TRAP220 is modulated by the antineoplastic agent 6-Mercaptopurine, and mediates the activation of the NR4A subgroup of nuclear receptors

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Abstract

The NR4A1–3 (Nur77, NURR1 and NOR-1) subfamily of nuclear hormone receptors (NRs) has been implicated in Parkinson's disease, schizophrenia, manic depression, atherogenesis, Alzheimer's disease, rheumatoid arthritis, cancer and apoptosis. This has driven investigations into the mechanism of action, and the identification of small molecule regulators, that may provide the platform for pharmaceutical and therapeutic exploitation. Recently, we found that the purine antimetabolite 6-Mercaptopurine (6-MP), which is widely used as an anti-neoplastic and anti-inflammatory drug, modulated the NR4A1–3 subfamily. Interestingly, the agonist-mediated activation did not involve modulation of primary coactivators' (e.g. p300 and SRC-2/GRIP-1) activity and/or recruitment. However, the role of the subsequently recruited coactivators, for example CARM-1 and TRAP220, in 6-MP-mediated activation of the NR4A1–3 subfamily remains obscure. In this study we demonstrate that 6-MP modulates the activity of the coactivator TRAP220 in a dose-dependent manner. Moreover, we demonstrate that TRAP220 potentiates NOR-1-mediated transactivation, and interacts with the NR4A1–3 subgroup in an AF-1-dependent manner in a cellular context. The region of TRAP220 that mediated 6-MP activation and NR4A interaction was delimited to amino acids 1–800, and operates independently of the critical PKC and PKA phosphorylation sites. Interestingly, TRAP220 expression does not increase the relative induction by 6-MP, however the absolute level of NOR-1-mediated trans-activation is increased. This study demonstrates that 6-MP modulates the activity of the NR4A subgroup, and the coactivator TRAP220.

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Introduction

Nuclear hormone receptors (NRs) function as ligand-activated transcription factors that regulate gene expression involved in reproduction, development and general metabolism (Gronemeyer & Laudet 1995). NRs function as the pipeline between environmental stimuli and gene expression, effecting physiological changes. The importance of NRs in human physiology is underscored by the extensive range of therapeutics that have been created to combat disorders associated with dysfunctional hormone signalling. These diseases affect every discipline of medicine (Chawla *et al.* 2001). The importance of the NR4A1–3 (Nur77, NURR1 and NOR-1) subfamily of NRs as potential therapeutic targets for disease was made through the discoveries that Nur77, NURR1 and NOR-1 were implicated in Parkinson's disease, schizophrenia, manic depression, atherogenesis, Alzheimer's disease, rheumatoid arthritis, cancer and apoptosis (Murphy & Conneely 1997, Borghaei *et al.* 1998, Saucedo-Cardenas *et al.* 1998, Backman *et al.* 1999, Labelle *et al.* 1999, Schimmel *et al.* 1999, Buervenich *et al.* 2000, Newman *et al.* 2000, Chen *et al.* 2001, Monajemi *et al.* 2001, Arkenbout *et al.* 2002,

Liu *et al.* 2002, McEvoy *et al.* 2002, Maltais *et al.* 2002, Wu *et al.* 2002, Kim *et al.* 2003, Le *et al.* 2003).

All members of the NR superfamily display a highly-conserved structural organisation, with an amino terminal region AB that encodes activation function 1 (AF-1); followed by the C region which encodes the DNA binding domain (DBD); a linker region D and the C-terminal E region. The DE region encodes the ligand binding domain (LBD) and a transcriptional domain, denoted as activation function 2 (AF-2) (Gronemeyer & Laudet 1995). The NR4A1–3 subfamily of orphan nuclear receptors is well conserved in the DBD (~91–95%) and the C-terminal LBD (~60%), but divergent in the N-terminal AB region (e.g. AF-1).

A decade ago, gene products were identified that appeared to belong to the nuclear receptor superfamily on the basis of their nucleic acid sequence identity. The endogenous signalling molecules which bound to these proteins were unknown and thus the term 'orphan receptor' was adopted. Thus the orphans forecast an enormous yet unexploited opportunity for the discovery of important new therapeutic agents.

6-Mercaptopurine (6-MP) belongs to the thiopurines, a group of substances structurally related to endogenous

purine bases like adenine, guanine, and hypoxanthine. Azathioprine (parent compound of 6-MP) and other thiopurine drugs have been administered in the treatment of leukaemia and a number of autoimmune conditions, such as lupus erythematosus, pemphigus, and myasthenia gravis. More recently their use has been extended to the management of patients with chronic inflammatory bowel diseases (IBDs) including Crohn's disease and ulcerative colitis, and also multiple sclerosis. However, the precise mechanism of action of the thiopurine drugs remains unclear, despite more than 40 years of use. (Cara *et al.* 2004). It has been shown that this purine anti-metabolite has anti-proliferative and cytotoxic effects resulting primarily from the inhibition of purine *de novo* biosynthesis at multiple steps and incorporation into nucleic acids as thioguanine nucleotides (Cara *et al.* 2004).

Our previous work has expanded the function of purine anti-metabolites by demonstrating that 6-MP targets the NR4A1–3 subfamily of NRs and regulates its activity (Ordentlich *et al.* 2003, Wansa *et al.* 2003). This suggested that the signalling pathways that inhibit proliferation via inhibition of *de novo* purine and/or nucleic acid biosynthesis are involved in the regulation NR4A1–3 activity. The absence of a conventional ligand binding pocket in the NR4A1–3 LBD may account for the inability of 6-MP to directly bind to the NR4A1–3 subfamily (Baker *et al.* 2003, Wang *et al.* 2003). 6-MP is a specific activator of the NR4A1–3 subfamily of NRs, as FXR, RXR, LXR α , ROR α and ER α are not activated by 6-MP (Ordentlich *et al.* 2003). Furthermore general transactivators (GALVP16), and the myogenic transactivators (MyoD, Myogenin and MFE2C), and the coactivators steroid receptor coactivator-2 (SRC-2) and p300 are not activated by 6-MP (Wansa *et al.* 2003).

Ligand-dependent transcriptional activation by NRs is a process that involves a stepwise recruitment of various coactivators to the promoters of hormone-regulated genes assembled into chromatin. Ligand-dependent recruitment of the p160 factors (SRCs) in concert with other factors such as CREB binding protein (CBP), p300, p300/CBP-associated factor (PCAF), and coactivator-associated arginine methyltransferases 1 (CARM1) bring histone acetyltransferase and histone methylation activity to NR complexes. The resulting modification of chromatin structure facilitates the recruitment of the thyroid receptor-associated proteins/vitamin D receptor-interaction proteins (TRAP/DRIP) complex, helping to recruit RNA polymerase II to the promoters of the hormone regulated-genes. (Glass & Rosenfeld 2000).

In this study, we determined whether the auxiliary coactivators CARM1 and TRAP220/DRIP205 could be modulated by 6-MP. The results here identify 6-MP as a regulator of TRAP220 activity. Moreover, we demonstrate that TRAP220 interacts with the NOR-1

and Nur77 in an AF-1-dependent manner. The region of TRAP220 that mediates 6-MP activation was delimited to amino acids 1–800. This study demonstrates that 6-MP not only mediates activation of the NR4A1–3 subfamily, but also modulates TRAP220 activity.

