Nucleophosmin acts as a novel AP2 α -binding transcriptional corepressor during cell differentiation

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Nucleophosmin (NPM) is an important nucleolar phosphoprotein with pleiotropic functions in various cellular processes. In this study, we have further examined the largely uncharacterized role of NPM in transcriptional regulation by uncovering novel NPM-binding transcriptional factors. Among potential interactors, we found that activating protein transcription factor 2 (AP2) α forms a complex with NPM during retinoic-acid-induced cell differentiation. We show that this complex is recruited to the promoters of certain retinoic-acid-responsive genes, including NPM itself. Such binding of AP2 α , and consequent recruitment of NPM, is selective and dependent on a consensus AP2 α -binding sequence. Remarkably, suppression of NPM by RNA interference alleviates the repression of gene expression mediated by retinoic acid and AP2 α . Our findings further show that, on promoter binding, NPM probably exerts its repressive effect by inducing a change in local chromatin structure that also engages histone deacetylases. This study unveils a hitherto unrecognized transcriptional corepressor function of the NPM protein, and highlights a novel mechanism by which NPM regulates cell growth and differentiation.

Keywords: NPM; AP2; retinoic acid; differentiation; corepressor
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INTRODUCTION

Nucleophosmin (NPM) is a multifunctional nucleolar phosphoprotein that has crucial roles in the control of different aspects

of cell growth and homeostasis, such as ribosome biogenesis, centrosome duplication, cell cycle progression, apoptosis and cell differentiation (Grisendi *et al*, 2006; Naoe *et al*, 2006). Cumulative evidence has indicated that one aspect of cell growth control in which NPM might be involved is transcriptional regulation. Its role in this regard is partly mediated by interaction with several transcription factors, including YY1 transcription factor (YY1), nuclear factor- κ B (NF- κ B), alternate reading frame tumour-suppressor protein (ARF) and interferon regulatory factor-1 (IRF1; Inouye & Seto, 1994; Kondo *et al*, 1997; Dhar *et al*, 2004; Korgaonkar *et al*, 2005). These studies all point to the potentially vital function of NPM in regulating gene expression at the transcriptional level. However, the exact role and underlying mechanism remain largely undefined.

In this study, we first carried out a transcription factor–transcription factor (TF–TF)-binding array analysis to identify possible NPM-binding partners. Results from this assay confirmed previously identified interactions (YY1 and NF- κ B), and also uncovered novel associated factors. Among these proteins, activating protein transcription factor 2 (AP2) α was chosen for further study on the basis of its interaction with NPM. The choice was also made on the basis of its potential link with retinoic-acid-mediated gene expression, under which NPM was previously found to be regulated (Yung, 2004).

AP2 is a developmentally regulated transcription factor that interacts with inducible viral and cellular enhancer elements to positively and negatively regulate expression of selected genes (Williams & Tjian, 1991; Mohibullah *et al*, 1999). AP2 factors bind to the consensus sequence 5'-GCCNNNGGC-3' present in the regulatory regions of several genes, which are involved in a large spectrum of important biological functions, such as cell proliferation and development (Schorle *et al*, 1996; Nottoli *et al*, 1998). AP2 α is one of the five highly homologous AP2 isoforms that are encoded by different retinoic-acid-responsive genes (Tummala *et al*, 2003).

In the present study, we show that during retinoic-acid-induced cell differentiation, AP2 α recruits NPM to the promoters of certain retinoic-acid-responsive genes, such as *p120*, heat shock protein 60 gene (*Hsp60*), *b-Myb* and even *NPM* itself.

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We further demonstrate that NPM is directly involved in mediating gene expression induced by retinoic acid signalling, thus acting as a negative co-regulator. Finally, our results indicate that one possible mode of action of NPM might be to mediate a chromatin structure alteration that also involves histone deacetylase 1/2 (HDAC1/2).

