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Repression of *Dmp1* and *Arf* transcription by anthracyclins: critical roles of the NF-κB subunit p65

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Abstract

Both genotoxic and oncogenic stress activates the nuclear factor-kappa B (NF-κB) and p53 proteins; however, the p53 activity is antagonized by NF-κB signaling. Dmp1 is a Myb-like transcription factor that activates the Arf-p53 pathway. The *Dmp1* promoter was activated by a classical NF-κB activator tumor necrosis factor α, but repressed by treatment of cells with non-classical NF-κB activators, anthracyclins and UV-C. p65 and other subsets of NF-κB proteins were bound to the *Dmp1* promoter following anthracyclin/UV-C treatment of rodent fibroblasts. This resulted in the downregulation of Dmp1 mRNA and protein. Repression of the *Dmp1* transcription by anthracyclins depended on the unique NF-κB site on the promoter. Downregulation of p65 significantly attenuated the repression of the *Dmp1* promoter by anthracyclins/UV-C. The amount of Dmp1 bound to the *Arf* promoter decreased significantly upon anthracyclin treatment; this, in turn, downregulated the Arf levels. Repression of the *Arf* promoter by p65 or anthracyclins depended on Dmp1, which was significantly attenuated in $Dmp1^{-/-}$ cells. Both $Dmp1^{-/-}$and $Arf^{-/-}$cells showed resistance to anthracyclin-induced cell death compared to wild-type cells; non-immortalized p65-knockdown cells were much more sensitive. Thus, the Dmp1-Arf pathway is repressed by p65 in response to genotoxic stress, which implicates a novel mechanism of p53 inactivation by NF-κB.

Keywords

Dmp1; Arf; p53; NF-κB; anthracyclin; UV-C

Introduction

The nuclear factor-kappa B (NF-κB) transcription factors were originally isolated and characterized for controlling gene expression and function in the immune system (Hayden and Ghosh, 2004). However, the importance of NF-κB in modulating stress signaling, cellular cycles, apoptosis and carcinogenesis has also been well documented (Karin *et al*., 2002; Hayden and Ghosh, 2004; Karin, 2006). In unstimulated cells, the NF-κB dimers are bound by inhibitory IκB molecules and stay in the cytoplasm. Since transcriptional activation by NF-κB requires its nuclear translocation, signal-induced degradation of IκB molecules is considered to be critical in NF-κB activation (Hayden and Ghosh, 2004). There are five distinct subunits of NF-κB, namely p65 (relA), relB, c-rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2). Among these, p50:p65 heterodimers are most abundantly expressed subunits in mammalian cells while p52 and c-rel subunits are mainly detected in hemato-poietic/lymphoid

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cells (Hayden and Ghosh, 2004, Karin *et al*., 2002). Owing to the presence of a strong transactivation domain, p65 is responsible for most NF-κB transcriptional activity.

Generally, NF-κB is a well-documented anti-apoptotic factor, which is simultaneously activated with p53 in response to chemotherapeutic agents' DNA damage, oncogenes and by tumor necrosis factor α (TNFα) (for review see, Perkins, 2004). These transcription factors modulate each other's activities. For instance, the *p53* promoter is activated by NF-κB in response to TNFα and to anticancer drugs. NF-κB plays essential roles in p53-mediated programmed cell death (Ryan *et al*., 2000). On the other hand, p53 stabilization is decreased upon NF-κB activation by chemotherapeutic reagents through the upregulation of Mdm2 (Tergaonkar *et al*., 2002). Competition for limiting pools of transcriptional coactivators p300 and CBP has also been reported to mediate a mutual repression between NF-κB and p53 (Webster and Perkins, 1999). The third mechanism of antagonism involves the activation of anti-apoptotic genes by NF-κB, which competes with proapoptotic pathways activated by p53 (Tergaonkar *et al*., 2002; Perkins, 2004). Indeed, many human cancers have constitutive activation of NF-κB and inhibition of NF-κB has been documented to lead to increased efficacy of anti-cancer drugs (Karin *et al*., 2002; Karin, 2006).

The activity of p53 is positively regulated by $p19^{Arf}$ ($p14^{ARF}$ in humans) in response to oncogenic stress. $p19^{Arf}$ is an alternative reading frame gene product generated from the *Ink4a/Arf* locus (Sherr, 2001; Lowe and Sherr, 2003). Since the single genetic locus encodes two independent tumor suppressor proteins that regulate the p53 and the Rb pathways, this locus is often disrupted in human cancer (Ruas and Peters, 1998). *Arf* is induced by potentially harmful growth-promoting signals stemming from overexpression of various oncoproteins (Sherr, 2001; Lowe and Sherr, 2003). This drives incipient cancer cells to undergo p53-dependent and p53-independent cell cycle arrest or apoptosis, providing a powerful mode of tumor suppression (Sherr 2001, 2006). Among known *Arf* activators, the Dmp1 transcription factor (cyclin D binding myb-like protein-1, also called Dmtf1) is a unique tumor suppressor (Inoue *et al*., 2000, 2001, 2007). Dmp1 was originally isolated in a yeast two-hybrid screen of a murine T-lymphocyte library with cyclin D2 as bait. Importantly, Dmp1 directly binds to the *Arf* promoter to activate its expression, thereby inducing p53-dependent cell cycle arrest (Inoue *et al*., 1999). *Dmp1*-null mice are prone to spontaneous tumor development, which was accelerated when the animals were neonatally treated with ionizing radiation or dimethylbenzanthracene (Inoue *et al*., 2000, 2001). *Dmp1* is haplo-insufficient for tumor suppression and is a physiological regulator of the Arf-p53 pathway *in vivo* (Inoue *et al*., 2001). The Dmp1 promoter is activated by the oncogenic Ras-Raf-MEK-ERK pathway, and the induction of *Arf* by Ras is Dmp1-dependent (Sreeramaneni *et al*., 2005). On the other hand, the *Dmp1* promoter is repressed by overexpression of E2Fs and physiological mitogenic signaling. Thus, Dmp1 is a novel marker of cells that have exited from the cell cycle (Mallakin *et al*., 2006).

In the present study, we examined the roles of NF-κB in the regulation of the Dmp1-Arf tumor suppressor pathway, especially through the non-classical NF-κB pathways activated by anthracyclins and UV-C. Here we demonstrate that NF-κB directly binds and inhibits the *Dmp1* promoter and decreases its expression in response to genotoxic stress.

