection. 48h post-transfection dishes were washed in PBS and scraped in RIPA buffer with protease inhibitor cocktail and rotated at 4°C for 20 minutes before centrifuging at 14000 rpm for 10 minutes to pellet debris. Supernatant was quantified and brought to 2mg/mL in RIPA and precleared with washed protein A/G-agarose beads (Santa Cruz) for 1h at 4°C, followed by bead removal and immunoprecipitation overnight with 1:100 20S core subunit antibody (Calbiochem, San Diego, CA), 1:1000 V5 antibody (Invitrogen), or 1:100 Flag antibody-conjugated agarose beads (Sigma). Endogenous IP studies were performed using the same antibodies as were used in Western Blot studies, at 1:100 concentration. The next day fresh A/G-agarose beads were added for 2h to bind antibodies and samples were washed twice in RIPA buffer before boiling 5 min in 2X SDS loading buffer.

**Quantitative PCR**

Cells were cultured in 10cm dishes and harvested using RNeasy (Qiagen) to purify total RNA, and DNA degraded using Turbo DNA-free (Ambion, Austin, TX). After confirming quality via spectrophotometry and on agarose gel, RNA (25–100ng) was used as a template for qPCR with appropriate TaqMan probes (Applied Biosystems, Foster City, CA) according to manufacturer protocols, n=3 per group normalized against internal controls (Rox) and also external controls (GusB for mouse keratinocytes) on ABI 7500 Real-Time PCR system.

**In-vitro translation and 20S proteasomal degradation of translated protein**

Proteins were translated from lab-generated plasmids using TnT in-vitro translation kit (Promega, Madison, WI) with cold (non-radioactive) methionine added. Purified 20S proteasomal complex was purchased from Sigma and dissolved in DMSO. NQO1-V5 and pcDNA-C/EBPα plasmid DNA were translated for 90 minutes at 30°C, and 2 micrograms of this was incubated with 20S proteasome in Degradation Buffer (100 mmol/L Tris-Cl (pH 7.5), containing 150 mmol/L NaCl, 5 mmol/L MgCl₂, and 2 mmol/L DTT) as previously described (Gong et al., 2007) for various time points and quenched via freezing at ~80°C. Samples were separated on 12% SDS-PAGE and immunoblotted. Blots were probed with V5-HRP (to detect NQO1-V5), 20Sα5 and p63 (4A4-HRP) antibodies. In related experiments, in vitro translated NQO1 without or with NADH and NQO1 inhibitor dicoumarol were included and 20S degradation of p63 experiments repeated. Dicoumarol was dissolved in 3% NaOH in deionized water for use, and NADH was dissolved in degradation buffer.

**Immunohistochemistry**

All animals were euthanized with isofluorane vapors according to IACUC-approved protocols and skin was removed using scissors after shaving with cordless clippers to remove excess hairs. Shaved skin patches were fixed in formalin solution (Sigma) overnight and afterwards placed into cassettes and embedded in paraffin at the University of Maryland Dermatopathology Laboratory. Paraffin blocks were sliced and sections were affixed to
charged slides. Slides were hydrated in xylene, 100% EtOH, 95%, 90%, 70%, 30%, followed by peroxidase blocking with 3% H2O2/MeOH, Retrievagen antigen retrieval (BD Pharmingen, Mississauga, ON, Canada) at ~95°C using steam for 30 minutes and using the Vectastain ABC-AP Kit (Vector Labs, Burlingame, CA) with primary antibodies (1:500 C/EBPα 14AA) for 1h in a dark humidified chamber, followed by staining with Vector Red Alkaline Phosphatase Substrate Kit I (Vector Labs), and hematoxylin counterstaining for 3 minutes, all according to manufacturers recommendations (Sigma), followed by sealing under coverslips with aqueous Vectamount (Vector Labs). Slides were photographed with Nikon Eclipse 80i upright microscope under Plan Apo 20x/0.75 DIC N2 WD 1.0 and Plan Apo 40x/0.95 DIC M/N2 WD 0.14 objectives, and all fields were rotated as necessary to match. Nikon NIS-Elements BR 3.0 software was used with the microscope, along with DS-Fi1 bright-field camera. All slide photography was done at room temperature and all files saved as jpeg format with each field subjected to ‘auto white balance’ before capturing, with no other manipulation.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**References**