RESULTS

AP2 α was identified as a candidate NPM-binding partner

To carry out a thorough examination of the transcriptional function of NPM, a TF–TF array analysis was carried out to identify NPM-associated candidates (see the supplementary information online). Results from this assay confirmed previously identified interactions, such as with YY1 and NF- κ B (data not shown), verifying the feasibility of this particular experimental system. Interestingly, this blot further uncovered AP2 α specifically as a novel NPM-associated factor.

To illustrate further the potential interaction of NPM with AP2 α , coimmunoprecipitation experiments were carried out. Endogenous NPM was found to be specifically coimmunoprecipitated with AP2 α (Fig 1A, lane 1) as well as ectopically expressed haemagglutinin (HA)–AP2 α (Fig 1B, lane 2). Conversely, endogenous AP2 α was also detected in the complexes pulled down by an NPM antibody (Fig 1A, lane 3). Moreover, in cells that transiently coexpressed HA–AP2 α and Flag–NPM, we were able to detect co-precipitation of HA–AP2 α by the Flag antibody (Fig 1B, lane 5). Further immunoprecipitation experiments using deletion constructs of AP2 α have shown that NPM might interact with AP2 α through its carboxyl terminus (supplementary Fig S1 online). To assess further association of these two proteins, we carried out a GST (glutathione S-transferase) pull-down assay with GST-fused recombinant NPM (Fig 1C, upper panel, lane 3) or AP2 α (lower panel, lane 6), and subsequently found that endogenous AP2 α and NPM could respectively be detected in the co-precipitates. In conjunction with the TF–TF array assay, these *in vitro* and *in vivo* binding experiments verify the existence of an NPM–AP2 α complex in the cell.

NPM and AP2 α are recruited to gene promoters

Next, we sought to address the transcriptional function of the NPM–AP2 α complex. Previous reports demonstrated that both NPM and AP2 α are targets of retinoic-acid during retinoic-acid-induced cell differentiation (Luscher *et al*, 1989; Yung, 2004). The responsiveness of their expression to retinoic acid treatment was analysed and confirmed. This test showed an increase in AP2 α , but a downregulation of NPM, at both protein and messenger RNA levels in the presence of retinoic acid (Fig 2B; supplementary Fig S2A online). Both proteins have been proposed to confer retinoic acid responsiveness to cells undergoing differentiation. We then explored further the potential involvement of these proteins in gene regulation during differentiation. First, we used the transcription element search software (TESS) analysis to look for the presence of AP2 α -binding consensus sequence in retinoic-acid-responsive gene promoters (Song *et al*, 2003). During this process, we inadvertently discovered that the *NPM* promoter surprisingly has such sites (supplementary Fig S2B online). On the basis of these observations, we therefore examined the functional relationship between AP2 α and NPM by first studying the retinoic-acid-mediated regulation of the *NPM* promoter. To

Fig 1 | Identification of AP2 α as a nucleophosmin-binding partner.

(A) Retinoic-acid-treated HL-60 cell extracts were subjected to immunoprecipitation by AP2 α (lane 1), NPM (lane 3) or control mouse IgG (lane 2) antibodies. The presence of AP2 α (upper panel) or NPM (lower) in these immunocomplexes was detected by immunoblotting. Extract input (lane 4) is 1/40 of the immunoprecipitation. LC, antibody light chain. (B) Lysates from HeLa cells transiently overexpressing HA–AP2 α alone (lanes 1–3) or HA–AP2 α + Flag–NPM (lanes 4–6) were immunoprecipitated with HA (lane 2), Flag (lane 5) or control mouse IgG (lanes 3,4) antibody. The lysates (lanes 1,6) and the immunocomplexes were then analysed by immunoblotting using HA (lanes 1–6), and Flag (lanes 4–6) or NPM (lanes 1–3) antibodies. (C) GST (lanes 2,5), GST-fused and NPM (lane 3) and GST–AP2 α (lane 6) were used as baits in the *in vitro* pull-down assay (as seen on Coomassie blue-stained gels). Nuclear extracts of retinoic-acid-treated HL-60 cells were subjected to binding reaction. The presence of bound AP2 α (top) or NPM (bottom) was detected by immunoblotting. Input (lanes 1,4) is 1/40 of pulldown. AP2, activating protein transcription factor 2; GST, glutathione S-transferase; HA, haemagglutinin; NPM, nucleophosmin.

