Oncogene (2009) 28, 4075–4094

www.nature.com/onc

ORIGINAL ARTICLE

The neuroblastoma tumour-suppressor TrkAI and its oncogenic alternative TrkAIII splice variant exhibit geldanamycin-sensitive interactions with Hsp90 in human neuroblastoma cells

AR Farina[1,4], A Tacconelli[1,4], L Cappabianca[1], G Cea[1], A Chioda[1], A Romanelli[2], S Pensato[2], C Pedone[2], A Gulino[3] and AR Mackay[1]

[1]*Department of Experimental Medicine, University of L'Aquila, L'Aquila, Italy;* [2]*Department of Biological Science, University of Naples 'Federico II', Naples, Italy and* [3]*Department of Experimental Medicine, University of Rome 'La Sapienza', Rome, Italy*

Hsp90 chaperones stabilize many tyrosine kinases including several oncogenes, which are inhibited or induced to degrade by the Hsp90 inhibitor geldanamycin (GA). As a consequence, GA has been developed for future chemotherapeutic use in several tumour types including neuroblastoma (NB). Alternative splicing of the neurotrophin receptor tyrosine kinase TrkA may have a pivotal function in regulating NB behaviour, with reports suggesting that tumour-suppressing signals from TrkA may be converted to oncogenic signals by stress-regulated alternative TrkAIII splicing. Within this context, it is important to know whether Hsp90 interacts with TrkA variants in NB cells and how GA influences this. Here, we report that both TrkAI and TrkAIII are Hsp90 clients in human NB cells. TrkAI exhibits GA-sensitive interaction with Hsp90 required for receptor endoplasmic reticulum export, maturation, cell surface stabilization and ligand-mediated activation, whereas TrkAIII exhibits GA-sensitive interactions with Hsp90 required for spontaneous activity and to a lesser extent stability. We show that GA inhibits proliferation and induces apoptosis of TrkAI expressing NB cells, whereas TrkAIII reduces the sensitivity of NB cells to GA-induced elimination. Our data suggest that GA-sensitive interactions with Hsp90 are critical for both TrkAI tumour suppressor and TrkAIII oncogenic function in NB and that TrkAIII expression exerts a negative impact on GA-induced NB cell eradication, which can be counteracted by a novel TrkAIII-specific peptide nucleic acid inhibitor.
Oncogene (2009) **28,** 4075–4094; doi:10.1038/onc.2009.256; published online 7 September 2009

Keywords: neuroblastoma; TrkAI; TrkAIII; Hsp90; geldanamycin

Correspondence: AR Mackay, Department of Experimental Medicine, Section of Molecular Pathology, University of L'Aquila, Coppito 2, Via Vetoio, L'Aquila, AQ 67100, Italy.
E-mail: andrewreay.mackay@univaq.it
[4]These authors contributed equally to this work.
Received 20 October 2008; revised 30 April 2009; accepted 21 July 2009; published online 7 September 2009

Introduction

The Hsp90 family is comprised of abundant cytoplasmic ATPases Hsp90α and Hsp90β and the endoplasmic reticulum (ER) paralogue Grp94 (Richter *et al*., 2001; Rosser *et al*., 2004; Frey *et al*., 2007). These chaperones have essential functions in the folding, maturation, activity and stability of numerous client proteins, including many protein tyrosine kinases (Neckers, 2002; Citri *et al*., 2006). The essential function of Hsp90 in stabilizing client proteins is reflected by induction of client protein degradation by the specific Hsp90 inhibitor ansamycin antibiotic geldanamycin (GA). This quinone occupies the N-terminal ATP/ADP-binding site of Hsp90 homodimers, abrogating ATP/ADP binding, inhibiting ATPase activity and interfering with ATPase-dependent client–protein interactions (Grenert *et al*., 1997, 1999; Onuoha *et al*., 2007), which frequently results in client–protein dissociation and ubiquitin-dependent degradation (Schneider *et al*., 1996; Stebbins *et al*., 1997; Connell *et al*., 2001; Xu *et al*., 2002; Zhou *et al*., 2003). Hsp90 proteins also exhibit generalized passive chaperone function with some client proteins, involving an alternative ATP/ADP-independent-binding site (Shankovich *et al*., 1992; Jakob *et al*., 1995; Scheibel *et al*., 1998).