Adler J, Reuven N, Kahana C, Shaul Y. c-Fos proteasomal degradation is activated by a default mechanism, and its regulation by NAD(P)H:quinone oxidoreductase 1 determines C-Fos serum response kinetics. Mol Cell Biol. 2010; 30:3767–3778. [PubMed: 20498278]

Ahn KS, Sethi G, Jain AK, Jaiswal AK, Aggarwal BB. Genetic deletion of NAD(P)H:quinone oxidoreductase 1 abrogates activation of nuclear factor-kappaB, IkappaBalpha kinase, c-Jun N-terminal kinase, Akt, p38, and p44/42 mitogen-activated protein kinases and potentiates apoptosis. J Biol Chem. 2006; 281:19798–19808. [PubMed: 16682409]

Asher G, Loten J, Cohen B, Sachs L, Shaul Y. Regulation of p53 stability and p53-dependent apoptosis by NADH quinone oxidoreductase 1. Proc Natl Acad Sci USA. 2001; 98:1188–1193. [PubMed: 11158615]

Asher G, Loten J, Sachs L, Kahana C, Shaul Y. Mdm-2 and ubiquitin-independent p53 proteasomal degradation regulated by NQO1. Proc Natl Acad Sci USA. 2002; 99:13125–13130. [PubMed: 12232053]

Asher G, Tsvetkov P, Kahana C, Shaul Y. A mechanism of ubiquitin-independent proteasomal degradation of the tumor suppressors p53 and p73. Genes Dev. 2005; 19:316–321. [PubMed: 15687255]

Garate M, Wong RPC, Campos EI, Wang Y, Gang Li. NAD(P)H:quinone oxidoreductase 1 inhibits the proteasomal degradation of the tumour suppressor p33ING1b. EMBO Reports. 2008; 9:576–581. [PubMed: 18388957]
Gong X, Kole L, Iskander K, Jaiswal AK. NRH:quinone oxidoreductase 2 and NAD(P)H:quinone oxidoreductase 1 protect tumor suppressor p53 against 20S proteasomal degradation leading to stabilization and activation of p53. Cancer Res. 2007; 67:5380–5388. [PubMed: 17545619]

Higashikawa K, Yoneda S, Tobiume K, Taki M, Shigeishi H, Kamata N. Snail-induced down-regulation of deltaNp63alpha acquires invasive phenotype of human squamous cell carcinoma. Cancer Res. 2007; 67:9207–9213. [PubMed: 17909026]

Iskander K, Gaikwad A, Paquet M, Long DJ, Brayton C, Barrios R, et al. Lower induction of p53 and decreased apoptosis in NQO1-null mice leads to increased sensitivity of chemical-induced carcinogenesis. Cancer Res. 2005; 65:2054–2058. [PubMed: 15781611]

Jaiswal AK, Bell DW, Radjendirane V, Testa JR. Localization of human NQO1 gene to chromosome 16q22 and NQO2-6p25 and associated polymorphism. Pharmacogenetics. 1999; 9:413–418. [PubMed: 10471077]

Kaspar JW, Niture SK, Jaiswal AK. Nrf2:INrf2 (Keap1) signaling in oxidative stress. Free Radic Biol Med. 2009; 47:1304–1309. [PubMed: 19666107]

King KE, Weinberg WC. P63: defining roles in morphogenesis, homeostasis, and neoplasia of the epidermis. Mol Carcinog. 2007; 46:716–724. [PubMed: 17477357]

Long DJ II, Jaiswal AK. NRH:quinone oxidoreductase2 (NQO2). Chemico-Biol Interact. 2000; 129:99–112.