this end, we used chromatin immunoprecipitation (ChIP) analysis to assess endogenous promoter occupancy. ChIP analysis was carried out on chromatin prepared from retinoic-acid-treated HL-60 cells. Our results indicate that, concomitant with differentiation-associated promoter downregulation, recruitment of AP2 α to the promoter is evident (Fig 2A; supplementary Fig S3A online). Intriguingly, NPM was also found *in vivo* in its own promoter, with kinetics of occupancy similar to AP2 α (Fig 2A; supplementary Fig S3A online). Conversely, c-Myc and YY1, two known transcriptional activators of *NPM* (Zeller *et al*, 2001; Yung, 2004), became dissociated from the *NPM* promoter region (supplementary Fig S2C online). Such loss of binding might presumably be attributed to the decrease in their expression levels on retinoic acid treatment (Fig 2B; supplementary Fig S2A online). Taken together, these studies show a dynamic occupancy of the *NPM* gene promoter by different factors during differentiation.

Fig 2 | Certain retinoic-acid-downregulated promoters that harbour AP2α-binding sites are occupied by both AP2α and nucleophosmin. (A) Chromatin was prepared from HL-60 cells previously treated with the indicated amounts of retinoic acid (0, 1 or 5 μM) for 48 h or 72 h as shown. ChIP analysis was carried out using control antibodies (IgG) or antibodies against AP2α or NPM. The graph represents the quantitative determination of bound DNA in the NPM and AP2α immunoprecipitates, as determined by quantitative RT-PCR. Data presented are normalized to input DNA (shown as a percentage), and the mean ± s.d. values from at least six experiments are shown. (B) HL-60 cells were treated with 0, 1 or 5 μM retinoic acid for 72 h. RNA was isolated from these cells and subjected to RT-PCR with primers specific to the corresponding retinoic-acid-responsive gene sequences (3-PGD; 3-phosphoglycerate dehydrogenase). The expression level of β-actin acts as a loading control (bottom). (C) ChIP analysis and quantification of promoter binding were carried out as in (A), using specific antibodies for AP2α (left) or NPM (right) and primers specific to *Hsp60*, *b-Myb*, *3-PGD* and *p120*. AP2, activating protein transcription factor 2; ChIP, chromatin immunoprecipitation; *Hsp60*, heat shock protein 60 gene; NPM, nucleophosmin; RT-PCR, reverse transcription-PCR.

To confirm our finding that NPM might act as a partner of the AP2α transcription repressor, we looked for other genes that were associated with retinoic-acid-induced differentiation (Song *et al*, 2003) and that might be targets of AP2α. Through TESS analysis, regulatory regions of the *Hsp60*, nucleolar protein *p120* and *b-Myb* genes were found to have putative AP2α-binding sites. Using reverse transcription-PCR (RT-PCR), our results confirmed that these genes were transcriptionally downregulated during retinoic-acid-induced differentiation (Fig 2B). Interestingly, concomitant with transcriptional repression, both NPM and AP2α were recruited in a similar manner to all these promoters (Fig 2C; supplementary Fig S3B online). By contrast, no recruitment of AP2α to the 3-phosphoglycerate dehydrogenase (*3-PGD*) gene promoter was observed (Fig 2C; supplementary Fig S3B online)—this gene was also responsive to retinoic acid (Fig 2B) but lacked the AP2α site. We also noted that NPM was correspondingly absent. Such colocalization at the promoters indicates functional involvement of NPM in the AP2α-mediated transcription repression.

AP2α recruits NPM to retinoic acid-induced promoters

To explain further the physical association of NPM with AP2α and their binding to the AP2 motif during retinoic-acid-induced

differentiation, we carried out the biotin-streptavidin pull-down assay (see Methods). The biotin-labelled 51-bp double-stranded oligonucleotides corresponding to -107 to -57 of the *NPM* promoter were incubated with HL-60 nuclear extracts. Our results showed that the presence of AP2α on the probe was dependent on retinoic acid induction as well as an intact AP2-binding motif (Fig 3A, upper panels). In addition, although NPM was incapable of associating with the probe under noninduction conditions, its binding was increased with retinoic acid treatment and was significantly disrupted by AP2α site mutations (Fig 3A, lower panels).

