

Differential effects of retinoic acid isomers on the expression of nuclear receptor co-regulators in neuroblastoma

Penny E. Lovat^{a,b}, Margherita Annicchiarico-Petruzzelli^d, Marco Corazzari^d,
Mark G. Dobson^b, Archie J. Malcolm^c, Andy D.J. Pearson^a, Gerry Melino^d,
Christopher P.F. Redfern^{b,*}

^aDepartment of Child Health, Medical School, University of Newcastle, Newcastle Upon Tyne NE2 4HH, UK

^bDepartment of Medicine, Medical School, University of Newcastle, Newcastle Upon Tyne NE2 4HH, UK

^cDepartment of Pathology, Medical School, University of Newcastle, Newcastle Upon Tyne NE2 4HH, UK

^dIDI-IRCCS laboratory, Department of Experimental Medicine, University of Rome, Tor Vergata, Rome 00133, Italy

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Abstract Retinoic acid modulates growth and induces differentiation and apoptosis of neuroblastoma cells *in vitro*, with the *all-trans* and *9-cis* isomers having different biological properties. Transcriptional activation in response to retinoic acid isomers is mediated by retinoic acid receptors and retinoid X receptors. The differential expression of co-activators and co-repressors which preferentially interact with retinoic acid receptors or retinoid X receptors may be a mechanism leading to different cellular responses to *9-cis* and *all-trans* retinoic acid. To test this hypothesis, we have studied the expression of the nuclear receptor co-regulators TIF1 α , TIF1 β , SUG1 and SMRT in the N-type and S-type neuroblastoma cell lines SH SY 5Y and SH S EP. Transcripts for all four co-regulators were expressed in these neuroblastoma cells. The expression of TIF1 α , TIF1 β and SUG1 did not change in response to retinoic acid; however, SMRT was induced in both neuroblastoma cell lines, but particularly by *all-trans* retinoic acid in SH S EP cells. An additional co-activator, Trip3, was isolated by differential mRNA display and shown to be preferentially induced by *9-cis* retinoic acid in SH SY 5Y and SH S EP cells. These data suggest that retinoic acid isomer-specific induction of nuclear receptor co-regulators may determine, in part, the differential biological effects of retinoic acid isomers.

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Key words: Neuroblastoma; Co-activator; Co-repressor; *All-trans* retinoic acid; *9-cis* retinoic acid; SMRT; Trip3; TIF1

1. Introduction

Retinoic acid (RA) induces differentiation, growth arrest and apoptosis in many neuroblastoma cell lines [1,2] and in recent clinical trials with *13-cis* RA, the overall survival rates for children treated for residual neuroblastoma following bone marrow transplantation were significantly increased from 20 to 40% [3,4]. *In vitro* studies have shown that other RA isomers have a greater biological activity than *13-cis* RA, particularly *9-cis* RA which also has distinct biological properties [5,6]. The effects of RA are mediated by nuclear receptors (RARs), ligand-dependent transcriptional regulators functioning as heterodimers with auxiliary factors referred to as retinoid X receptors (RXRs) [7,8]. In this context, RXRs may function independently of ligand, but they can

also bind *9-cis* RA with a high affinity [9] and regulate gene transcription either as *9-cis* RA-dependent RXR homodimers [10] or as heterodimers with other receptors [11,12].

The ligand-dependent activation of genes via RXRs, RARs or other nuclear receptors depends on co-activators that interact with the receptor and the pre-initiation transcription complex. For example, the co-activator SUG1 interacts with the RAR partner of RAR/RXR heterodimers in a ligand-dependent manner [13] whereas the co-activator TIF1 α apparently interacts synergistically with both RAR and RXR heterodimer partners [13,14]. These co-activators may act either as 'bridging' molecules between receptors and the basal transcriptional machinery, or facilitate changes in the chromatin structure to allow transcriptional activation. The transcriptional activity of unliganded nuclear receptors is inhibited by co-repressors such as SMRT which preferentially bind to RARs and partially to RXRs in the absence of ligand [15]. Binding of ligand to RAR reduces the interaction with SMRT, whereas the binding of ligand to RXR has less effect [15]. TIF1 β is a co-regulator related to TIF1 α but lacks the nuclear receptor interacting domain of TIF1 α and may function as a co-repressor via the KRAB domain [13]. However, more-recent data suggest that TIF1 β enhances the expression of glucocorticoid regulated genes in a ligand- and response element-dependent manner [16].