Results

Responsiveness of the *Dmp1* promoter to p65 and atypical NF-κB activators

We studied the responsiveness of the *Dmp1* promoter to p50 and p65, the most ubiquitously expressed subunits of NF-κB in mammalian cells. Although p50 had little effect on the *Dmp1* promoter (data not shown), p65 efficiently inhibited its transcription (~fivefold) (Figure 1a). Conversely, the *Twist* promoter was activated considerably by p65, demonstrating that

repression of the *Dmp1* promoter by p65 was very specific (Figure 1b). The *Dmp1* promoter was efficiently repressed by all of the non-classical NF-κB activators (doxorubicin (DOX), daunorubicin (DNR)) or by UV-C, although the effect was more striking with anthracyclins (~75% repression) than with UV-C (~50% repression) (Figure 1c). Interestingly, the *Dmp1* promoter was activated by stimulation of murine embryonic fibroblasts (MEFs) with TNFα (Figure 1c). To study the degree to which repression of the Dmp1 promoter by anthracyclins depended on NF-κB, a reporter assay was performed by transfecting the cells with a p65 expression vector (Figure 1d). The basal *Dmp1* promoter levels decreased in cells transfected with p65 and the promoter became less responsive to DOX, DNR or TNFα (Figure 1d). The responsiveness of the *Dmp1* promoter to TNFα or genotoxic stress was completely lost by mutation of the unique NF-κB site of the promoter (−374 *Nsi*I NF-κB mut) or in the deletion construct that lacks this unique NF-κB site (−88 *Apa*I) (Figure 1e, Supplementary Figure 1). Furthermore, the repression of the *Dmp1* promoter was completely (DOX, DNR) or partially (UV-C) reversed by the addition of Trichostatin A to the tissue culture media, implicating the involvement of histone deacetylases in *Dmp1* promoter repression (Figure 1f). These results suggest that transcriptional repression of the *Dmp1* promoter by DOX, DNR or UV-C depends on the unique NF-κB binding site on the *Dmp1* promoter and involves histone deacetylases.

NF-κB binds to the *Dmp1* promoter upon TNFα and anthracyclin treatment

To confirm the binding of NF-κB proteins to the *Dmp1* promoter in response to TNFα and anthracyclins, an electrophoretic mobility shift assay (EMSA) was performed with a probe covering the NF-κB consensus sequence on the *Dmp1* promoter. When 3T3 cells were treated with TNFα, a specific complex A was formed and was supershifted with antibody to p65 (complex S, Figure 2a, middle panel). Other NF-κB proteins (p50, p52, relB and c-rel) were not found to associate with the *Dmp1* promoter upon TNFα treatment (Figure 2a, middle panel). When the cells were treated with 5 μM DOX for 90 min, complex A was generated, which was supershifted by incubating the complex with antibody to p65 (Figure 2a, right panel). This supershift was not observed with antibodies to p50, p52, relB or c-rel (Figure 2a, right panel). The pattern of EMSA and supershift assay with 1 μM DNR treatment was essentially identical with that of DOX, that is, p65 was the major component directly bound to the *Dmp1* promoter (data not shown). NF-κB binding to the *Dmp1* promoter was also studied by chromatin immunoprecipitation (ChIP) in MEFs (Figure 2b). p65 was the only subunit detected on the *Dmp1* promoter upon TNFα stimulation, as predicted from the EMSA (Figure 2b, second panel). p65 and relB were bound to the *Dmp1* promoter on DOX treatment of the cells, whereas p65 and c-rel were bound upon DNR treatment (Figure 2b, third and fourth panels). On the other hand, p52, p65 and c-rel were found on the *Dmp1* promoter upon UV-C treatment (Figure 2b, fifth panel). The specificity of NF-κB binding to the proximal region of the *Dmp1* promoter was confirmed by PCR amplification of the genomic DNA fragment located 2 kb upstream from the transcription initiation site as a control (Figure 2b, bottom panel). Taken together, our data suggest that (i) homodimers of p65 activate the *Dmp1* promoter in response to TNFα and (ii) subsets of NF-κB proteins, including p65, repress the *Dmp1* promoter upon anthracyclin and UV-C treatment of rodent fibroblasts.

Anthracyclin and UV-C treatment downregulate *Dmp1* and *Arf* in a p65-dependent fashion

Whether classical and non-classical NF-κB activators would affect *Dmp1* expression levels was then investigated. When 3T3 cells were treated with TNFα, *Dmp1* mRNA increased rapidly, peaking 15 min after treatment (Figure 3a, left panel). Conversely, treatment of wild-type MEFs with 5 μM DOX resulted in marked reduction of *Dmp1* mRNA by 4 h and was sustained through 8 h (Figure 3a, right panel). DNR (1 μM) and UV-C (40 J/m^2) treatment of MEFs affected *Dmp1* mRNA levels in a manner similar to DOX (Figure 3a). Real-time PCR demonstrated that *Dmp1* mRNA decreased to 20% with DOX, 10% with DNR and 18% with UV-C as compared to untreated cells (Figure 3b). These results were confirmed by

conventional semi-quantitative RT–PCR analysis (Supplementary Figure 2). The classical Dmp1 target *Arf* mRNA was also downregulated by DOX/DNR/UV-C treatment of MEFs at similar kinetics (Figure 3b). Next we examined whether the *Dmp1* promoter repression was dependent on p65 or on other NF-κB proteins by downregulating their expression with shRNA (Figure 3c). The level of *Dmp1* mRNA did not change significantly in non-immortalized *p65*-knockdown cells with any of the genotoxic treatments (Figure 3d, left panel). The level of *Arf* mRNA either remained unchanged or was paradoxically increased, in *p65*-knockdown cells with genotoxic stress (Figure 3d, right panel). Conversely, *Dmp1* was downregulated in *p52*- or *relB*-knockdown cells treated with DOX or DNR (Supplementary Figure 2d). These data suggest that downregulation of *Dmp1* or *Arf* mRNA by anthracyclins/UV-C is dependent on p65. We then examined the kinetics of Dmp1 and Arf protein expression following genotoxic stress (Figure 4). Dmp1 protein was significantly downregulated (80–90% decrease) at 6–32 h with DOX and 8–32 h with DNR treatment (Figures 4a and b, top panels). $p19^{Arf}$ was also downregulated with DOX/DNR at 6–32 h after exposure (second panels). p53 transiently increased at 4 h with DOX or DNR treatment and then declined when both Dmp1 and Arf were downregulated (third panels). A striking decline in the expression of the p53 target, $p21^{Cip1}$ was observed 6–32 h after anthracyclin treatment (fourth panels). In contrast, Dmp1 and $p19^{Arf}$ protein levels did not change significantly with DOX or DNR treatment in *p65*-knockdown cells (Figures 4c and d, top and second panels). Although there was significant accumulation of p53 in *p65*-knockdown cells, there was no decrease of p53 and its target $p21^{Cip1}$ 8–16 h after DOX or DNR exposure (third and fourth panels). Collectively, these results indicate that both Dmp1 and Arf protein expression are downregulated by anthracyclins, which are dependent on p65.