We next addressed the question of how the AP2α-NPM complex is recruited to its regulated promoters. First, we demonstrated the coexistence of NPM and AP2α at the *NPM*, *p120* and *Hsp60* promoters by sequential ChIP assays (Fig 3B, compare lanes 2 and 3). Next, we monitored the promoter binding of NPM and AP2α, respectively, in the AP2α RNA interference (RNAi) and NPM-RNAi cells (supplementary Fig S4 online). We subsequently found that NPM dissociates from the responsive promoters in the context of downregulated AP2α (Fig 3C, left graph; supplementary Fig S5 online). Conversely, knockdown of NPM had no effect on AP2α recruitment (Fig 3C, right graph). These ChIP data are consistent with the above pull-down assays,

Fig 3 | Retinoic-acid-induced promoter recruitment of nucleophosmin depends on AP2α. (A) Biotin-labelled oligonucleotide pull-down assay was carried out as described in Methods. Presence of bound AP2α (top) and NPM (bottom) was detected by immunoblotting analysis. (B) Coexistence of AP2α and NPM on promoters was demonstrated by a sequential ChIP experiment. Chromatin was first immunoprecipitated by AP2α antibody (lane 1). A second round of ChIP was carried out using control (lane 2) or NPM (lane 3) antibody. PCR analysis of binding to the *NPM* (top), *Hsp60* (middle) and *p120* (bottom) promoters is shown. (C) Chromatin was prepared from the control, AP2α^{RNAi} (left) or NPM^{RNAi} (right) cells. ChIP was carried out to probe for the *NPM* and *p120* promoter DNA fragment in the NPM (left) or AP2α (right) immunocomplexes. Quantification determination, as assessed by RT-PCR, was carried out as in Fig 2A ($n=6$; $**P<0.01$). AP2, activating protein transcription factor 2; ChIP, chromatin immunoprecipitation; *Hsp60*, heat shock protein 60 gene; NPM, nucleophosmin; RT-PCR, reverse transcription-PCR.

and collectively indicate that the presence of AP2α-binding sites, and therefore AP2α, is indispensable for the recruitment of NPM to particular retinoic-acid-responsive regulatory loci during differentiation.

NPM acts as an AP2α corepressor

To delineate further the functional significance of NPM in AP2α-mediated gene expression, we next sought to determine whether NPM functions as a corepressor. First, RT-PCR was carried out to examine the expression profile of retinoic-acid-responsive genes in the HL-60 cells that harbour NPM-targeting short interfering RNA (siRNA). Compared with the control, we discovered that knockdown of NPM resulted in greater accumulation of the *Hsp60*, *p120* and *b-Myb* transcripts that were previously found to be downregulated in the presence of retinoic acid (Fig 4A, top three panels). This observation indicates a reduction in retinoic-acid-mediated repression of these genes. Furthermore, reduction in transcriptional repression was not detected for the *3-PGD* gene (Fig 4A). This indicates that the effect of NPM was specific to retinoic-acid-responsive, AP2α-bound (and consequently,

NPM-bound) promoters (see the supplementary Fig S6 online for further discussion).

Further ChIP experiments demonstrated that retinoic-acid-mediated downregulation of promoters coincided with a reduction in the levels of a promoter-associated epigenetic mark (acetylated histone H4) and RNA polymerase II (Fig 4B, compare lanes 2 and 3 with lane 1). Promoters also undergo a retinoic-acid-responsive increase in the repression-associated mark (dimethyl-lysine 9 of histone H3), implying establishment of a more condensed chromatin state (Fig 4C; supplementary Fig S7A online). We next compared the levels of these marks at the retinoic-acid-responsive promoters in wild-type cells compared with NPM RNAi cells. We subsequently found that RNAi knockdown of NPM led to relatively elevated levels of acetylated H4 and bound RNA polymerase II at the promoters (Fig 4B). Conversely, there is a decrease in H3-lysine 9 dimethylation (Fig 4C; supplementary Fig S7A online). These data are consistent with a reversal of retinoic-acid-responsive transcriptional repression, and further emphasize the importance of NPM in this regulatory process.