Since TIF1 α or SUG1 differ in their interactions with RARs and RXRs the relative expression of these two co-activators may determine differential responses to RA isomers. Furthermore, since RAR β is induced by RA in neuroblastoma cells [5,17] it may be predicted that the overall levels of expression of co-repressors would also be regulated by RA to maintain cellular homeostasis with respect to the ligand-dependence of RARs. We have thus investigated the expression levels and responses to different retinoic acid isomers of co-regulators TIF1 α , SUG1, TIF1 β and SMRT in the SH SY 5Y and SH S EP cell lines, representing neuroblast (N-type) and substrate adherent (S-type) neuroblastoma cells derived from the mixed parental cell line SK N SH (in which both phenotypes are represented) [18], and report the identification of an additional retinoic acid-induced co-activator expressed in these neuroblastoma cells.

2. Materials and methods

2.1. Cell culture and treatment with retinoic acid isomers

SH SY 5Y (N-type) and SH S EP (S-type) neuroblastoma cells were

*Corresponding author. Fax: (44) (191) 2228129.

E-mail: chris.redfern@ncl.ac.uk

grown in Dulbecco's modification of Eagles medium, supplemented with 10% fetal bovine serum (Sera Lab, Crawley, UK) (culture medium) in a humidified atmosphere of 5% CO₂ in air. Prior to treatment with either 9-*cis* or all-*trans* RA, 8 × 10⁶ cells were seeded into 75 cm² tissue culture flasks (Costar, Bucks, UK) and cultured overnight. 9-*cis* or all-*trans* RA (Sigma Chemical Company, Poole, UK) were added in ethanol to a final concentration of 10⁻⁶ M. An equal volume of ethanol (<0.1% of the culture volume) was used to treat the control cells. Cultures were incubated in the presence of retinoids for 2–48 h prior to RNA extraction.

2.2. Extraction of RNA, size fractionation and Northern blotting

Total cellular RNA for Northern blotting was extracted by lysing cells in a guanidinium isothiocyanate solution followed by extraction with 1:1 phenol/chloroform [19]. RNA was concentrated by precipitation in isopropanol and size fractionated (20 µg per track) through glyoxal/DMSO gels [20], transferred to nylon membranes (Hybond, Amersham Bucks, UK) by vacuum blotting and fixed by treatment with UV light. For analysis by differential mRNA display, total cellular RNA was prepared from SH SY 5Y cells by lysis with guanidine thiocyanate and ultracentrifugation [21] at 230 000 × *g* for 3 h at 18°C (Beckman TLA 120.2 fixed angle rotor). Pelleted RNA was extracted three times with 1:1 phenol:chloroform [22], once with chloroform and ethanol precipitated at -20°C. After digestion with RNase-free DNase (Message Clean, Genhunter Corp., Nashville, TN, USA), RNA was stored in 3 µl aliquots at a concentration of 1 µg/µl at -20°C. For the RNase protection assays, total cytoplasmic RNA was extracted [22].

2.3. Probing of Northern blots with [³²P]-labelled cDNA probes

Northern blots were probed with [³²P]dCTP (Amersham)-labelled cDNA probes for SMRT, SUG1, TIF1β, TIF1α and GAPDH (loading control) [23] or with a riboprobe for Trip3 isolated from differential display experiments. Pre-hybridization and hybridization of the Trip3 riboprobe, prepared as for RNase protection assays was performed at 60°C using 7 × 10⁴ cpm in 15 ml of hybridization buffer. cDNA probes were inserts from EST clones obtained from I.M.A.G.E. consortium (LLNL) and their identities were confirmed by sequence analysis (Table 1). Blots were exposed to X-ray film and the autoradiographic images scanned for the measurement of band intensity using Quantiscan software (Version 1.5, Elsevier Biosoft). Band intensities were corrected for GAPDH loading and expressed relative to control, untreated cells. The data were analyzed using robust, non-parametric statistics and are presented in the figures as medians and ranges.

2.4. Differential mRNA display

2 µg RNA was reverse transcribed using, poly (T₁₈A, G, or C) primers, Superscript II reverse transcriptase (Life Technologies, Paisley, UK) and the RNA Image mRNA Differential Display system (GeneHunter) [24]. Primers for PCR were poly(T₁₈A) and CCGAATTCTGGTTCCAAAACCG primers for the upstream or 5' primer. PCR products were analyzed by electrophoresis through non-reducing sequencing gels and autoradiographed. Differentially-expressed bands were reamplified by PCR and cloned into the pCR2.1 cloning vector (Invitrogen, Leek, The Netherlands). After transformation into *Escherichia coli* (TOP10F') and the preparation of plasmid DNA, cloned inserts were sequenced in forward and reverse orientation using an Applied Biosystems DNA sequencer in the University of Newcastle Facility for Molecular Biology [24]. Sequence data were compared against sequences in the non-redundant Genbank and EST databases using the BLASTN program [25].