Material and methods

Transient transfections

Proliferating C2C12 cells were grown in DMEM supplemented with 10% Serum supreme foetal calf serum (Biowhittaker, Edward Kellar Pty Ltd, Hallam, Victoria, Australia) in 6% CO₂. Cells grown in 12-well dishes to 50% confluence were transiently transfected with 1 μ g (per well) POMC-TK-LUC (NurRE-5_{POMC}-tk-LUC) reporter plasmid (Maira *et al.* 1999) together with 0.16 μ g (per well) pSG5-NOR-1-FL or pSG5 cotransfected with pcDNA-TRAP220 (0.01, 0.1 and 0.3 μ g per well) alone using a DOTAP/Metafectene (Biotex Laboratories, GmbH, Munich, Germany) liposome mixture in HEBS (42 mM HEPES, 275 mM NaCl, 10 mM KCl, 0.4 mM Na₂HPO₄ and 11 mM Dextrose, pH 7.1). The DNA/DOTAP/Metafectene mixture was added to the cells in 1 ml phenol red free DMEM, containing 10% charcoal-stripped foetal calf serum and incubated for 14 h. Medium was replaced 16–24 h later and/or 6-MP (Sigma) was added and cells grown for a further 24–48 h. Cells were harvested after 48 h and assayed for luciferase activity.

GAL4 hybrid assay

C2C12 cells were passaged into 12-well plates and transfected at 50–80% confluence with the reporter, G5E1b-LUC (0.33–1 μ g per well), and 0.16 μ g (per well) of the GAL chimeric constructs SV40-GAL4 DBD, CMV-GAL4 DBD, GAL-NOR-1-FL, CMV-GAL-CARM1, GAL-TRAP220 and the chimeric constructs GAL-TRAP220-aa1–1000, GAL-TRAP220-aa1–800, GAL-TRAP220-aa1–300, GAL-TRAP220-aa330–660, GAL-TRAP220-aa660–970, GAL-TRAP220-aa970–1300, GAL-TRAP220-aa330–1567, GAL-TRAP220-aa1290–1567, GAL-TRAP220-aa800–1567, GAL-TRAP220S656A/S657A, GAL-TRAP220S756A/S757A and GAL-TRAP220S796A/S797A. Furthermore, C2C12 cells were transfected with 0.33–1 μ g (per well) reporter G5E1b-LUC and 0.16 μ g (per well) of the GAL chimeric constructs CMV-GAL4 DBD, CMV-GAL-NOR-1-FL and GAL-TRAP220aa-1–1000 and were then cotransfected with either pcDNA-SRC-2, pcDNA-TRAP220 or SG5-NOR-FL (0.66 μ g per well) using DOTAP/Metafectene liposome mixture in HEBS per well. The DNA/DOTAP/Metafectene mixture was added to the cells in 1 ml of DMEM, containing 10%

FCS, and incubated for 14 h. Medium was replaced 16–24 h later and/or 6-MP (Sigma) was added and cells grown a further 24–48 h. Cells were harvested after 48 h and assayed for luciferase activity. Phenol red-free DMEM containing 10% CSF was used for transfections with 6-MP in the procedure, as described previously (Wansa *et al.* 2002, 2003).

Mammalian two-hybrid assay

Plasmids (1 µg per well G5E1b-LUC reporter and 0.33 µg per well GAL4 DBD or GAL-TRAP220 chimeric constructs) were co-transfected/expressed in human choriocarcinoma JEG3 cells grown in 12-well plates with either VP160, VP16-NOR-1 or VP16-Nur77 chimeric constructs (0.33 µg per well) in DMEM containing 5% charcoal-stripped FCS by the DOTAP/metafectene-mediated procedure as described previously, then assayed with respect to their ability to transactivate the reporter (G5E1b-LUC) (Chen *et al.* 2000).

Luciferase assays

Luciferase activity was assayed using a Lucite kit (PerkinElmer Pty Ltd, Rowville, Victoria, Australia) according to the manufacturer's instructions. Briefly, cells were washed once in PBS and resuspended in 80 µl phenol red-free DMEM and 80 µl Lucite substrate buffer. Cell lysates were transferred to a 96-well plate and relative luciferase units were measured for 5 sec in a Wallac Trilux 1450 microbeta luminometer (Perkin-Elmer, Wansa *et al.* 2002, 2003).

Plasmids, plasmid construction and primer sequences

The expression plasmids SV40 GAL0 (Kato *et al.* 1990), CMVGAL0, (Casanova *et al.* 1994, Willy *et al.* 1995), pCMV-GAL-CARM1, pCMV-SRC-2 (Chen *et al.* 2000, 2002), pcDNA-TRAP220 (Rachez & Freedman 2000), GAL-NOR-FL (Wansa *et al.* 2003) and the reporter plasmid G5E1b-LUC (Lillie & Green 1989) have been described elsewhere. All primers used in this study were obtained from GeneWorks Pty Ltd, Thebarton, South Australia, Australia. CMV-GAL-NOR-1-FL was constructed by excising NOR1 cDNA from pSG5-NOR-FL and recloning into the *EcoRI* site of the CMVGAL0 vector. 5' GCG **GTC GAC** AT ATG CCC TGC GTG CAA GCC CAG and 3' GCG **GTC GAC** TCA GAA AGG CAG GGT GTC AAG GAA primers containing *SalI* sites were used to PCR NOR-1 cDNA; and 5' GCG **GTC GAC** AT ATG CCC TGT ATT CAA GCT CAA and 3' GCG **GTC GAC** TCA GAA AGA CAA TGT GTC CAT primers containing *SalI* sites were used

to PCR Nur77 cDNA from pSG5-NOR-FL and pSG5-Nur77-FL expression plasmids, respectively. The PCR products were cloned into the *SalI* site of SV40-VP160. 5' GCG **GTC GAC** AT ATG CCC TGC GTG CAA GCC CAG and 3' GCG **GTC GAC** TCA TGT GCC TTC CCC AGA TGA TGA were used to PCR and clone NOR-1-AB and 5' GCG **GTC GAC** AT ATG GTT AAG GAA GTT GTG CGT and 3' GCG GTC GAC TCA GAA AGG CAG GGT GTC AAG GAA primers were used to PCR and clone NOR-1-DE. 5' GCG **GTC GAC** AT ATG CCC TGT ATT CAA GCT CAA and 3' GCG **GTC GAC** TCA CTC GCT GCC ACC TGA AGC CCC were used to PCR and clone Nur77-AB and 5' GCG **GTC GAC** AT ATG CGG CGG AAC CGC TGC CAG and 3' GCG **GTC GAC** TCA GAA AGA CAA TGT GTC CAT were used to PCR and clone Nur77-DE. PCR products were amplified from pSG5-NOR-1-FL and pSG5-Nur77-FL expression plasmids and the cloned *SalI* site of SV40-VP160. Bold type indicates restriction enzyme sites.

Primers used for PCR amplification of TRAP220 sub-domains cloned into SV40 GAL0 were: TRAP220aa1–800 GMUQ 805 5' GCG **GTC GAC** ATA TGA GTT CTC TCC TGG AAC G and GMUQ 807 3' GCG **GTC GAC** CTA ATG CCC AGA GCT TGA AG; TRAP220aa1–330- GMUQ 808 5' **CCG TCG ATA** CCA TGA GTT CTC TCC TGG AAC G and GMUQ 809 3' GCG GAG CTC CTA CTG AGT GAT CAG TTC ATA C; TRAP220aa800–1567- GMUQ 810 5' **CCA TCG ATA** CCA TGT CTC AGA GTA CCC and GMUQ 811 3' GCG **GAG CTC** CTA ATT CCC AAT CAG GGC C; TRAP220aa1290–1567- GMUQ 812 5' **CCA TCG ATA** CCA TGG TCA TAG ATA AAC TG and GMUQ 811 3' TRAP220aa330–660- GMUQ 813; 5' GCG **GAA TTC** ACC ATG TTT GAG CTA TCA AAG G and GMUQ 814 3' GCG **GAA TTC** CTA GCG GGG TGA GCC GGA; TRAP220aa 660–970- GMUQ 815 5' GCG **GAA TTC** ACC ATG GAA ATA TGC TCG GGG and GMUQ 816 3' GCG **GAA TTC** CTA GGT GCC ATT GCC TTC C; TRAP220aa970–1300- GMUQ 817 5' GCG **GAA TTC** ACC ATG AGT AAT AGT ACT CTC and GMUQ 818 3' GCG **GAA TTC** CTA GAC AAC CCC ATG CTT C; TRAP220aa330–1567- GMUQ 813 5' and GMUQ 819 3' GCG **GAA TTC** CTA ATT CCC AAT CAG GGC. TRAP220aa1–1000 has been described elsewhere (Harris *et al.* 2002). Bold type indicates the nucleotides mutated on TRAP220.