Long DJ II, Waikel RL, Wang XJ, Perlaky L, Roop DR, Jaiswal AK. NAD(P)H:quinine oxidoreductase 1 deficiency increases susceptibility to benzo(a)pyrene induced mouse skin carcinogenesis. Cancer Res. 2000; 60:5913–5915. [PubMed: 11085502]

Long DJ II, Waikel RL, Wang XJ, Roop DR, Jaiswal AK. NAD(P)H:quinine oxidoreductase 1 deficiency and increased susceptibility to 7,12-dimethylbenz[a]-anthracene-induced carcinogenesis in mouse skin. J Natl Cancer Inst. 2011; 93:1166–1170. [PubMed: 11481389]

Mills AA, Zheng B, Wang XJ, Vogel H, Roop DR, Bradley A. p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature. 1999; 398:708–713. [PubMed: 10227293]

Moll UM, Slade N. p63 and p73: Roles in development and tumor formation. MolCancer Res. 2004; 2:371–386.

Patrick BA, Gong X, Jaiswal AK. Disruption of NAD(P)H:quinone oxidoreductase 1 gene in mice leads to 20S proteasomal degradation of p63 resulting in thinning of epithelium and chemical-induced skin cancer. Oncogene. 2011; 30:1098–107. [PubMed: 21042282]

Pietsch EC, Sykes SM, McMahon SB, Murphy SE. The p53 family and programmed cell death. Oncogene. 2008; 27:6507–6521. [PubMed: 18955976]

Radjendirane V, Joseph P, Lee YH, Kimura S, Klein-Szanto AJ, Gonzalez FJ, et al. Disruption of the DT diaphorase (NQO1) gene in mice leads to increased menadione toxicity. J Biol Chem. 1998; 273:7382–7389. [PubMed: 9516435]

Ross D. Quinone reductases multitasking in the metabolic world. Drug Metab Rev. 2004; 36:639–654. [PubMed: 15554240]

Siegel D, McGuinness SS, Winski SL, Ross D. Genotype-phenotype relationships in studies of a polymorphism in NAD(P)H:quinone oxidoreductase 1. Pharmacogenetics. 1999; 9:113–121. [PubMed: 10208650]

Traver RD, Horikoshi T, Danenberg KD, Stadlbaur THW, Danenberg PV, Ross D, et al. NAD(P)H:quinone oxidoreductase gene expression in human colon carcinoma cells: Characterization of a mutation which modulates DT-diaphorase activity and mitomycin sensitivity. Cancer Res. 1992; 52:797–802. [PubMed: 1737339]

Vasiliou V, Ross D, Nebert DW. Update of the NAD(P)H:quinone oxidoreductase (NQO) gene family. Hum Genomics. 2006; 2:329–335. [PubMed: 16595077]