2.5. RNase protection assays

RNase protection assays were performed using the Ambion RNase protection assay kit (Ambion, AMS Biotechnology, Oxford). 1 µg of plasmid cDNA containing a Trip3 insert was linearized and a riboprobe prepared in a reaction mix consisting of T7 polymerase, 5 × buffer, 4 µl of 2.5 M ribonucleotide triphosphates (GTP, UTP and ATP), 1 µl of RNasin (40 U/µl), 2 µl 100 mM dithiothreitol, 2 µl of distilled water and 5 µl of radio-labelled [³²P]CTP (Amersham). After incubation for 1 h at 37°C, 2 U of DNase I were added and the reaction was incubated for a further 15 min at 37°C. After extraction with 1:1 phenol:chloroform and once with chloroform, the riboprobe (7 × 10⁴ counts/reaction) and test RNA (7.5 µg) were precipitated together at -20°C for 15 min with 0.5 M sodium acetate and 2.5 volumes of absolute ethanol, centrifuged at 13 000 rpm for 10 min at room temperature and redissolved in 20 µl of solution A from the RNase protection assay kit. The samples were then heated briefly for 3–4 min at 95°C before being hybridized overnight at 43°C. Following digestion with RNase A (1:50 dilution of stock) for 30 min at 37°C, samples were precipitated (-20°C for 15 min), resuspended in loading buffer and separated by electrophoresis through 0.4 mm 6% non-reducing sequencing gels. Dried gels were autoradiographed at -80°C overnight.

3. Results

3.1. Expression of TIF1α, TIF1β, SUG1 and SMRT in SH SY 5Y and SH S EP neuroblastoma cells

The expression of TIF1β, SUG1 and SMRT was readily detectable in SH SY 5Y and SH S EP cells (Figs. 1–3): SMRT mRNA was visible as a band running above the 28S rRNA, consistent with its reported transcript size of 5.9–6.0 kb [26], TIF1β mRNA was approximately 3.0 kb in length, running between 18S and 28S rRNA, whereas the SUG1 probe detected a single transcript between 18S rRNA and the 1.3 kb GAPDH mRNA (reported transcript size 1.4 kb [27]) (Fig. 1). TIF1α was also expressed but was only just detectable on Northern blots (data not shown) as a band running just ahead of 28S rRNA, consistent with its reported size of 4.5 kb [28]. Compared to TIF1α, SUG1 and TIF1β gave strong signals on Northern blots, suggesting that they were expressed at a higher level than TIF1α in these cells.

After treatment of SH S EP cells for up to 48 h with either all-*trans* or 9-*cis* RA at 10⁻⁶ M, there was a clear induction of SMRT mRNA in response to RA relative to control, ethanol-treated cells (data of Fig. 1 and two additional independent experiments: Kruskal-Wallis one-way 'analysis of variance' on control, 9-*cis* and all-*trans* RA-treated cells, all time points combined, *P* < 0.001: all-*trans* RA versus control, Mann-Whitney U-test, *P* < 0.002). Induction of SMRT mRNA was detectable after 2 h and reached up to 5-fold after 16–24 h with all-*trans* RA, before declining over the next 24 h (Fig. 2). Although SMRT was induced in response to both 9-*cis* and all-*trans* RA, all-*trans* RA gave a significantly greater induction than 9-*cis* RA overall (9-*cis* RA versus all-*trans* RA, all time points combined, Mann-Whitney U-test *P* < 0.05): 9-*cis* RA induced SMRT after 2 h but SMRT mRNA levels

Table 1
EST clones used for probes

Probe	Insert (kb)	Extent ¹	Source ²	Clone ID	EST name
TIF1α	1.6	3'UTR+ <0.5 CDS	IMAGE	446686	zx58a12.r1
TIF1β	1.0	3'UTR+0.25 CDS	IMAGE	265340	yx52a11.r1
SUG1	1.3	3'UTR+CDS	ATCC		EST188567
SMRT	0.5	3'UTR	IMAGE	145024	yi74g09.r1

¹UTR, untranslated region; CDS, coding sequence.