Dmp1 plays critical roles in transcriptional repression of the Arf promoter by p65

Although, we observed downregulation of $p19^{Arf}$ in response to DOX, DNR or UV-C, the *Arf* promoter lacks typical NF-κB binding sequences (Inoue *et al*., 1999). Therefore, we hypothesized that Dmp1 has essential roles in the downregulation of $p19^{Arf}$ with these treatments. A significant level of endogenous Dmp1 was bound to the *Arf* promoter before treatment of wild-type MEFs (Figure 5a). Interestingly, Dmp1 became undetectable on the *Arf* promoter 2–4 h after DOX treatment. Accordingly, the wild-type *Arf* promoter was significantly repressed by overexpression of p65 in 3T3 cells (Figure 5b, wild-type). Disruption of the Dmp1/Ets locus on the *Arf* promoter abolished its responsiveness to p65 (Figure 5b, Dmp1/Ets M). We then mutated the *Arf* promoter to the sequence that is recognized by Dmp1 (CCCGTATGC) or by Ets family proteins (CCCGGAAGC). The GTATGC mutant was repressed by p65, while the GGAAGC mutant was slightly activated by p65 (Figure 5b). These results suggest that Dmp1 is crucial for *Arf* promoter repression by p65. These data were confirmed by an independent experiment employing $Dmp1^{+/+}$ and $Dmp1^{-/-}$ MEFs. Overexpression of p65 as well as treatment of cells with DNR or DOX repressed the *Arf* promoter in wild-type cells (Figure 5c, left panel). The *Arf* promoter was unresponsive or significantly less responsive to p65 or DNR/DOX treatment in $Dmp1^{-/-}$ MEFs (Figure 5c, right panel). Taken together, our data suggest that anthracyclins downregulate the *Arf* levels through downregulation of Dmp1.

Both Dmp1-null and Arf-null cells are resistant to anthracyclin-induced cell death

To study the biological significance of the downregulation of the Dmp1-Arf pathway in response to genotoxic stress, wild-type, *Dmp1*-null, *Arf*-null and *p65*-knockdown cells were treated with DOX and DNR to study their survival (Figures 6a and b). Ninety-eight % and 95 percent of wild-type cells died after 64 h with 5 μM DOX or 1 μM DNR (dark blue lines) treatment, respectively. In $Dmp1^{-/-}$ and $Arf^{-/-}$ cells, the number of cells initially increased with both DOX and DNR treatment and then began to undergo apoptotic cell death; however, >20% of the cells still survived after 64 hours of exposure (Figure 6, pink lines for $Dmp1^{-/-}$

and green lines for *Arf*$^{-/-}$). A genomic DNA fragmentation assay confirmed that the cells were undergoing apoptosis (data not shown). No significant difference in the survival of *Dmp1*$^{-/-}$ cells versus the survival of *Arf*$^{-/-}$ cells with 5 μM DOX treatment was observed. Arf$^{-/-}$ cells showed more drug resistance than *Dmp1*$^{-/-}$ cells with 1 μM DNR treatment (Figure 6, green and pink lines), consistent with the results of the reporter assay shown in Figure 5c. Importantly, the freshly established *p65*-knockdown (*p65*KD) cells were very sensitive to DOX or DNR; all cells were dead within 36–48 h of drug treatment (Figure 6, light blue). The sensitivity of the p65 KD cells to DOX/DNR reverted to normal MEF levels after expression of p65 by retroviral infection (Figure 6, broken light blue lines; Figure 6c), suggesting critical roles for p65 in preventing anthracyclin-induced apoptotic cell death. On the other hand, the immortalized *p65*-knockdown cells (*p65* KD^{im}) were extremely resistant to DOX or DNR treatment (Figure 6, brown lines) and the sensitivity of the cells to DOX/DNR did not change significantly after p65 restoration (survival curves not shown). These results suggest that the absence of Dmp1 or p19^{Arf} is associated with relative resistance of the cells to anthracyclins and thus, normal cells escape from anthracyclin-induced cell death by downregulating the activity of the Dmp1-Arf-p53 pathway.

Our current hypothesis regarding the downregulation of the Dmp1-Arf pathway by atypical NF-κB activators is summarized in Figure 6d. In wild-type cells, the p65 subunit of NF-κB is directly or indirectly activated by anthracyclins or by UV-C, which, in turn binds to the *Dmp1* promoter and decreases its expression. This results in the downregulation of p19^{Arf}, which will attenuate the apoptotic cell death mediated by p53. On the other hand, the non-immortalized *p65*-knockdown cells are more sensitive to anthracyclins/UV-C-induced cell death due to the increased activity of the Arf-p53 pathway. The p53 activity is also regulated by genotoxic stimuli through phosphorylation by ATM (Bakkenist and Kastan, 2004; Kurz *et al*., 2004).

Discussion

The classical NF-κB activators, such as inflammatory cytokines and some classes of cytotoxic agents, induce a form of NF-κB to promote anti-apoptotic gene expression (Karin *et al*., 2002; Karin, 2006). On the other hand, the second class of activators, which include anthracyclins and UV-C, induce NF-κB to act as repressors of anti-apoptotic gene expression through p65 phosphorylation and association with HDACs (Campbell *et al*., 2004). Thus, NF-κB is an important mediator of apoptotic cell death as well. Our data show that both *Dmp1*$^{-/-}$ and *Arf*$^{-/-}$ MEFs are more resistant to DOX or DNR-induced cell death than wild-type cells, suggesting the proapoptotic roles of the Dmp1-Arf pathway in programmed cell death. Consistently, it was reported that p14^{ARF} expression sensitizes normal and cancer cells to apoptosis in the presence of DOX, although p14^{ARF} did not induce cell death by itself (Gallagher *et al*., 2005). Therefore, atypical NF-κB activators downregulate both proapoptotic (Dmp1, Arf) and anti-apoptotic (Bcl-xL, XIAP and A20) genes and thus, the roles of NF-κB in response to genotoxic stress are more intricate than previously believed.