Site directed mutagenesis

Using Stratagene Quick change site directed mutagenesis kit as per manufacturers' instructions (Stratagene, Cedar Creek, Texas, USA), GAL-TRAP220-aa1–800 chimeras

that simultaneously carried double amino acid mutations were constructed. The primers were TRAP220S656A/S657A-5' C CCT TTA GAA AGG CAG AAC **GCC GCT TCC GGC TCA CCC CG** and 3' CG GGG TGA GCC GGA AGC **GGC GTT CTG CCT TTC TAA AGG G**; TRAP220S756A/S757A- 5' GG ATG GTC CGA CTA GCC GCT TCA GAC AGC ATT GGC CC and 3' GG **GCC AAT GCT GTC TGA AGC GGC TAG TCG GAC CAT CC**; TRAP220S796A/S797A – 5' CC CCT CTT CGA GAT **GCT GCA AGC TCT GGG CAT TCT CAG** and 3' CTG AGA ATG CCC AGA GCT TGC **AGC ATC TCG AAG AGG GG**.

Results

6-MP modulates the activity of TRAP220/DRIP205

Previously we demonstrated that general transcriptional activators, myogenic trans-activators, and the primary coactivators, SRC-2 and p300, were not 6-MP targets (Wansa *et al.* 2003).

In the current study we investigated whether any subsequently recruited cofactors in the NR4A activation could be modulated by 6-MP. We utilised the GAL4 hybrid system, whereby putative activation domains are fused to the DBD of the yeast transcription factor GAL4. If these regions encode a modular activation domain, they complement the GAL4 DBD to produce a functional transactivator and induce the transcription of the GAL-responsive reporter construct G5E1b-LUC. G5E1b-LUC contains five copies of the GAL4 binding site upstream of a minimal E1b promoter. The coactivators, CARM1 and TRAP220, were fused to the GAL4 DBD and were examined for the ability of these chimeras to regulate the expression of the G5E1 bLUC reporter in C2C12 myogenic cells in the presence and absence of 6-MP (Fig. 1A). We observed that 6-MP did not activate the cofactor CARM1, however, the coactivator TRAP220 which contained amino acids 1–1000 was significantly activated by 6-MP (Fig. 1B). This suggested that 6-MP modulated the activity of TRAP220.

To investigate the dose-dependent nature of 6-MP-mediated modulation of TRAP220 we examined the ability of TRAP220 relative to NOR-1 to be activated by 0.1–100 μ M 6-MP. GAL-NOR-FL and GAL-TRAP220 were responsive to 6-MP at concentrations of 10 μ M and the activity of these chimeras was further potentiated at concentrations of 33 μ M, 50 μ M and 100 μ M (Fig. 1C). Thus, NOR-1 and TRAP220 were both modulated by 6-MP. The EC₅₀ values were in the range of 33–50 μ M, however, at those concentrations 6-MP produced a more efficacious activation of NOR-1 relative to TRAP220.

Figure 1 6-MP modulates the activity of TRAP220/DRIP205. (A) A diagrammatic representation of the Gal4 hybrid assay is shown. (B and C) GAL-TRAP220-aa1–1000, GAL-CARM1 and GAL-NOR-1-FL (0.16 μ g) were co-transfected into C2C12 proliferating myoblasts together with the reporter plasmid G5E1b-LUC (0.83 μ g) in presence and absence of 50 μ M of 6-MP. (C) Dose response activity to 6-MP is shown for GAL4-DBD, GAL-NOR1-FL and GAL-TRAP220-aa1–1000 at concentrations of 0.1, 0.3, 1, 3.3, 10, 33, 50 and 100 μ M. Fold induction is expressed as the difference in fold activation obtained after cotransfection of a GAL-chimeric construct in the presence of 6-MP relative to the same GAL-chimeric construct transfected in the absence of 6-MP. The mean luciferase fold activation/induction and s.d. values (bars) were derived from a minimum of 2–3 independent triplicate experiments.

The region between amino acids 1–800 of TRAP220 mediates the activation by 6-MP

In order to further characterise the region of TRAP220 that is activated by 6-MP, we constructed a comprehensive set of TRAP220 deletions by PCR and cloned these segments into the GAL4 DBD (Fig. 2A) and examined the ability of these chimeras to regulate the expression/transcription of the G5E1b-LUC reporter in the presence and absence of 6-MP. In the GAL4 hybrid

Figure 2 The region between amino acids 1–800 of TRAP220 mediates activation by 6-MP. (A) Diagrammatic representation of SV40-GAL-TRAP220 chimeric constructs. (B) GAL-TRAP220-aa1–1000, GAL-TRAP220-aa1–800, GAL-TRAP220-aa800–1567, GAL-TRAP220-aa1–330, GAL-TRAP220-aa330–660, GAL-TRAP220-aa660–970, GAL-TRAP220-aa970–1300, GAL-TRAP220-aa330–1567 and GAL-TRAP220-aa1290–1567 (0.16 μ g) were cotransfected with the reporter plasmid G5E1b-LUC (0.83 μ g) into C2C12 proliferating myoblasts. Fold activation is expressed relative to luciferase activity obtained after cotransfection of the GAL4 DBD alone in the absence of 6-MP arbitrarily set at 1. The mean luciferase fold activation/induction and s.d. values (bars) were derived from a minimum of 2–3 independent triplicate experiments.

system we observed, as before (Figs 1B and 1C) the region between amino acids 1–1000 of TRAP220 retained the potential to be activated significantly by 6-MP. Further delimiting of TRAP220 demonstrated that the region activated by 6-MP was between amino acids 1–800, which was activated 18-fold over the TRAP220-mediated transcription of the reporter gene

in the absence of 6-MP (Fig. 2B). The importance of amino acids 1–800 of TRAP220 in mediating 6-MP activation was reinforced by the observation that domains downstream of amino acids 800 were not activated by 6-MP. For example, deletions containing amino acid regions 660–970, 970–1300, 300–1567 and 1290–1567 of TRAP220 did not mediate 6-MP

Figure 3 The characterized protein kinase A and C sites in TRAP220 are not involved in 6-MP-mediated modulation of TRAP220 activity. (A) Schematic diagram of the TRAP220 showing PKA and PKC sites and the LXXLL signature motifs (NR1 and NR2). (B) GAL-TRAP220-aa1–800, GAL-TRAP220-S656A/S657A, GAL-TRAP220-S756A/S757A and GAL-TRAP220-S796A/S797A chimeras (0.16μg) were cotransfected with the reporter plasmid G5E1b-LUC (0.83μg) into C2C12 proliferating myoblasts. Fold activation is expressed relative to luciferase activity obtained after cotransfection of the GAL4 DBD alone in the absence of 6-MP arbitrarily set at 1. The mean luciferase fold activation and s.d. values (bars) were derived from a minimum of 2–3 independent triplicate experiments.

activation. Furthermore, additional delimitation of the first 800 amino acids into smaller segments spanning amino acids, 1–300 and 300–660 TRAP220 were not activated by 6-MP (Figs 2A and B).