Zhang Y, Kessler TW, Cho C, Posner GH, Talalay P. Anticarcinogenic activities of sulforaphane and structurally related synthetic norbornyl isothiocyanates. Proc Natl Acad Sci USA. 1994; 91:3147–3150. [PubMed: 8159717]
Figure 1.
NQO1 controls C/EBPα to regulate p63. A) Expression of C/EBPα corresponds to NQO1 levels in skin tissue and tumor-derived keratinocytes. Homogenates from Wild type (WT) and NQO1−/− mice, along with lysates from tumor-derived keratinocytes, were separated on 10% SDS-PAGE gels and probed with antibodies against NQO1, C/EBPα, p63, and GAPDH. Bar graphs beneath blots represent densitometric analysis of C/EBPα expression levels. B) p63 expression in skin tissue corresponds to C/EBPα expression. Skin from age-matched male 7–9 week old mice were harvested and prepared for immunohistochemical study, and probed with antibodies against C/EBPα and p63. Expression of each protein is visualized as red area. C) Modulation of NQO1 levels leads to corresponding modulation of C/EBPα and p63 levels in cells. WT mouse kerainocytes were transfected with pCDNA-NQO1-V5 (upper panels) or siRNA against NQO1 (lower panels). After 48h cells were harvested and lysates analyzed by SDS-PAGE and immunoblotting with indicated antibodies. D) Modulation of C/EBPα leads to corresponding modulation of p63 levels in cells. WT mouse kerainocytes were transfected with pCDNA-C/EBPα-V5 (upper panels) or siRNA against C/EBPα (lower panels). After 48h cells were harvested and lysates immunoblotted. E) C/EBPα control of p63 expression is downstream to NQO1 control of C/EBPα. WT mouse kerainocytes were transfected with pCDNA-C/EBPα-V5 and/or siRNA against NQO1. After 48h lysates were separated on SDS PAGE followed by immunoblotting and probing with indicated antibodies. Bar graph to right represents densitometric analysis of C/EBPα and p63 expression.
NQO1 and C/EBPα regulates p63 gene expression. A) p63 promoter construct is regulated by C/EBPα. 2.3Kb and 0.5Kb constructs were overexpressed in WT mouse keratinocytes along with either control vector or pCDNA-C/EBPα-V5 and luciferase activation was measured. B) −185 C/EBPα binding element binds with endogenous C/EBPα protein. Chromatin precipitation assay was performed on WT keratinocytes cultured under normal conditions, using PCR primers specific for 4 putative C/EBPα binding sites. C) Disruption of −185 C/EBPα binding site alone is sufficient to compromise NQO1- and C/EBPα-mediated p63 promoter activation. WT mouse keratinocytes were transfected with p63 promoter constructs containing either WT or mutated versions of each putative C/EBPα binding site along with overexpression of NQO1-V5 (white boxes) or C/EBPα-V5 (grey boxes) and analyzed for luciferase activity. The lysates were immunoblotted to show level of expression of NQO1-V5 and C/EBPα-V5. Bar graph represents p63 promoter activation. D) WT but not mutant NQO1 and C/EBPα act in concert to regulate p63 promoter activity. Left panel, 0.5Kb p63 promoter was transfected in WT mouse keratinocytes along with NQO1-V5 and/or C/EBPα-V5. Right panel, WT mouse keratinocytes were transfected with 0.5Kb p63 promoter construct and WT or inactive Y127/129A mutant NQO1-V5 plasmid. The transfected cells were immunoblotted and analyzed for luciferase activity. E) C/EBPα mediated induction of p63 promoter activity is enhanced by chemical agents. WT mouse keratinocytes were transfected with p63 promoter construct and either control plasmid or C/
EBPα-V5 plasmid and exposed to either vehicle control, Benzopyrene (left panel), or tert-butylhydroquinone (right panel) and analyzed for luciferase activity and immunoblotted for NQO1 and C/EBPα.