Our results indicated that the response of immortalized *p65*-knockdown cells to genotoxic drugs was completely different from that of non-immortalized cells. It has been reported that immortalized *p65*-null MEFs are more resistant to UV-C-induced cell death than wild-type cells (Campbell *et al*., 2004). In agreement, our experiments also showed that spontaneously immortalized *p65*-knockdown MEFs were very resistant to DOX/DNR-induced cell death. However, non-immortalized *p65*-knockdown cells were much more sensitive to DOX/DNR-induced apoptosis (Figure 6). Therefore, p65 appears to behave as an anti-apoptotic protein in non-immortalized cells. Our results are consistent with the previous report that showed increased cell death and p53 induction with DOX treatment in *IKK1/2*-null cells that lacked detectable NF-κB activity (Tergaonkar *et al*., 2002). Since immortalization of *p65*-knockout

cells always involves inactivation of $p19^{Arf}$ or p53 (Gapuzan *et al*., 2005), the responsiveness of *p65*-knockdown cells to anthracyclins/UV-C is completely different depending on the integrity of the Arf-p53 pathway.

Although different NF-κB family proteins bind to the *Dmp1* promoter in response to DOX, DNR or UV-C signaling, downregulation of p65 was sufficient to abolish the inhibitory effects of these geno-toxic stimuli on the Dmp1-Arf pathway. On the other hand, downregulation of other NF-κB proteins, such as relB or p52, had little effect on the *Dmp1* promoter repression although they were clearly detected on the *Dmp1* promoter by ChIP. Since p65 was the only NF-κB subunit directly bound to the *Dmp1* promoter by EMSA in response to DOX/DNR, we speculate that other NF-κB proteins bind to the *Dmp1* promoter indirectly through their interaction with p65. The only exception to this hypothesis would be relB binding to the *Dmp1* promoter, since it has been reported that relB does not make heterodimers with p65 (Hayden and Ghosh, 2004). It is possible that relB binds to the *Dmp1* promoter indirectly as an intertwined homodimer (Huang *et al*, 2005) or by making heterodimers with residual levels of p50 or p52 (Figure 2b).

DOX treatment of cells stimulates nuclear accumulation and phosphorylation of p53, a process of which is mediated by ATM (Kurz *et al*., 2004). Our data show that Dmp1-Arf and p53 are differentially regulated by anthracyclins, within at least 4 h after drug treatment; however, both p53 and $p21^{Cip1}$ decreased when Dmp1 and $p19^{Arf}$ were downregulated. On the other hand, p53 or $p21^{Cip1}$ was not downregulated in *p65*-knockdown cells, where both Dmp1 and $p19^{Arf}$ remain elevated and the cells underwent extensive apoptosis 24–36 h after the genotoxic drug treatment when approximately 20% of wild-type cells survived. Thus, downregulation of the Dmp1–Arf pathway by p65 appears to mediate protection of normal cells from the extensive cell death induced by genotoxic drugs. This mechanism will be especially important with respect to the side effects of chemotherapeutic agents in normal tissues. The major mechanisms of action of anthracyclins have been considered to be stabilization of topoisomerase IIα cleavage complexes and generation of reactive oxygen intermediates (DeVita *et al*., 2005). The former causes protein-linked double- and single-stranded DNA breaks, which are converted into cytotoxic DNA damage and cell death. Thus, cancer cells are generally much more sensitive to anthracyclins than normal tissues even when they have *ARF* deletions or *p53* mutations simply because they divide more frequently than normal cells.

Both the TNFα-responsive element and the anthra-cyclin/UV-C-responsive elements were mapped to the unique NF-κB site on the *Dmp1* promoter. p65 was the major player in stimulation of the *Dmp1* promoter by TNFα, although other subsets of NF-κB were also detected on the promoter in response to DOX/DNR/UV-C. Our data agree with previous reports that suggested the involvement of subunits of NF-κB other than the classical p50:p65 heterodimer following geno-toxic and other stresses (Rocha *et al*., 2003; Saccani *et al*., 2003). Thus, fine differences in the NF-κB subunits that are bound to the *Dmp1* promoter could explain differences in the responsiveness of the *Dmp1* promoter to TNFα and genotoxic stress. It has been reported that TNF stimulation of cells activates the JNK/SAPK, p38MAPK and Jak-STAT pathways in addition to the classical IKK-IκB-NF-κB pathway (Watts, 2005; Yoshimura, 2006). Since the *Dmp1* promoter was repressed solely by overexpressing p65, p65 might interact with transcription factors that act downstream of these signaling pathways to activate *Dmp1* transcription in response to TNFα. Although it is not clear which other factors are involved in the *Dmp1* promoter activation by TNFα, apparently p65 plays a major role in this process since the responsive element was clearly mapped to the unique NF-κB site on the *Dmp1* promoter and direct binding of p65 was confirmed by EMSA.

In conclusion, our present study identified a novel pathway that directly links NF-κB activation and Dmp1–Arf signaling. Since Arf is a positive regulator of p53, our study implicates a novel mechanism of p53 inactivation by NF-κB.

Materials and methods

Electrophoretic mobility-shift assay

To detect proteins that bind to the NF-κB consensus sequence on the murine *Dmp1* promoter, lysates were prepared from 3T3 cells treated with 20 ng/ml TNFα for 30 min, 1 μM DNR for 90 min or with 5 μM DOX for 90 min. The nuclear lysate was incubated with [α-^{32}P]-labeled oligonucleotide probe covering the NF-κB consensus sequence of the murine *Dmp1* promoter obtained by annealing sense oligonucleotide 5′-ACGCCTGCCAAGGGAAGACCCTCCTTTACGTCT-3′ and its reverse complementary sequence (the NF-κB consensus sequence is underlined). For competition assays, a 100-fold excess of unlabeled oligonucleotides was added to reaction mixtures before probe incubation. To verify the identity of the proteins in shifted complexes, reaction mixtures were incubated with control non-immune rabbit serum or with specific antibodies to p50 (sc-7178x), p52 (sc-7386x), p65 (sc-7151x and sc-372x), relB (sc-226x) or c-rel (sc-71x) proteins.