The characterized protein kinase A and C sites in TRAP220 are not involved in 6-MP mediated modulation of TRAP220 activity

It has been proposed that phosphorylation regulates p300/CBP and SRC-1 coactivator function (Janknecht & Nordheim 1996, Rowan *et al.* 2000, See *et al.* 2001, Gusterson *et al.* 2002, McKenna & O'Malley 2002a). Moreover, *in vitro* and *in vivo* approaches identified that TRAP220 is phosphorylated, the major phosphorylation sites were subsequently identified to be protein

kinase A (PKA), C (PKC) and mitogen-activated protein kinase (MAPK) sites. Furthermore, the effect of MAPK activation on TRAP220-mediated PPARγ transcription suggested that this kinase signal pathway positively influences TRAP220 function (Misra *et al.* 2002).

The region of TRAP220 (amino acids 1–800) that mediates 6-MP activation contains the defined PKC and PKA phosphorylation sites (Fig. 3A). We mutated these PKA and PKC sites within TRAP220 and investigated the effect of the mutations on 6-MP-mediated activation of TRAP220. We changed the two critical serines in each of the three defined kinase motifs to alanines. We observed that the mutants GAL-TRAP220-S656A/S657A, GAL-TRAP220-S756A/S757A and GAL-TRAP220-S796A/S797A did not compromise the ability of 6-MP to modulate the activity of TRAP220 (Fig. 3B).

TRAP220 expression stimulates the activity of NOR-1

Our previous studies had demonstrated the direct *in vitro* binding of SRC-2 and TRAP220 to Nur77 and NOR-1. Furthermore, we demonstrated that SRC-2 modulated the activity of Nur77 and NOR-1 (Wansa *et al.* 2003). Activation of gene expression by the classical nuclear hormone receptors is dependent on the recruitment of the SRCs that recruit a number of cofactors in a sequential manner that possess intrinsic histone acetyltransferase (HAT) activity, and precipitate the assembly of a higher order structure that includes the TRAP/DRIP protein complex that regulates localised nucleosome structure (Glass & Rosenfeld 2000). The key member of the TRAP/DRIP complex is the TRAP220/DRIP205 subunit that has also been shown to interact with many NRs, such as vitamin D receptor (VDR), thyroid receptor (TR), androgen receptor (AR), estrogen receptor (ER), glucocorticoid receptor (GR) and peroxisome proliferator activated-receptor (PPAR γ) (Zhu *et al.* 1997, Hittelman *et al.* 1999, Sharma & Fondell 2002, Burakov *et al.* 2000, Ren *et al.* 2000, Wang *et al.* 2002, Acevedo & Kraus 2003).

Hence, we wished to investigate whether TRAP220 modulated the activity of NOR-1 in the GAL4 hybrid system. In these assays the activity of NOR-1 is independent of its binding to its cognate binding motifs. If TRAP220 regulates the transcriptional activity of NOR-1, then the potential of the GAL4-NOR-1-FL fusions to trans-activate gene expression should be increased in this assay (Fig. 4A). C2C12 cells were cotransfected with GAL-NOR-1-FL and the G5E1b-LUC reporter in the presence and absence of an SRC-2 or TRAP220 expression vector (Fig. 4B). Transfection of GAL-NOR-1-FL efficiently induced transcription relative to the GAL4 DBD, this level of activity was stimulated by the addition of SRC-2 by ~ 3 -fold (Fig. 4B) as we had previously shown (Wansa *et al.* 2003). Furthermore, we observed that NOR-1 activity was also stimulated in the presence of TRAP220 by ~ 3 fold, this suggested that NOR-1-mediated transcriptional activation is coactivated by TRAP220.

Subsequently, we examined whether TRAP220 potentiates NOR-1-mediated activation of a native NR4A response element. We utilized a heterologous reporter gene that contains native NOR-1 response elements i.e. the NurRE-5 POMC-tk-LUC reporter contains five copies of the naturally occurring NR4A response element (TGATATTTACCTCCAAATGCCA) from the pro-opiomelanocortin (POMC) gene, (Fig. 4C). This native response element is responsive to physiological stimuli, binds NR4A dimers and efficaciously responds to NR4A mediated transactivation (Maira *et al.* 1999). C2C12 muscle cells were cotransfected with the reporter plasmid NurRE-5 POMC-tk-LUC and

the NOR-1 expression vector. We observed efficient NOR-1-dependent transactivation of the NurRE-5 POMC reporter by ~ 60 -fold (Fig. 4D) as previously reported. Moreover, increasing concentrations of TRAP220 expression plasmid potentiated NOR-1-mediated activation of the native response element by ~ 4 – 9 -fold, consistent with the GAL4 hybrid assay (Fig. 4A). Higher concentrations of TRAP220 resulted in squelching of NOR-1-mediated transcription (data not shown).

In addition, 6-MP induced NOR-1-mediated transactivation of the NurREPOMC reporter by >5 -fold, as previously reported (Wansa *et al.* 2003). Interestingly, TRAP220 expression did not further potentiate the induction of NOR-mediated transactivation by 6-MP, however, the absolute level of NOR-1 mediated reporter gene activation was certainly increased in the presence of TRAP220 and 6-MP (Fig. 4D). These experiments indicate NOR-1-mediated transcriptional activation is potentiated by TRAP220.

NOR-1 and Nur77 interact with TRAP220 in an AF-1-dependent manner in a cellular context: amino acids 1–800 of TRAP220 are necessary for nuclear receptor recruitment

Our previous studies demonstrated that NOR-1 could recruit TRAP220 *in vitro*. To investigate whether NOR-1 and Nur77 could interact with TRAP220 in a cellular context *in vivo* we utilised the mammalian two-hybrid assay. In these experiments the yeast GAL4 DBD domain is fused to TRAP220 and expressed in transfected JEG-3 cells with NOR-1 or Nur77 linked to the transactivation domain (AD) of the herpes simplex virus, VP16. Transactivation of a GAL4-dependent reporter gene (G5E1 bLUC) is only achieved when the coexpressed proteins interact physically (Fig. 5A).

JEG-3 cells (and not the C2C12 muscle cells) were utilized for the mammalian two-hybrid assays, because the activity of NOR-1, Nur77 and TRAP220 is high in C2C12, COS-1 and 293 cells (data not shown) (Castro *et al.* 1999, Wansa *et al.* 2002, 2003). Furthermore, Castro *et al.* (1999) reported that the NR4A activity in this cell line is very weak, consequently background activity is low, thus enabling *in vivo* interactions to be observed (and increasing the sensitivity of the assay). This feature of JEG-3 cells was previously exploited in the mammalian two-hybrid assays involving this subgroup of receptors (Perlmann & Jansson 1995).

We observed that coexpression of VP16-NOR-1 and VP16-Nur77 effectively and significantly trans-activated GAL-TRAP220 by ~ 5 - and 15 -fold, respectively (Fig. 5B), in concordance with our GST-pulldown studies (Wansa *et al.* 2002, 2003).

To determine the regions of NOR-1 and Nur77 that mediated the *in vivo* interactions with TRAP220, we

performed mammalian two-hybrid experiments in which the N and C-terminal regions of NOR-1 and Nur77 (for example, NOR-1-AB, NOR-1-DE, Nur77-AB or Nur77-DE) were linked to the transactivation domain of the herpes simplex virus, VP16. We observed that VP16-NOR-1-AB and VP16-Nur77-AB

effectively and significantly transactivated GAL-TRAP220 activity by ~5-fold. In contrast, the C-terminal DE regions of these receptors, in an atypical manner, failed to complement the activity of TRAP220 (Figs 5C and 5D). This is consistent with the AF-1-dependent nature of NOR-1 activity.

However, we did not observe increased NOR-1-mediated recruitment of TRAP220 in the presence of 6-MP (Fig. 6A). To further determine whether 6-MP could potentiate TRAP220-induced NOR-1-mediated transcription we utilized and further exploited the GAL4 hybrid assay (Fig. 6B). As demonstrated in Figure 4A, NOR-1 activity was stimulated in the presence of TRAP220, in this reverse assay TRAP220 activity was increased by NOR-1 co-expression by ~3-fold (Fig. 6C). As observed initially, TRAP220 activity was stimulated by 6-MP (Figs 1B and 6C), however NOR-1 expression did not increase the inducibility of TRAP220 activity by 6-MP. Although the absolute level of activation (relative to the GAL4 DBD) was clearly increased.