²IMAGE, I.M.A.G.E. Consortium, Lawrence Livermore National Laboratory, Livermore, CA; ATCC, American Tissue Culture Collection.

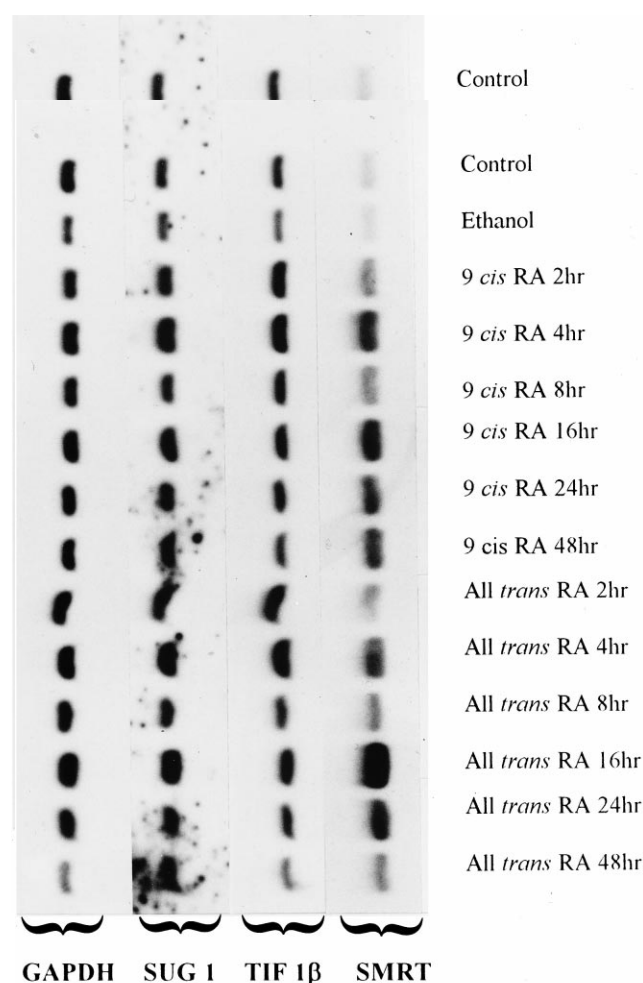


Fig. 1. Northern blot of SMRT, TIF1 β , SUG1 and GAPDH expression in SH S EP neuroblastoma cells treated for 2, 4, 8, 16, 24 or 48 h with 1 μ M 9-*cis* or all-*trans* RA. Quantification of these data are summarized in Fig. 2 together with the data from two additional independent experiments.

reached a plateau after 4 h with little subsequent change. Conversely, there was no significant induction of either SUG1 or TIF1 β (Fig. 2) or TIF1 α (data not shown) over 48 h of treatment with 9-*cis* or all-*trans* RA. SH SY 5Y cells showed a similar pattern of SMRT induction relative to TIF1 β and SUG1 but in these cells there was no significant difference between all-*trans* and 9-*cis* RA (Fig. 3).

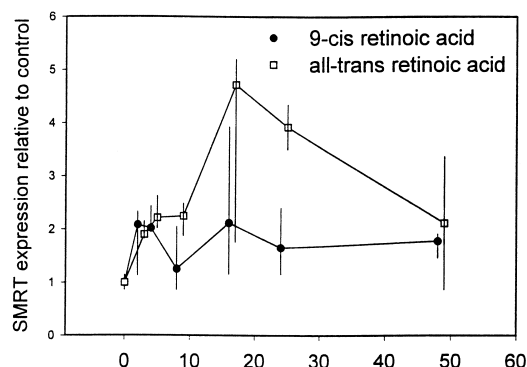
3.2. Identification of Trip3 as a RA regulated co-activator in neuroblastoma cells

A differential display analysis carried out to identify the genes regulated by different RA isomers resulted in the identification of a 135 bp cDNA fragment preferentially induced by 1 μ M 9-*cis* RA after 6 h. A BLASTN search of the EMBL/Genbank database [25] with the sequence of this cDNA gave an exact match to bases 166–300 of the putative co-activator Trip3 (EMBL accession number L40410) [29]. Since this is within the encoding sequence of Trip3 and not the 3'UTR as expected from the differential display strategy, this cDNA presumably resulted from internal priming during the cDNA synthesis or PCR steps.