NPM-mediated epigenetic alteration involves HDAC1/2

Having established that NPM acts as a corepressor linked directly to chromatin state modulation, we next aimed to determine the underlying mechanism. Towards this end, we targeted the HDAC enzymes on the basis of the above observation that promoter-associated histone acetylation underwent NPM-dependent changes. Among the HDACs tested (HDAC1–HDAC8), only interaction with HDAC1 and HDAC2 was consistently observed in reciprocal coimmunoprecipitation experiments (Fig 5A; data not shown). Furthermore, subsequent ChIP assays demonstrated that HDAC1/2 occupied AP2α-targeted promoter in response to retinoic acid treatment (Fig 5B; supplementary Fig S7B online). Such recruitment was dependent on NPM as promoter binding was diminished in the NPM RNAi cells (Fig 5B; supplementary Fig S7B online). These results indicate that one likely mode of repressive action of NPM might lie in the recruitment of HDACs to the targeted promoters, which ultimately leads to a repressed local chromatin structure.

DISCUSSION

In this work, we reported the transcriptional role of the AP2α–NPM complex during retinoic-acid-induced differentiation of the leukaemic HL-60 cell line, and demonstrated a chromatin-based mechanism through which NPM might act as a corepressor. Interestingly, a role of NPM in retinoic-acid-associated transcriptional regulation was reported previously, albeit in a pathological context (Redner *et al*, 1996, 1997). Redner *et al* (2000) further demonstrated that the t(5;17) acute promyelocytic leukaemia fusion protein NPM–RAR acts as a transcriptional activator or repressor of retinoic-acid-responsive genes, depending on the context of the promoter. NPM seems to have an analogous mode of involvement in both the leukaemia-associated and the AP2α-targeted transcriptional programmes. In this regard, NPM acts as a regulator of retinoic-acid-responsive, sequence-specific transcription factors. However, the two systems have different underlying molecular mechanisms. Although the NPM–RAR fusion proteins might have both positive and negative transcriptional properties, our study indicated that NPM primarily acts

Fig 4 | RNA interference depletion of nucleophosmin alleviates transcriptional repression of AP2α-regulated genes during retinoic acid treatment. (A) Control (lanes 1–3) or *NPM*^{RNAi} (lanes 4–6) HL-60 stable lines were treated with 1 or 5 μM retinoic acid (RA) for 72 h. The mRNA level of the specified genes in these cells was examined by RT-PCR and measured by quantitative RT-PCR (see Methods). The bar graph is shown with the normalized values in the mock-treated cells represented as 100, and with the mean ± s.d. values from three independent experiments. (B) Chromatin fragments were prepared from retinoic-acid-treated control (lanes 1–3) or *NPM*^{RNAi} (lanes 4–6) HL-60 cells. ChIP was carried out with control (IgG), acetyl-H4 or RNA pol II antibody, as denoted on the left. Quantitative determination of the bound DNA, carried out with RT-PCR, is depicted by the bar graphs on the right. The mean ± s.d. values from at least six experiments are shown. (C) ChIP and quantitative RT-PCR analyses were carried out as in (B), but with control (IgG) or dimethylated-H3 (Lys9) antibody (as indicated by H3K9me2). Statistical significance of the indicated comparisons: **P* < 0.05; ***P* < 0.01; ****P* < 0.001. 3-PGD, 3-phosphoglycerate dehydrogenase; AP2, activating protein transcription factor 2; ChIP, chromatin immunoprecipitation; *Hsp60*, heat shock protein 60 gene; NPM, nucleophosmin; RNAi, RNA interference; RT-PCR, reverse transcription-PCR.