Western blotting

For analysis of Dmp1, p19^{Arf}, p53, p21^{Cip1} and actin, affinity–purified polyclonal antibodies to the mouse Dmp1 (RAX, Mallakin *et al*., 2006), p19^{Arf} (sc-32748, Santa Cruz Biotechnology, Santa Cruz, CA, USA), p53 (sc-6243G), p21^{Cip1} (sc-6246) or Actin (sc-1615), were used, followed by incubation of the filters with HRP-conjugated second antibodies, reaction with the enhanced ECL detection kit (PerkinElmer, Boston, MA, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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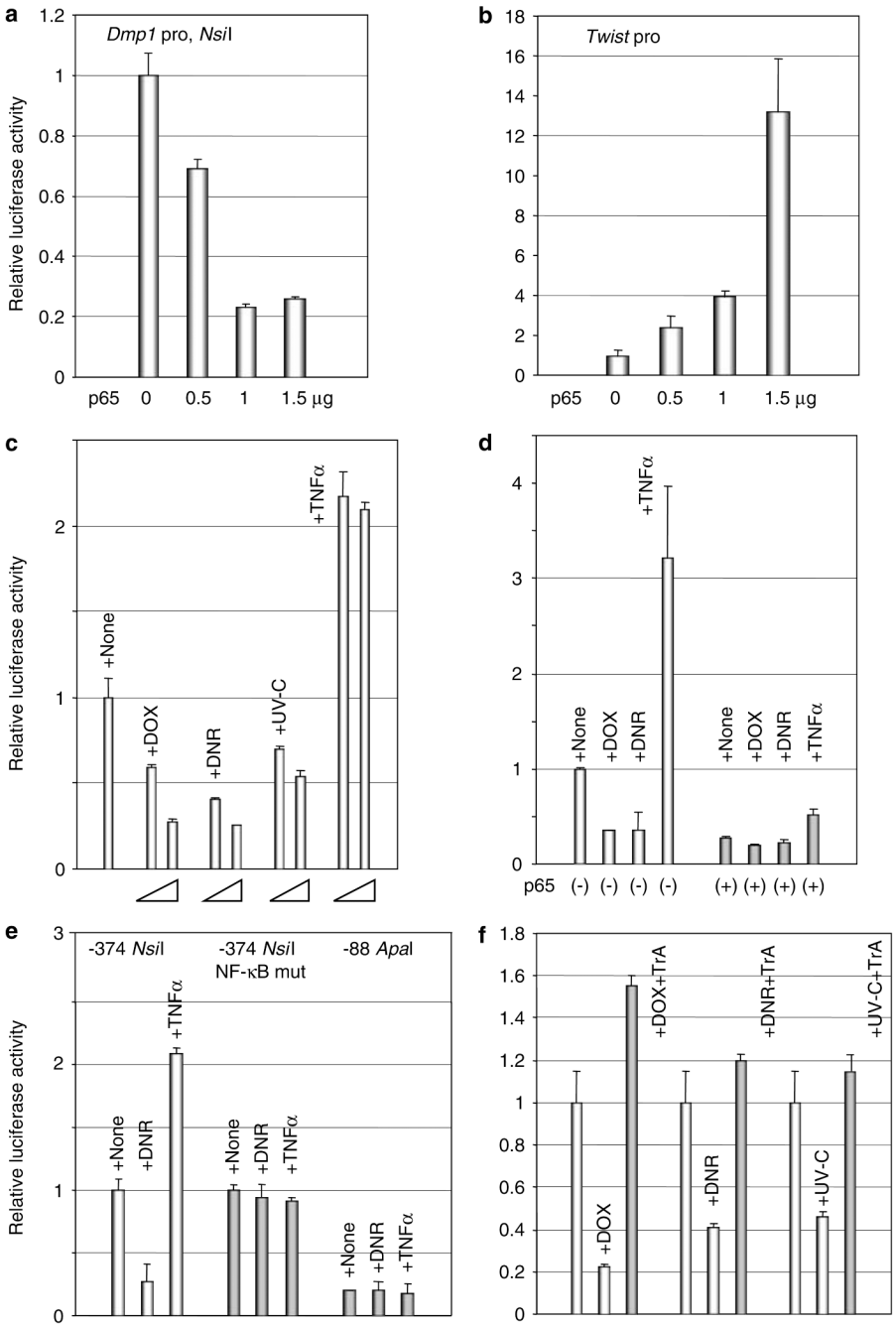

Figure 1.

Responsiveness of the murine *Dmp1* promoter to anthracyclins and UV-C. (**a**) The *Dmp1* promoter (−374 *Nsi*I fragment; Supplementary Figure 1) is repressed by p65 overexpression in 3T3 cells. (**b**) The classical nuclear factor-kappa B (NF-*κ*B) target, the *Twist* promoter is strongly activated by p65. (**c**) Responsiveness of the *Dmp1* promoter to doxorubicin (DOX), daunorubicin (DNR), UV-C or to tumor necrosis factor*α* (TNF*α*). NIH 3T3 cells were transfected with the −374 *Nsi*I construct and were treated with DOX (2–5 μM), DNR (1–2 μM), UV-C (20–40 J/m^2) or TNF*α* (10–20 ng/ml). Reporter assays were performed 14 h after the treatment of cells with DOX, DNR or UV-C and 2 h for TNF*α*. (**d**) The responsiveness of the *Dmp1* promoter to anthracyclins and UV-C is subverted by overexpressing p65. (**e**)

Repression and activation of the *Dmp1* promoter by DNR and TNFα are dependent on the unique NF-κB site on the promoter. (**f**) Repression of the *Dmp1* promoter by DOX, DNR or UV-C is histone deacetylase-dependent. The *Dmp1* promoter assay was conducted in the presence (black bars) or absence (white bars) of the HDAC inhibitor, Trichostatin A (TrA).