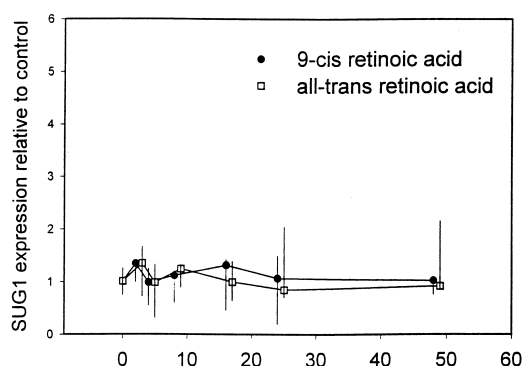
Induction of Trip3 in response to 9-*cis* RA was confirmed

by RNase protection assays using the 135 bp Trip3 cDNA cloned into the pCR2.1 vector, as a probe. In three independent experiments, SH SY 5Y cells were treated with 1 μ M all-*trans* or 9-*cis* RA for 6 h. Relative to control cells, Trip3 was consistently induced by 9-*cis* RA: all-*trans* RA also induced Trip3 but compared to induction by 9-*cis* RA the response was minimal (Fig. 4). Trip3 was also expressed in SH S EP cells and markedly induced by 9-*cis* RA but to a lesser extent by all-*trans* RA (Fig. 4).

SMRT expression in retinoic acid-treated SH S EP cells



SUG1 expression in retinoic acid-treated SH S EP cells



Tif1 β expression in retinoic acid-treated SH S EP cells

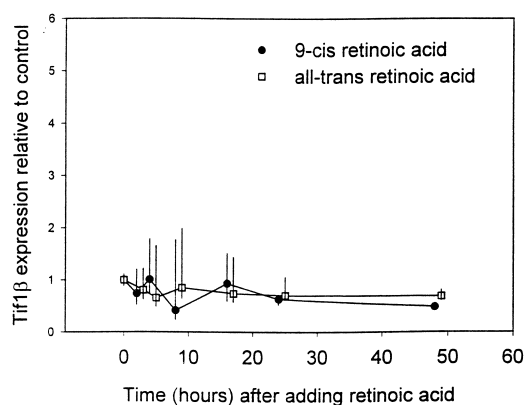
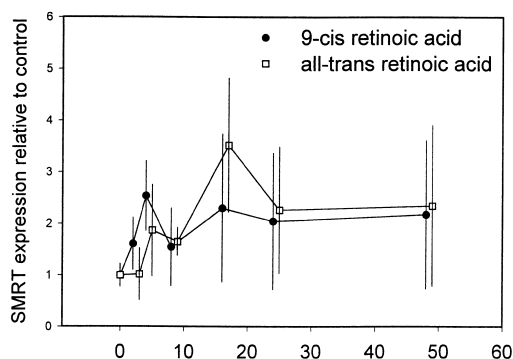


Fig. 2. Time course of SMRT, SUG1 and TIF1 β expression in SH S EP S-type neuroblastoma cells. Each point is the median induction relative to control ($t=0$) cells (error bars are the ranges) for three replicates (data of Fig. 1 and two additional independent experiments), except controls for which $n=6$.

SMRT expression in retinoic acid-treated SH SY 5Y cells



SUG1 expression in retinoic acid-treated SH SY 5Y cells

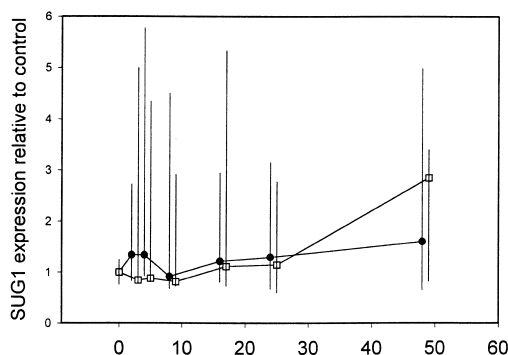
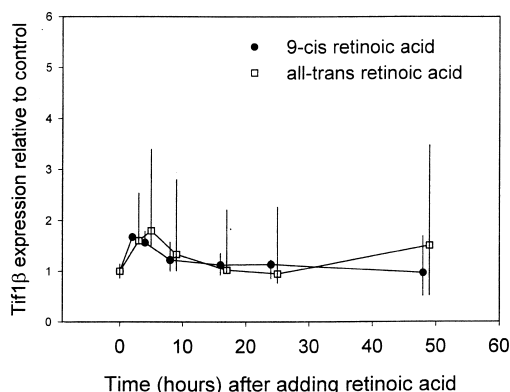
Tif1 β expression in retinoic acid-treated SH SY 5Y cells

Fig. 3. Time course of SMRT, SUG1 and TIF1 β expression in N-type SH SY 5Y neuroblastoma cells. Each point is the median induction relative to control (error bars are the ranges) for three replicates, except controls where $n=6$ and the SMRT results where each point represents the mean of duplicate experiments.