F) C/EBPα binding to −185 C/EBPα binding site on p63 promoter is modulated by known modulators of NQO1 activity. WT keratinocytes were exposed to either vehicle control, NQO1 inducer benzopyrene (top panel), or NQO1 inhibitor dicoumarol (bottom panel) for 12h before ChIP assay was performed using PCR primers against −185 C/EBPα binding site.
Figure 3.
NQO1 with cofactor NADH stabilizes C/EBPα against 20S proteasomal degradation. A) CEBPα levels in NQO1-null keratinocytes are modulated by proteasomal degradation. WT and NQO1-null keratinocytes were exposed to proteasome inhibitor MG-132 for 6h, lysed and separated on SDS-PAGE and immunoblotted with indicated antibodies. Bar graph beneath represents densitometric analysis of C/EBPα band. B) Loss of NQO1-mediated C/EBPα is modulated via proteasomal degradation. WT mouse keratinocytes were transfected with either control or NQO1-specific siRNA for 48h followed by incubation with vehicle or MG-132 for 6h. Lysates were separated on SDS-PAGE and immunoblotted with indicated antibodies. C) C/EBPα levels are strongly modulated by 20S proteasomal degradation. pcDNA-V5 was used in TnT in vitro translation to produce recombinant protein, which was incubated with 2 micrograms purified 20S proteasomal complex in degradation buffer for various time points. Reactions were halted by freezing and immediately separated via SDS-PAGE followed by immunoblot analysis. D) NQO1 with cofactor NADH is sufficient to rescue C/EBPα levels from 20S proteasomal degradation. C/EBPα protein was incubated with purified 20S proteasomal complex along with either vehicle, purified NQO1, or NQO1 with reduced NADH cofactor for 1h before halting reaction. The mixtures were analyzed by SDS-PAGE/immunoblotting. E) Inhibition of NQO1 disrupts NQO1-mediated protection of C/EBPα from 20S proteasomal degradation. In vitro degradation assay was performed as in D, with addition of experimental group containing 100 micromolar dicoumarol. F) WT but not NADH binding mutant NQO1 is capable of protection of C/EBPα against purified 20S proteasomal degradation. In vitro degradation assay was performed against recombinant C/EBPα protein containing equal amounts of either WT recombinant NQO1 or various mutant NQO1 species.
Figure 4.
Interaction between NQO1, C/EBPα, and 20S proteasome. A) Interaction between NQO1 and C/EBPα. Endogenous interactions (upper and middle panels). Endogenous NQO1 and C/EBPα were immunoprecipitated from wild type (WT) and NQO1-null (NQO1−/−) keratinocytes and immunoblotted against NQO1 and C/EBPα. Overexpressed interactions (lower panels). FLAG-NQO1 and C/EBPα-V5 were co-transfected in Hepa-1 cells and immunoprecipitated/immunoblotted with antibodies as indicated. B) Interaction between C/EBPα and 20S. Endogenous interactions (upper and middle panels). Endogenous 20S and C/EBPα were immunoprecipitated from WT and NQO1-null keratinocytes and blotted against 20S and C/EBPα. Overexpressed interactions (lower panels). Flag-C/EBPα was transfected in Hepa-1 cells and immunoprecipitated/immunoblotted with Flag and 20S antibodies. C) Interaction between NQO1 and 20S. Endogenous interactions (upper and middle panels). Endogenous NQO1 and 20S were immunoprecipitated from WT and NQO1-null keratinocytes and blotted against NQO1 and 20S. Overexpressed interactions (lower panels). NQO1-V5 was transfected in Hepa-1 cells and immunoprecipitated/immunoblotted with 20S and V5 antibodies.
Chemical induction of NQO1 leads to C/EBPα stabilization and increase in p63 gene expression in wild type (WT) but not in NQO1-null (1−/−) mice and keratinocytes. A) Benzo(a)pyrene (B[a]P) induction of NQO1 causes modulation of C/EBPα and Δ Np63. Wild type (WT) and NQO1-null (1−/−) mice and keratinocytes were exposed to 1800 nmol B[a]P for 8h and lysates analyzed by SDS-PAGE and immunoblotting with antibodies as indicated. B–C) B[a]P induction of NQO1 modulates C/EBPα levels and both RNA and protein levels of Δ Np63. Keratinocytes were exposed to B[a]P in a dose-(B) and time-(C) dependent manner and lysates were analyzed by immunoblotting and RNA subjected to Real Time PCR for Δ Np63α RNA levels (C, lower panels).
Figure 6.
Hypothetical model of NQO1 control of C/EBPα stability to regulate p63 expression. In wild-type cells, where NQO1 is present, NQO1 is able to directly protect C/EBPα protein against 20S proteasome-mediated degradation, and thereby allow C/EBPα to upregulate p63 expression and p63-downstream processes, leading to keratinocyte differentiation. In NQO1-null cells, lack of NQO1 allows C/EBPα to be efficiently degraded by 20S proteasomal complex, thereby leading to lower steady-state levels of C/EBPα, and therefore lower steady-state activation of C/EBPα sites on p63 promoter and less p63 expression, finally resulting in decreased signaling through p63-downstream processes leading to less keratinocyte differentiation.