We progressed to further utilize this assay to delimit the region of TRAP220 that mediates interaction with NOR-1 and Nur77 employing the mammalian two-hybrid assay. Interestingly, the region between amino acids 1–800 of TRAP220, which mediates transcriptional activation by 6-MP, is also necessary to support the cellular interaction with NOR-1 and Nur77. We observed that VP16-NOR-1-FL and VP16-Nur77-FL significantly transactivated GAL-TRAP220-aa1–800 activity by ~3-fold and ~11-fold, respectively (Fig. 7). The regions of TRAP220 that were previously shown to not be modulated by 6-MP (Fig. 2B) did not interact with NOR-1 and Nur77, thus demonstrating the importance of the region between amino acids 1–800 of

Figure 4 TRAP220 potentiates the activity of NOR-1 (A) A diagrammatic representation of the GAL4 hybrid assay is shown. This assay was used to determine the effect of SRC-2/GRIP-1 TRAP220 expression on NOR-1-mediated transactivation of gene expression. pCMV-GAL-NOR-1-FL (0.16 µg) was co-transfected with the reporter plasmid G5E1b-LUC (1µg) into C2C12 proliferating myoblasts in the presence and absence of the co-transfected expression plasmid encoding pcDNA-SRC-2 or pcDNA-TRAP220 (2µg per 3 wells). Fold activation is expressed relative to luciferase activity obtained after cotransfection of the pCMVGAL4 DBD alone arbitrarily set at 1. The mean luciferase fold activation and s.d. values (bars) were derived from a minimum of 2 independent triplicate experiments. (C) A diagrammatic representation of the reporter gene assay. (D) SG5-NOR-1-FL (0.16µg) pcDNA-TRAP220 (0.1, 0.3 and 1 µg per 3 wells) were co-transfected into C2C12 proliferating myoblasts together with the reporter gene NurRE-5-tk-Luc in the presence and absence of 100µM 6-MP. Fold activation is expressed relative to luciferase activity obtained after cotransfection of pSG5 alone, arbitrarily set at 1. The mean luciferase fold activation values and standard deviations (bars) were derived from a minimum of 2–3 independent triplicate experiments.

TRAP220 for modulation by 6-MP, as well as mediating interactions with NOR-1 and Nur-77 (Fig. 7).

These mammalian two-hybrid experiments clearly demonstrate that NOR-1 and Nur77 significantly interact with TRAP220 in a cellular context in an AF-1/AB region-dependent manner. This is concordant with the *in vitro* GST-pulldown assays that showed that the AB regions of NOR-1 and Nur77 mediate coactivator recruitment, and that the LBD interacts poorly with coactivators (Wansa *et al.* 2002, 2003). Moreover, we demonstrated that a TRAP220 deletion mutant containing only the NR-interacting regions (LXXLL motifs) did not interact with NOR-1 and Nur77. This is in contrast to other NRs, where coactivators interact with NRs namely through key interactions with residues in the LBD domain that form part of a charged clamp that accommodates the coactivator LXXLL α -helix motifs within a hydrophobic cleft in the LBD, conversely NOR-1 and Nur77 were shown to have unusually hydrophilic coactivator binding interfaces, thus accounting for the inability of NOR-1 and Nur77 LBDs to efficiently recruit TRAP220 (data not shown).

Discussion

In this study we have provided evidence that the purine anti-metabolite 6-MP, which has anti-proliferative, anti-cancer and anti-inflammatory properties, stimulated the activity of the coactivator TRAP220. The 6-MP-mediated activation of NOR-1 and TRAP220 is observed at an EC₅₀ of 33–50 μ M, and increases in a dose-dependent manner. We could not obtain saturating concentrations of 6-MP on NOR-1 and TRAP220 activation because at 6-MP concentrations of >100 μ M we observed a dose-dependent increase of >2-fold in the activity of the basal GAL4 DBD. This is consistent with the observations from Ordentlich and colleagues (Ordentlich *et al.* 2003). The range of 6-MP concentrations required for inhibition of purine biosynthesis varies greatly from cell to cell, with sensitive cell lines

Figure 5 TRAP220 directly interact with the AF-1 domain of NOR-1 and Nur77 *in vivo*. (A) Diagrammatic representation of the mammalian two-hybrid assay used to determine the interaction and cooperativity of TRAP220, NOR-1 and Nur77 in a cellular context. (B) GAL-TRAP220-aa1–1000 (0.33 μ g), VP16-NOR-1-FL, VP16-NOR-1-AB, VP16-NOR-1-DE, VP16-Nur77-FL, VP16-Nur77-AB and VP16-Nur77-DE (0.33 μ g) were cotransfected with the reporter plasmid G5E1b-LUC (1 μ g) in JEG3 cells. Fold activation is expressed relative to luciferase activity obtained after cotransfection of the SV40-GAL4 DBD and the SV40-VP16AD plasmids alone, arbitrarily set at 1. The mean luciferase fold activation and s.d. values (bars) were derived from a minimum of 2–3 independent triplicate experiments.

such as human lymphoblastic Molt F4 requiring 0.5–10 μM and some cell lines being resistant at concentrations above 100 μM (Ordentlich *et al.* 2003). Therefore, most biological effects of 6-MP observed to date occur in the micro molar range, and activation of NOR-1 and TRAP220 is concordant with the clinical utilization of this drug.

Transcriptional activation of NRs involves the stepwise recruitment and binding of coactivators to target gene promoters (McKenna & O'Malley 2002*b*). Initial recruitment of SRCs, p300/CBP, PCAF and CARM1 by ligand-activated NRs leads to subsequent

conscription of the TRAP/DRIP complex (McKenna & O'Malley 2002*a*). The single TRAP subunit, TRAP220, directly contacts TR, VDR, GR and ER in a ligand-dependent manner and is thought to anchor the TRAP/DRIP complex to DNA-bound NRs. We demonstrated that TRAP220 potentiates NOR-1-mediated transactivation. In addition, NOR-1 and Nur77 interact with TRAP220 in an AF-1-dependent manner in a cellular context. Interestingly TRAP220 expression does not increase the extent of inducibility by 6-MP. However, the absolute level of NOR-1-mediated activation is increased, suggesting additive rather than synergistic outcomes of 6-MP-mediated activation of NOR-1 and TRAP220.

TRAP220 is a member of a multi subunit complex, composed of at least 16 different polypeptides, ranging in size from ~15 to 240 kDa (Malik & Roeder 2000, Rachez & Freedman 2001). It has been shown that over expression of TRAP170 and TRAP100, members of the TRAP-complex also enhance ligand-dependent transcription by AR in cultured cells in a similar manner to TRAP220, indicating that other members of the TRAP-mediator complex are involved in binding and subsequent targeting of the TRAP-mediator complex to AR (Wang *et al.* 2002). Furthermore, TRAP170 interacts with the AF-1 domain of GR. It has been demonstrated that TRAP220 interacts with the GR ligand binding domain in a hormone-dependent manner and facilitates GR transactivation in concert with TRAP170/DRIP150 (Hittelman *et al.* 1999). Whether other members of the TRAP-complex may be required for coactivation of NOR-1-mediated transcription in response to 6-MP needs to be further investigated.