Fig 5 | Histone deacetylase 1/2 contributes to nucleophosmin-mediated transcriptional repression. (A) Lysates from HeLa cells transiently overexpressing control vector (lane 1), Flag-HDAC1 (lane 2) or Flag-HDAC2 (lane 3) were immunoprecipitated with Flag or NPM antibody (as indicated). The cell lysates (top two panels) and the immunocomplexes were then analysed by immunoblotting. (B) ChIP was carried out as in Fig 4B, but with control (IgG), HDAC1 or HDAC2 antibody, as indicated. Quantitative determination of the bound *Hsp60* and *p120* promoter DNA is depicted by the bar graphs. Statistical significance of the indicated comparisons: ** $P < 0.01$; *** $P < 0.001$.

AP2, activating protein transcription factor 2; ChIP, chromatin immunoprecipitation; HDAC, histone deacetylase; *Hsp60*, heat shock protein 60 gene; NPM, nucleophosmin; RT-PCR, reverse transcription-PCR.

as a corepressor in AP2 α -targeted gene expression. Furthermore, Redner *et al* (2000) did not detect an interaction between NPM and corepressor. Despite this fact, we were able to identify the existence of the NPM-HDAC1/2 complexes and a role of HDAC1/2 in NPM-mediated repression (Fig 5). Intriguingly, the t(5;17) genomic translocation is not limited to generating only a variant form of transcription factor. It might also deplete the endogenous NPM protein pool from the AP2 α -mediated transcriptional network during retinoic acid signalling, thereby leading to de-repression of the target genes. Consequently, such grossly aberrant transcriptional regulation might be a possible underlying factor in the pathogenesis of acute promyelocytic leukaemia.

Although potential involvement of NPM in the transcriptional aspect of gene expression has previously been documented, its mode of action in this capacity is largely uncharacterized. Furthermore, it is intriguing that NPM acts as a transcriptional regulator that is also subject to autoregulation (for a discussion on

this issue, see supplementary information online). Although a nucleic-acid-binding ability has been ascribed to this protein (Okuwaki *et al*, 2002), evidence for sequence-specific DNA binding is lacking. On the basis of our data and that of others, one likely mode of NPM recruitment to the regulatory region of a gene might be through a transcription factor, such as AP2 α or NF- κ B (Dhar *et al*, 2004). Furthermore, as in the retinoic acid system, it is evident that NPM-mediated gene repression might be through a chromatin-based mechanism (Figs 4,5). Consistent with a role in epigenetic modulation, NPM has previously been known as a histone chaperone (Okuwaki *et al*, 2001). Intriguingly, recent results on the interaction of NPM with CTCF (CCCTC-binding factor) have implicated NPM in enhancer/promoter-blocking insulator activity (Yusufzai *et al*, 2004), providing further evidence that NPM might participate in transcriptional repression at a more global level.

METHODS

Cell culture and generation of stable lines. HL-60 leukaemic cells (in suspension) were grown as described previously (Yung, 2004). HL-60 cells that stably express siRNA were generated by electroporation followed by clone selection in the presence of G418 (1 mg/ml).

Antibodies. The monoclonal antibody to NPM was kindly provided by Dr P.K. Chan (Baylor College of Medicine, Houston, TX, USA). Rabbit polyclonal antisera against YY1, AP2 α and HDAC2 were obtained from Santa Cruz (CA, USA). c-Myc polyclonal, acetyl-histone H4 (ChIP grade), HDAC1 and dimethyl-histone H3 (lysine 9) antibodies were purchased from Upstate (VA, USA). To achieve unambiguous immunoblotting of AP2 α , the molecular size of which is similar to that of the immunoglobulin heavy chain, a TrueBlot rabbit secondary antibody (eBiosciences, San Diego, CA, USA) was used in the corresponding immunoblotting experiments. This antibody recognizes only the native form of immunoglobulin.

Other experiments. Generation of siRNAs, RNA isolation and RT-PCR, identification of transcription-factor-binding sites, ChIP, real-time PCR and biotin-streptavidin pull-down assay were carried out as described in the supplementary information online.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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