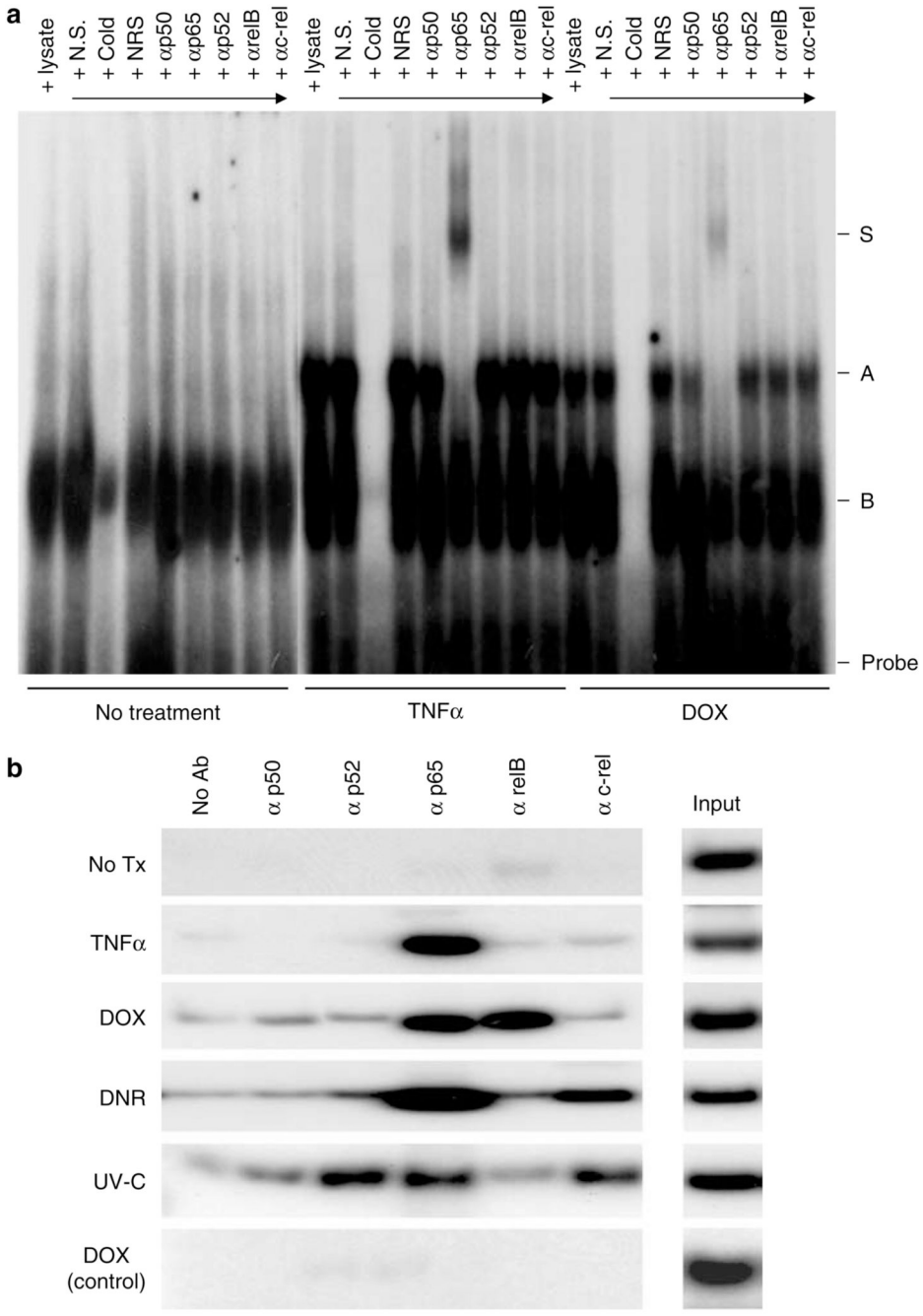

Figure 2.

Binding of nuclear factor-kappa B (NF-κB) proteins to the *Dmp1* promoter. (**a**) Left and middle panels: 3T3 cells were treated with or without 20 ng/ml of tumor necrosis factorα (TNFα) for 30 min and electrophoretic mobility shift assay (EMSA) was performed with oligonucleotides that covered the NF-κB consensus sequence (−229 to −238; Supplementary Figure 1a). NS: non-specific cold oligonucleotides; Cold: non-labeled oligonucleotide probe; NRS: normal rabbit serum. Right panel: 3T3 cells were treated with 5 μM doxorubicin for 90 min and EMSA was performed with the same probe. (**b**) Detection of the NF-κB proteins on the *Dmp1* promoter by chromatin immunoprecipitation (ChIP) assay. Wild-type murine embryonic fibroblasts before crisis were treated with TNFα, DOX, DNR or with UV-C and ChIP assays were

performed with specific antibodies to NF-κB proteins with primers covering the possible NF-κB sequence on the *Dmp1* promoter. No significant signals were obtained with control primers that amplify the *Dmp1* promoter sequence 2 kb upstream from the NF-κB site (DOX, control). Input indicates the relative intensity of signals from the standardized aliquot of the total input chromatin (0.2%).

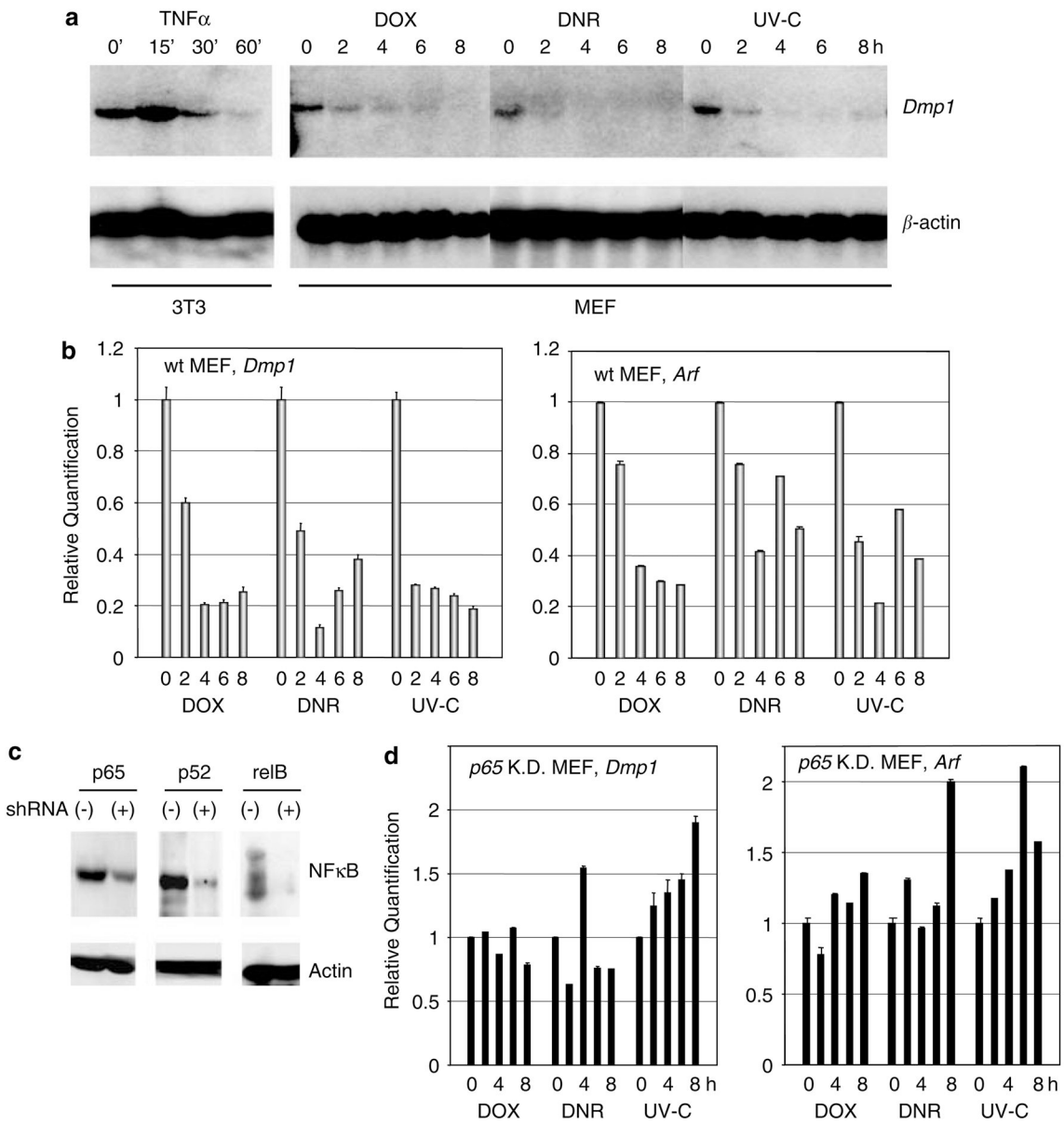

Figure 3.