4. Discussion

The results of this study show that SMRT, SUG1, TIF1 α and TIF1 β were all expressed in S-type SH S EP and N-type SH SY 5Y neuroblastoma cells, although expression of TIF1 α was barely detectable. SMRT and SUG1 (Trip1) are also expressed in the SK N BE, SMS KCNR and IMR32 neuroblastoma cell lines [30]. Since RAR- β is induced by RA in neuroblastoma cells [5,17], the induction of SMRT in response to all-*trans* and 9-*cis* RA supports the idea that co-repressor expression is co-induced to maintain ligand-dependence of particular nuclear receptors. SMRT induction in re-

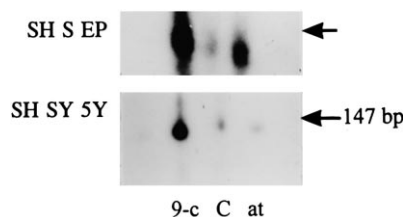


Fig. 4. The RNase protection assay for transcripts complementary to Trip3. RNA (7.5 μ g) was extracted from cells treated for 6 h with 1 μ M all-*trans* (at) or 9-*cis* RA (9c) or control ethanol (C) and hybridized with [32 P]-labelled riboprobes complementary to Trip3. The protected 135 base fragment corresponding to Trip3 ran just below the 147 base marker (arrow). An apparent difference in size between bands in the SH S EP cell experiment is a gel running artifact.

sponse to RA was rapid, detectable within 2 h, implying a direct transcriptional effect, although there are, as yet, no data identifying retinoic acid response elements within the SMRT promoter. Whether the induction of co-repressors is a general phenomenon linking changes in nuclear receptor expression with concomitant changes in co-repressor expression, is not known at present. As well as potentially maintaining homeostasis between nuclear receptors and co-repressors, the induction of SMRT by RA may have other consequences. For example, when overexpressed in primary synovial fibroblasts, SMRT inhibits the expression of matrix metalloproteinase-1 independently of hormone response elements [31]. Thus, this may be a mechanism whereby RA has indirect, RAR-independent effects on cellular responses. Furthermore, since the agonist/antagonist activity of synthetic receptor ligands such as anti-oestrogens (tamoxifen, 4-hydroxytamoxifen) and anti-progestins (RU486), can be modulated by altering the co-repressor/co-activator ratio [32,33], the induction of SMRT in response to RA may have important consequences for reducing unwanted agonist activity of anti-oestrogens and anti-progestins in a clinical setting. This implies that SMRT may also have a role in modulating the agonist/antagonist activities of synthetic retinoids.

Since our data show no apparent change in the expression of the co-activators SUG1 and TIF1 α in response to either RA isomer, the level of these co-activators could limit ligand-dependent gene regulation, but further studies will be required to examine the stoichiometry of co-activators and nuclear receptors. SUG1 preferentially interacts with RARs in RAR-RXR heterodimers [13,14] and the relatively high level of expression of SUG1 compared to TIF1 α in these neuroblastoma cells suggests that responses to RA will be mediated mainly via the RAR partner. However, other co-activators may be important in mediating ligand-dependent effects via both RARs and RXRs and this question needs to be addressed in neuroblastoma cells by performing two hybrid screens to assess qualitatively and quantitatively which co-regulators are important in mediating retinoid responses. Trip3 is a potential co-activator that may mediate RA-dependent effects. Other than the fact that Trip3 is expressed in a range of tissues, including neural tissue, and that it contains a transcriptional activation domain [29], little is known of the function of this gene product. The fact that Trip3 was preferentially induced by 9-*cis* RA relative to all-*trans* RA, whereas SMRT was preferentially induced by all-*trans* RA in SH S EP cells, suggests that differential regulation of co-regulators could be a factor underlying the differential bio-

logical effects of 9-*cis* RA and all-*trans* RA. This further emphasizes the importance of detailed studies of co-regulator stoichiometry to elucidate the mechanisms of different RA isomers and RA analogues in neuroblastoma cells.

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