The NR4A1–3 subfamily has been shown to be involved in the regulation of apoptosis in prostate, lung, gastric, breast and colon cancer cells (Li *et al.* 1998, Kang *et al.* 2000, Li *et al.* 2000, Ohkubo *et al.* 2000, Liu *et al.* 2002, Wu *et al.* 2002, Zhang 2002). Nur77 induces thymocyte apoptosis in a AF-1-dependent manner in

Figure 6 6-MP does not potentiate stimulation by TRAP220 in NOR-1-mediated transcription. GAL-TRAP220-aa1–1000, GAL-TRAP220-aa1–800 (0.33 μg), VP16-NOR-1-FL and VP16-Nur77-FL (0.33 μg) were cotransfected with the reporter plasmid G5E1b-LUC (1 μg) in JEG-3 cells. Fold activation is expressed relative to luciferase activity obtained after cotransfection of the SV40-GAL4 DBD and the SV40-VP16AD plasmids alone in the absence of 6-MP arbitrarily set at 1. (B) A diagrammatic representation of the GAL4 hybrid assay is shown (C) GAL-TRAP220-aa1–1000 (0.16 μg) and SG5-NOR-1-FL (2 μg per 3 wells) were co-transfected into C2C12 proliferating myoblasts together with the reporter gene G5E1b-LUC (1 μg) in the presence and absence of 50 μM of 6-MP. Fold activation is expressed relative to luciferase activity obtained after cotransfection of the GAL4 DBD and SG5 alone in the absence of 6-MP, arbitrarily set at 1. The mean luciferase fold activation and s.d. values (bars) were derived from a minimum of 2–3 independent triplicate experiments.

Figure 7 Amino acids 1–800 of TRAP220 are necessary for interaction with NOR-1 and Nur77. GAL-TRAP220-aa1–1000, GAL-TRAP220-aa1–800, GAL-TRAP220-aa1–330, GAL-TRAP220-aa330–660, GAL-TRAP220-aa660–970, GAL-TRAP220-aa970–1300 and GAL-TRAP220-aa330–1547 (0.16 µg) were cotransfected with the reporter plasmid G5E1b-LUC (0.83 µg) into JEG3 cells. Fold activation is expressed relative to luciferase activity obtained after cotransfection of the SV40 GAL4 DBD and the SV40-VP16AD plasmids alone, arbitrarily set at 1. The mean luciferase fold activation and s.d. values (bars) were derived from a minimum of 2–3 independent triplicate experiments.

transgenic mice (Kuang *et al.* 1999). TRAP220 has also been shown to regulate apoptosis through direct regulation of the tumour suppressor p53 (Frade *et al.* 2002). Interestingly, 6-MP targets the AB region of the NR4A1–3 subfamily and regulates the receptors' activity, furthermore TRAP220 interacts with the AB region of NOR-1 and Nur77 and stimulates NOR-1-mediated transcription. Whereas many of the steps involved in 6-MP-mediated cytotoxicity have been elucidated, it is possible that these effects can be enhanced or suppressed by transcriptional regulation of the NR4A1–3 subfamily.

6-MP is metabolized into the active derivative, 6-thio-inosine-mono-phosphate (6-TIMP), by hypoxanthine-guanine phosphoribosyltransferase (HGPRT), a key enzyme involved in the purine salvage

pathway. The 6-thio-IMP can be metabolized into 6-thioguanosine 5' monophosphate, which is then incorporated into DNA and RNA as 6-thio-guanosine triphosphate (6-thio-GTP, 6-TGN) resulting in eventual cytotoxicity and cell death (Cara *et al.* 2004). Incorporation of 6-TGNs into nucleic acids is considered the main mechanism of 6-MP cytotoxicity. 6-TIMP can be methylated by thiopurine methyltransferase (TPMT) to methyl thioinosine monophosphate (methyl-TIMP), a potent inhibitor of *de novo* purine biosynthesis which depletes the intracellular ATP pools (Cara *et al.* 2004). Most relevant to the observation presented in this study is the finding that 6-methyl-mercaptopurine riboside and 6-TGN are specific and effective inhibitors of protein kinase N, which has been implicated in nerve growth factor signalling (Volonte & Greene 1992,

Volonte *et al.* 1993). 6-methy-mecaptopurine riboside can also regulate tyrosine kinase signalling in angiogenesis (Presta *et al.* 1999). Furthermore, it has been shown that azathioprine and its metabolite 6-MP induced apoptosis of T cells in patients with Crohn's disease and control patients. Apoptosis induction required costimulation with CD28 and was mediated by a specific blockade of Rac1 activation through binding 6-TGN to Rac1 instead of GTP. The activation of Rac1 target genes such as mitogen-activated protein kinase/extracellular signal regulated kinase (MEK), NF-kappaB and bcl-x(L) was suppressed by azathioprine, leading to a mitochondrial pathway of apoptosis (Tiede *et al.* 2003).

The precedent set by these findings suggests that 6-MP may also regulate as yet unidentified activities in the cell that may specifically influence TRAP220 activity. Indeed, stimulation of CBP transcriptional coactivation by MAPK (Janknecht & Nordheim 1996, Liu *et al.* 1998), of p300-mediated transcription by mitogen-activated/extracellular response kinase kinase (MEKK1) (See *et al.* 2001, Gusterson *et al.* 2002), and SRC-1 function by ERK1 and ERK2 (Rowan *et al.* 2000) points to phosphorylation as a positive regulatory modification in coactivator activity. Furthermore, Ca²⁺/Calmodulin-dependent protein kinase IV (CaMKIV) enhances Nur77 transactivation in cotransfections in synergy with the CaMKIV responsive coactivator activating signal cointegrator-2 (ASC-2) (Sohn *et al.* 2001). Interestingly, TRAP220 contains PKC, PKA and MAPK sites that were shown to be phosphorylated by these kinases. The effect of MAPK activation on TRAP220 mediated PPAR γ transcription suggests that this kinase signalling pathway positively influences TRAP220 function (Misra *et al.* 2002). Interestingly, the region of TRAP220 (aa1–800) that mediates transactivation in response to 6-MP contains PKC and PKA phosphorylation sites, however mutation of these sites did not compromise the ability of 6-MP to activate TRAP220.

Apoptosis represents an effective way to eliminate cancerous cells and a variety of evidence suggests both the NR4A1–3 subfamily of nuclear receptors and TRAP220 to be implicated in cancer and apoptosis (Li *et al.* 1998, Zhu *et al.* 1999, Kang *et al.* 2000, Li *et al.* 2000, Ohkubo *et al.* 2000, Frade *et al.* 2002, Liu *et al.* 2002, Wu *et al.* 2002, Zhang 2002, Wada *et al.* 2004). Therefore, we speculate that there exists a cell signalling cascade that is sensitive to either levels of purine nucleotides or to regulation by 6-MP and that this pathway induces NR4A1–3 modulation. We suggest that 6-MP could act in concert with cellular signal transduction pathways to modulate NR4A-mediated transcription. In this regard, TRAP220 could serve to integrate the signalling cascade with NR4A-mediated transcription in response to 6-MP. Characterization of these signalling cascades has utility in the design of

pharmacological tools for the selective therapeutic regulation of the NR4A subgroup in treatment of Parkinson's disease, atherogenesis, Alzheimer's disease, rheumatoid arthritis and cancer.