Downregulation of the *Dmp1* and *Arf* by doxorubicin (DOX), daunorubicin (DNR) or UV-C depends on the p65 subunit of NF-*κ*B. (**a**) Detection of the *Dmp1* mRNA by northern blotting. NIH 3T3 or wild-type murine embryonic fibroblasts MEFs were treated with 20 ng/ml TNF*α*, 5 *μ*M DOX, 1 *μ*M DNR or with 40 J/m^2 UV-C and RNA was extracted at each time point. (**b**) Relative quantification of the *Dmp1* and *Arf* mRNA by real-time PCR. (**c**) Downregulation of NF-*κ*B proteins by shRNA. Wild-type MEFs were infected with retroviruses that express shRNA for NF-*κ*B genes, selected with puromycin and the NF-*κ*B proteins were detected by western blotting. (**d**) Relative quantitation of the *Dmp1* and *Arf* mRNA levels in *p65*-knockdown cells (*p65* KD) by real-time PCR.

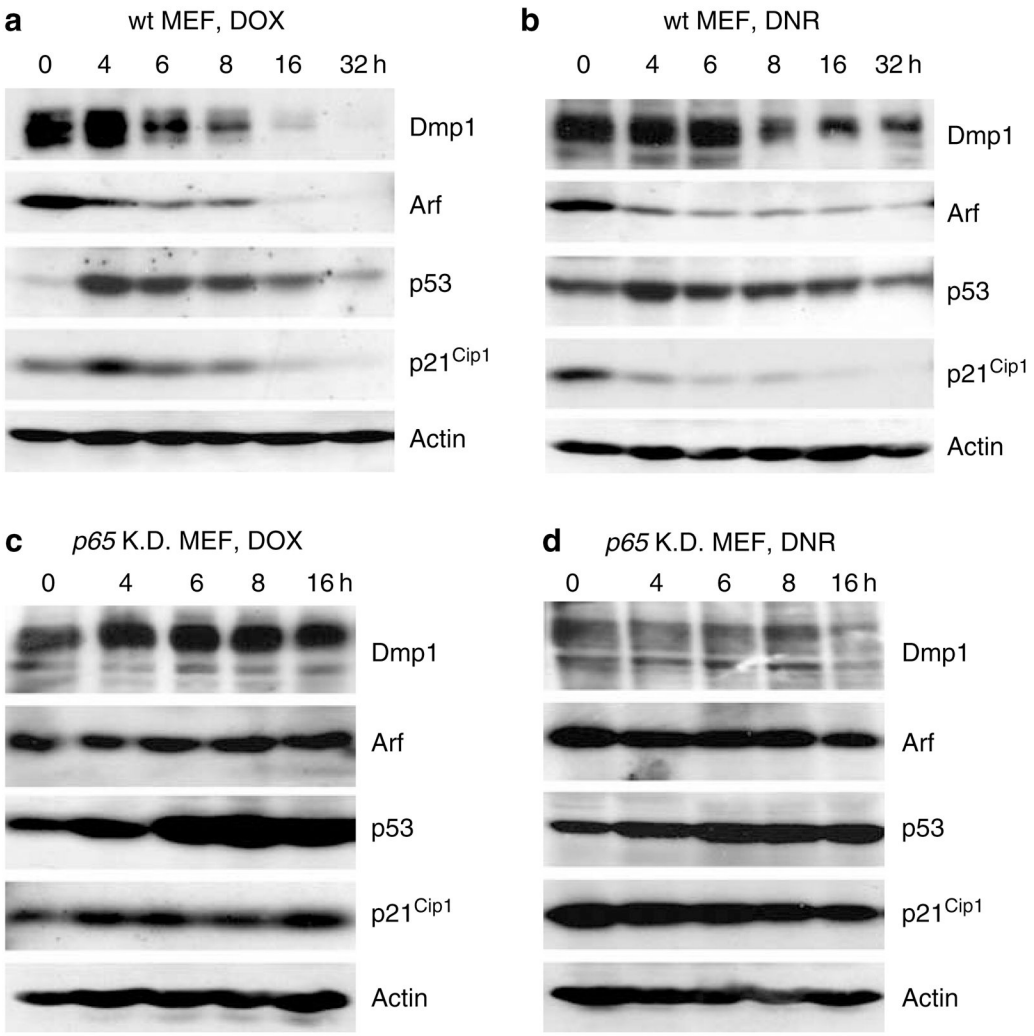

Figure 4.

Regulation of the Dmp1 and p19^{Arf} protein by nuclear factor-kappa B activators. (**a, b**) Both Dmp1 and p19^{Arf} proteins are downregulated in murine embryonic fibroblasts (MEFs) treated with 5 μM doxorubicin (DOX) (**a**) or with 1 μM daunorubicin (DNR) (**b**). Both p53 and its target protein p21^{Cip1} were downregulated when Dmp1 and p19^{Arf} decreased. (c, d) Non-immortal *p65*-knockdown MEFs treated with 5 μM DOX (**c**) or with 1 μM DNR (**d**). p53 and p21^{Cip1} were not downregulated after DOX/DNR treatment, reflecting the sustained activation of the Arf-p53 pathway.