References

- Acevedo ML & Kraus WL 2003 Mediator and p300/CBP-steroid receptor coactivator complexes have distinct roles, but function synergistically, during estrogen receptor alpha-dependent transcription with chromatin templates. *Molecular and Cellular Biology* **23** 335–348.
- Arkenbout EK, de Waard V, van Bragt M, van Achterberg TA, Grimbergen JM, Pichon B, Pannekoek H & de Vries CJ 2002 Protective function of transcription factor TR3 orphan receptor in atherogenesis: decreased lesion formation in carotid artery ligation model in TR3 transgenic mice. *Circulation* **106** 1530–1535.
- Backman C, Perlmann T, Wallen A, Hoffer BJ & Morales M 1999 A selective group of dopaminergic neurons express Nurr1 in the adult mouse brain. *Brain Research* **851** 125–132.
- Baker KD, Shewchuk LM, Kozlova T, Makishima M, Hassell A, Wisely B, Caravella JA, Lambert MH, Reinking JL, Krause H *et al.* 2003 The Drosophila orphan nuclear receptor DHR38 mediates an atypical ecdysteroid signaling pathway. *Cell* **113** 731–742.
- Borghaei RC, Sinai RS, Mochan E & Pease EA 1998 Induction of mitogen-inducible nuclear orphan receptor by interleukin 1 in human synovial and gingival fibroblasts. *Biochemical and biophysical research communications* **251** 334–338.
- Buervenich S, Carmine A, Arvidsson M, Xiang F, Zhang Z, Sydow O, Jonsson EG, Sedvall GC, Leonard S, Ross RG *et al.* 2000 NURR1 mutations in cases of schizophrenia and manic-depressive disorder. *American Journal of Medical Genetics* **96** 808–813.
- Burakov D, Wong CW, Rachez C, Cheskis BJ & Freedman LP 2000 Functional interactions between the estrogen receptor and DRIP205, a subunit of the heteromeric DRIP coactivator complex. *Journal of Biological Chemistry* **275** 20928–20934.
- Cara CJ, Pena AS, Sans M, Rodrigo L, Guerrero-Esteo M, Hinojosa J, Garcia-Paredes J & Guijarro LG 2004 Reviewing the mechanism of action of thiopurine drugs: towards a new paradigm in clinical practice. *Medical science monitor* **10** RA247–254.
- Casanova J, Helmer E, Selmi-Ruby S, Qi JS, Au-Fliegner M, Desai-Yajnik V, Koudinova N, Yarm F, Raaka BM & Samuels HH 1994 Functional evidence for ligand-dependent dissociation of thyroid hormone and retinoic acid receptors from an inhibitory cellular factor. *Molecular and Cellular Biology* **14** 5756–5765.
- Castro DS, Arvidsson M, Bondesson Bolin M & Perlmann, T 1999 Activity of the Nurr1 carboxyl-terminal domain depends on cell type and integrity of the activation function 2. *Journal of Biological Chemistry* **272** 37383–37390.
- Chawla A, Repa JJ, Evans RM & Mangelsdorf DJ 2001 Nuclear receptors and lipid physiology: opening the X-files. *Science* **294** 1866–1870.
- Chen SL, Dowhan DH, Hosking BM & Muscat GE 2000 The steroid receptor coactivator, GRIP-1, is necessary for MEF-2C-dependent gene expression and skeletal muscle differentiation. *Genes and Development* **14** 1209–1228.
- Chen SL, Loffler KA, Chen D, Stallcup MR & Muscat GE 2002 The coactivator associated arginine methyltransferase is necessary for muscle differentiation: CARM1 coactivates myocyte enhancer factor-2. *Journal of Biological Chemistry* **277** 4324–4333.
- Chen YH, Tsai MT, Shaw CK & Chen CH 2001 Mutation analysis of the human NR4A2 gene, an essential gene for midbrain dopaminergic neurogenesis, in schizophrenic patients. *American Journal of Medical Genetics* **105** 753–757.

- Frade R, Balbo M & Barel M 2002 RB18A regulates p53-dependent apoptosis. *Oncogene* **21** 861–866.
- Glass CK & Rosenfeld MG 2000 The coregulator exchange in transcriptional functions of nuclear receptors. *Genes and Development* **14** 121–141.
- Gronemeyer H & Laudet V 1995 Transcription factors 3: nuclear receptors. *Protein Profile* **2** 1173–1308.
- Gusterson R, Brar B, Faulkes D, Giordano A, Chrivia J & Latchman D 2002 The transcriptional co-activators CBP and p300 are activated via phenylephrine through the p42/p44 MAPK cascade. *Journal of Biological Chemistry* **277** 2517–2524.
- Harris JM, Lau P, Chen SL & Muscat GE 2002 Characterization of the retinoid orphan-related receptor- α coactivator binding interface: a structural basis for ligand-independent transcription. *Molecular Endocrinology* **16** 998–1012.
- Hittelman AB, Burakov D, Iniguez-Lluhi JA, Freedman LP & Garabedian MJ 1999 Differential regulation of glucocorticoid receptor transcriptional activation via AF-1-associated proteins. *Embo Journal* **18** 5380–5388.
- Janknecht R & Nordheim A 1996 MAP kinase-dependent transcriptional coactivation by Elk-1 and its cofactor CBP. *Biochemical and Biophysical Research Communications* **228** 831–837.
- Kang HJ, Song MJ, Choung SY, Kim SJ & Le MO 2000 Transcriptional induction of Nur77 by indomethacin that results in apoptosis of colon cancer cells. *Biological and Pharmaceutical Bulletin* **23** 815–819.
- Kato GJ, Barrett J, Villa-Garcia M & Dang CV 1990 An amino-terminal c-myc domain required for neoplastic transformation activates transcription. *Molecular and Cellular Biology* **10** 5914–5920.
- Kim KS, Kim CH, Hwang DY, Seo H, Chung S, Hong SJ, Lim JK, Anderson T & Isacson O 2003 Orphan nuclear receptor Nurr1 directly transactivates the promoter activity of the tyrosine hydroxylase gene in a cell-specific manner. *Journal of Neurochemistry* **85** 622–634.
- Kuang AA, Cado D & Winoto A 1999 Nur77 transcription activity correlates with its apoptotic function *in vivo*. *European Journal of Immunology* **29** 3722–3728.
- Labelle Y, Bussières J, Courjal F & Goldring MB 1999 The EWS/TEC fusion protein encoded by the t(9;22) chromosomal translocation in human chondrosarcomas is a highly potent transcriptional activator. *Oncogene* **18** 3303–3308.
- Le WD, Xu P, Jankovic J, Jiang H, Appel SH, Smith RG & Vassilatis DK 2003 Mutations in NR4A2 associated with familial Parkinson disease. *Nature Genetics* **33** 85–89.
- Li H, Kolluri SK, Gu J, Dawson MI, Cao X, Hobbs PD, Lin B, Chen G, Lu J, Lin F *et al.* 2000 Cytochrome C release and apoptosis induced by mitochondrial targeting of nuclear orphan receptor TR3. *Science* **289** 1159–1164.
- Li Y, Lin B, Agadir A, Liu R, Dawson MI, Reed JC, Fontana JA, Bost F, Hobbs PD, Zheng Y *et al.* 1998 Molecular determinants of AHPN (CD437)-induced growth arrest and apoptosis in human lung cancer cell lines. *Molecular and Cellular Biology* **18** 4719–4731.
- Lillie JW & Green MR 1989 Gene transcription: activator's target in sight. *Nature* **341** 279–280.
- Liu S, Wu Q, Ye XF, Cai JH, Huang ZW & Su WJ 2002 Induction of apoptosis by TPA and VP-16 is through translocation of TR3. *World Journal of Gastroenterology* **8** 446–450.
- Liu YZ, Chrivia JC & Latchman DS 1998 Nerve growth factor up-regulates the transcriptional activity of CBP through activation of the p42/p44(MAPK) cascade. *Journal of Biological Chemistry* **273** 32400–32407.
- McEvoy AN, Bresnihan B, Fitzgerald O & Murphy EP 2002 Corticotropin-releasing hormone signaling in synovial tissue vascular endothelium is mediated through the cAMP/CREB pathway. *Annals of the New York Academy of Sciences* **966** 119–130.
- McKenna NJ & O'Malley BW 2002a Minireview: nuclear receptor coactivators—an update. *Endocrinology* **143** 2461–2465.
- McKenna NJ & O'Malley BW 2002b Combinatorial control of gene expression by nuclear receptors and coregulators. *Cell* **108** 465–474.
- Maira M, Martens C, Philips A & Drouin J 1999 Heterodimerization between members of the Nur subfamily of orphan nuclear receptors as a novel mechanism for gene activation *Molecular and Cellular Biology* **19** 7549–7557.
- Malik S & Roeder RG 2000 Transcriptional regulation through Mediator-like coactivators in yeast and metazoan cells. *Trends in Biochemical Science* **25** 277–283.
- Maltais A, Filion C & Labelle Y 2002 The AF2 domain of the orphan nuclear receptor TEC is essential for the transcriptional activity of the oncogenic fusion protein EWS/TEC. *Cancer Letters* **183** 87–94.
- Misra P, Owuor ED, Li W, Yu S, Qi C, Meyer K, Zhu YJ, Rao MS, Kong AN & Reddy JK 2002 Phosphorylation of transcriptional coactivator peroxisome proliferator-activated receptor (PPAR)-binding protein (PBP). Stimulation of transcriptional regulation by mitogen-activated protein kinase. *Journal of Biological Chemistry* **277** 48745–48754.
- Monajemi H, Arkenbout EK & Pannekoek H 2001 Gene expression in atherogenesis. *Thrombosis and Haemostasis* **86** 404–412.
- Murphy EP & Conneely OM 1997 Neuroendocrine regulation of the hypothalamic pituitary adrenal axis by the nurr1/nur77 subfamily of nuclear receptors. *Molecular Endocrinology* **11** 39–47.
- Newman SJ, Bond B, Crook B, Darker J, Edge C & Maycox PR 2000 Neuron-specific localisation of the TR3 death receptor in Alzheimer's disease. *Brain Research* **857** 131–140.
- Ohkubo T, Ohkura N, Maruyama K, Sasaki K, Nagasaki K, Hanzawa H, Tsukada T & Yamaguchi K 2000 Early induction of the orphan nuclear receptor NOR-1 during cell death of the human breast cancer cell line MCF-7. *Molecular and Cellular Endocrinology* **162** 151–156.
- Ordentlich P, Yan Y, Zhou S & Heyman RA 2003 Identification of the antineoplastic agent 6-mercaptopurine as an activator of the orphan nuclear hormone receptor Nurr1. *Journal of Biological Chemistry* **278** 24791–24799.
- Perlmann T & Jansson L 1995 A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and NURR1. *Genes and Development* **9** 769–782.
- Presta M, Rusnati M, Belleri M, Morbidelli L, Ziche M & Ribatti D 1999 Purine analogue 6-methylmercaptopurine riboside inhibits early and late phases of the angiogenesis process. *Cancer Research* **59** 2417–2424.
- Rachez C & Freedman LP 2000 Mechanisms of gene regulation by vitamin D(3) receptor: a network of coactivator interactions. *Gene* **246** 9–21.
- Rachez C & Freedman LP 2001 Mediator complexes and transcription. *Current Opinion in Cellular Biology* **13** 274–280.
- Ren Y, Behre E, Ren Z, Zhang J, Wang Q & Fondell JD 2000 Specific structural motifs determine TRAP220 interactions with nuclear hormone receptors. *Molecular and Cellular Biology* **20** 5433–5446.
- Rowan BG, Weigel NL & O'Malley BW 2000 Phosphorylation of steroid receptor coactivator-1. Identification of the phosphorylation sites and phosphorylation through the mitogen-activated protein kinase pathway. *Journal of Biological Chemistry* **275** 4475–4483.
- Saucedo-Cardenas O, Quintana-Hau JD, Le WD, Smidt MP, Cox JJ, De Mayo F, Burbach JP & Conneely OM 1998 Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. *PNAS* **95** 4013–4018.
- Schimmel JJ, Crews L, Roffler-Tarlov S & Chikaraishi DM 1999 4-5 kb of the rat tyrosine hydroxylase 5' flanking sequence directs tissue specific expression during development and contains

- consensus sites for multiple transcription factors. *Brain Research and Molecular Brain Research* **74** 1–14.
- See RH, Calvo D, Shi Y, Kawa H, Luke MP & Yuan Z 2001 Stimulation of p300-mediated transcription by the kinase MEKK1. *Journal of Biological Chemistry* **276** 16310–16317.
- Sharma D & Fondell JD 2002 Ordered recruitment of histone acetyltransferases and the TRAP/Mediator complex to thyroid hormone-responsive promoters *in vivo*. *PNAS* **99** 7934–7939.
- Sohn YC, Kwak E, Na Y, Lee JW & Lee SK 2001 Silencing mediator of retinoid and thyroid hormone receptors and activating signal cointegrator-2 as transcriptional coregulators of the orphan nuclear receptor Nur77. *Journal of Biological Chemistry* **276** 43734–43739.
- Tiede I, Fritz G, Strand S, Poppe D, Dvorsky R, Strand D, Lehr HA, Wirtz S, Becker C, Atreya R *et al.* 2003 CD28-dependent Rac1 activation is the molecular target of azathioprine in primary human CD4+ T lymphocytes. *Journal of Clinical Investigation* **111** 1133–1145.
- Volonte C & Greene LA 1992 6-Methylmercaptapurine riboside is a potent and selective inhibitor of nerve growth factor-activated protein kinase N. *Journal of Neurochemistry* **58** 700–708.
- Volonte C, Ross AH & Greene LA 1993 Association of a purine-analogue-sensitive protein kinase activity with p75 nerve growth factor receptors. *Molecular Biology of the Cell* **4** 71–78.
- Wada O, Oishi H, Takada I, Yanagisawa J, Yano T & Kato S. 2004 BRCA1 function mediates a TRAP/DRIP complex through direct interaction with TRAP220. *Oncogene* **23** 6000–6005.
- Wang Q, Sharma D, Ren Y & Fondell JD 2002 A coregulatory role for the TRAP-mediator complex in androgen receptor-mediated gene expression. *Journal of Biological Chemistry* **277** 42852–42858.
- Wang Z, Benoit G, Liu J, Prasad S, Aarnisalo P, Liu X, Xu H, Walker NP & Perlmann T 2003 Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors. *Nature* **423** 555–560.
- Wansa KD, Harris JM & Muscat GE 2002 The activation function-1 domain of Nur77/NR4A1 mediates trans-activation, cell specificity, and coactivator recruitment. *Journal of Biological Chemistry* **277** 33001–33011.
- Wansa KD, Harris JM, Yan G, Ordentlich P & Muscat GE 2003 The AF-1 domain of the orphan nuclear receptor NOR-1 mediates trans-activation, coactivator recruitment, and activation by the purine anti-metabolite 6-mercaptopurine. *Journal of Biological Chemistry* **278** 24776–24790.
- Willy PJ, Umesono K, Ong ES, Evans RM, Heyman RA & Mangelsdorf DJ 1995 LXR, a nuclear receptor that defines a distinct retinoid response pathway. *Genes and Development* **9** 1033–1045.
- Wu Q, Liu S, Ye XF, Huang ZW & Su WJ 2002 Dual roles of Nur77 in selective regulation of apoptosis and cell cycle by TPA and ATRA in gastric cancer cells. *Carcinogenesis* **23** 1583–1592.
- Zhang XK 2002 Vitamin A and apoptosis in prostate cancer. *Endocrine Related Cancer* **9** 87–102.
- Zhu Y, Qi C, Jain S, Rao MS & Reddy JK 1997 Isolation and characterization of PBP, a protein that interacts with peroxisome proliferator-activated receptor. *Journal of Biological Chemistry* **272** 25500–25506.
- Zhu Y, Qi C, Jain S, Le Beau MM, Espinosa R 3rd, Atkins GB, Lazar MA, Yeldandi AV, Rao MS & Reddy JK. 1999 Amplification and overexpression of peroxisome proliferator-activated receptor binding protein (PBP/PPARBP) gene in breast cancer. *PNAS* **96** 10848–10853.

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