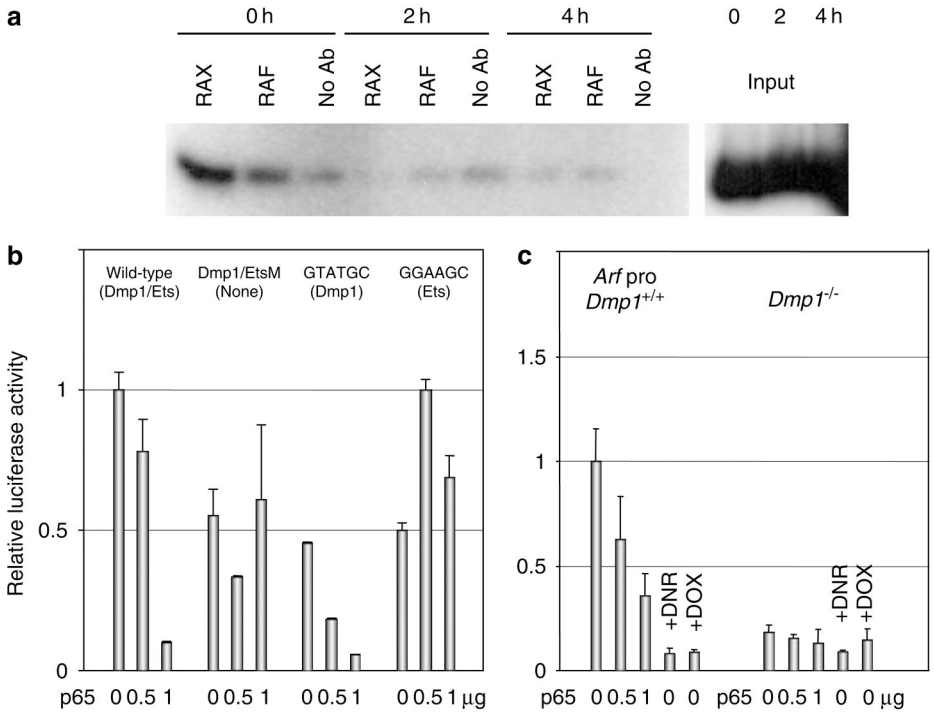

Figure 5.

The *Arf* promoter repression by anthracyclins depends on Dmp1. (**a**) Chromatin immunoprecipitation assay demonstrating the Dmp1 binding to the proximal promoter region of the *Arf* promoter. RAX and RAF designate the two different antibodies to Dmp1. Input indicates the relative intensity of signals obtained from 1% of the total chromatin. (**b**) The repression of the *Arf* promoter by p65 depends on the Dmp1/Ets site. The *Arf* promoter reporter assay was performed with −281 *BamHI* fragment with increasing amount of the p65 expression vectors. (**c**) Repression of the *Arf* promoter by p65 or by anthracyclins largely depends on Dmp1. The *Arf* promoter reporter assay was conducted in both wild-type and $Dmp1^{-/-}$ MEFs with increasing amount of the p65 expression vector or by treating the cells with doxorubicin or daunorubicin.

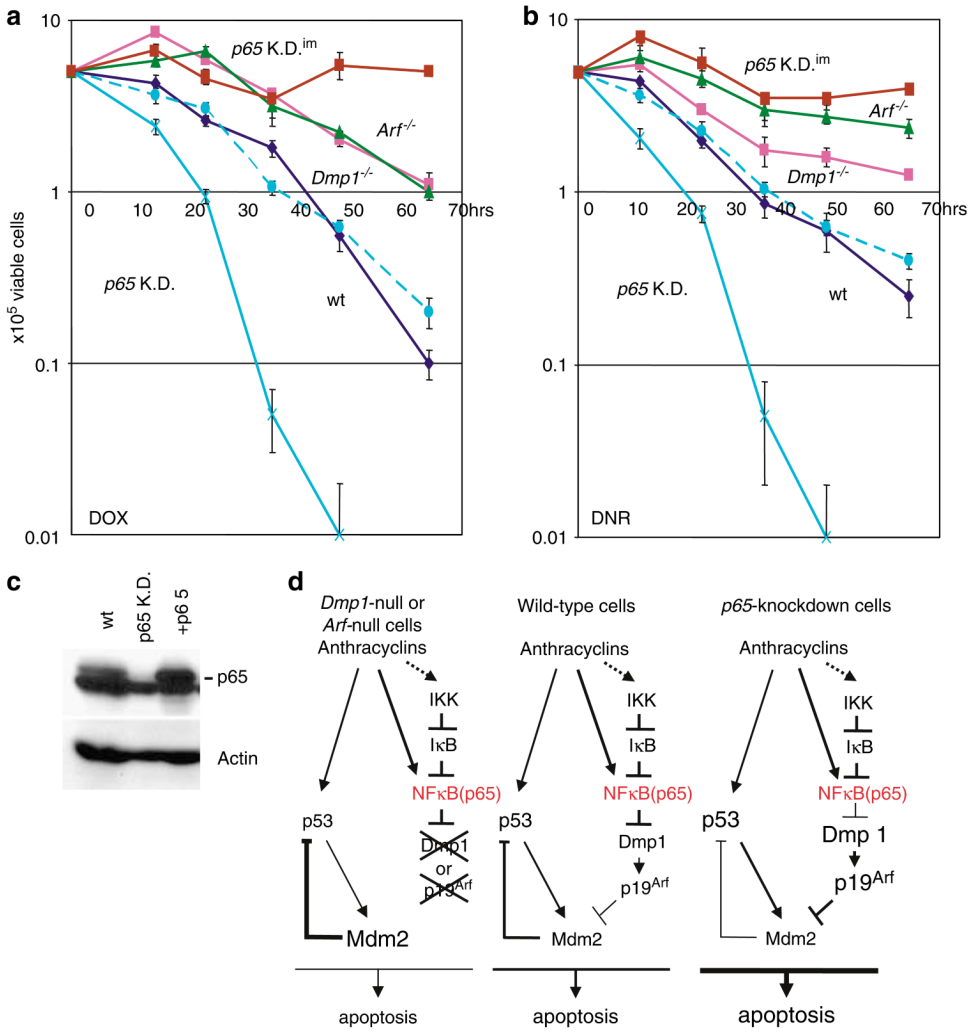

Figure 6.

Both *Dmp1*$^{-/-}$ and *Arf*$^{-/-}$ murine embryonic fibroblasts are resistant to anthracyclin-induced cell death. Wild-type (dark blue), *Dmp1*-null (pink), *Arf*-null (green) and *p65*-knockdown (light blue) cells were treated with either 5 μM doxorubicin (DOX) (a) or 1 μM daunorubicin (DNR) (**b**) to study their survival. Broken light blue lines: non-immortalized *p65*-knockdown cells restored with p65; brown lines: immortalized *p65*-knockdown cells. (**c**) Western blotting analysis shows restoration of p65 in *p65*-knockdown cells after retroviral infection. (**d**) Our current hypothesis regarding the downregulation of the Dmp1-Arf pathway by atypical NF-κB activators.