Hsp90 clients include the oncogenic kinases ErbB2 (Her2/neu), EGFRvIII, BCR-ABL, Flt3, NPM-ALK, PDGFRα, Src and Akt, which are either inhibited or induced to degrade by GA (Xu *et al*., 2001, 2002; Lavictoire *et al*., 2003; Zhou *et al*., 2003; Dai and Whitesell, 2005; Matei *et al*., 2007). As a consequence, GA has been evaluated and developed as a potential chemotherapeutic agent and GA analogues have recently completed Phase-I clinical trials in adult and paediatric tumours and the paediatric tumour neuroblastoma (NB) identified for potential future GA-analogue chemotherapy (Lòpez-Maderuelo *et al*., 2001; Kim *et al*., 2003; Escobar *et al*., 2005; Graner and Bigner, 2005; Grem *et al*., 2005; Bagatell *et al*., 2007; Erdmann *et al*., 2007; Ramanathan *et al*., 2007; Shen *et al*., 2007; Solit *et al*., 2007; Weigel *et al*., 2007; Xu and Neckers, 2007).

NBs represent the most frequent paediatric extracranial solid tumour and originate from neural crest cells during sympathetic nervous system development (reviewed in Pahlman and Hedborg, 2000). Consistent with this origin, NBs exhibit varying degrees of neurotrophin tyrosine kinase receptor TrkA expression, which regulates sympathetic nervous system development (Pahlman and Hedborg, 2000; Orike *et al*., 2001). TrkA expression in NB is considered to be clinically relevant and unveils a pivotal function for this receptor in regulating tumour behaviour (Nakagawara *et al*., 1992; Nakagawara, 2001). TrkA is expressed as either TrkAI, TrkAII or alternative TrkAIII splice variants (Tacconelli *et al*., 2006). TrkAI and TrkAII differ in exon 9 inclusion, are expressed as cell surface receptors and activated on nerve growth factor (NGF) or NT3 neurotrophin ligation. In contrast, the stress-regulated alternative TrkAIII splice variant bears a unique in-frame exons 6/7 deletion, which abrogates receptor cell surface expression and promotes intracellular accumulation and spontaneous activation (Tacconelli *et al*., 2004, 2005, 2006). In NB, wild-type TrkA (I/II) is expressed predominantly by low-stage tumours and exhibits tumour-suppressor activity in NB models (Matsushima and Bogenmann, 1990; Lavenius *et al*., 1995; Lucarelli *et al*., 1997; Nakagawara, 2001; Eggert *et al*., 2002; Tacconelli *et al*., 2004). In contrast, TrkAIII is expressed by advanced stage tumours and exhibits oncogenic activity in NB models (Tacconelli *et al*., 2004, 2005, 2006). This has led to the novel hypothesis that stress-regulated alternative TrkAIII splicing provides a mechanism through which tumour-suppressing signals from TrkAI may be converted to oncogenic signals from TrkAIII during tumour progression, and has identified TrkAIII as a novel potential therapeutic target. Within this context and considering future GA chemotherapeutic use, it is important to know how Hsp90 chaperones interact with alternative TrkA splice variants and how GA influence this interaction in NB cells.

In this study, we report that both TrkAI and TrkAIII are Hsp90 clients in human NB cells, involved in GA-sensitive interactions potentially critical for TrkAI tumour suppressor and TrkAIII oncogenic function. We also show that TrkAIII exerts a negative impact on GA-induced NB cell eradication that can be counteracted by a TrkAIII-specific peptide nucleic acid (PNA).

Results

GA and GA analogues destabilize cell surface TrkAI in NB cells

TrkAI SH-SY5Y transfectants exhibited predominant TrkAI localization to the cell surface and Golgi network (GN), with GN localization confirmed by overlapping expression with the cis-GN marker GM130 (Figure 1a). Overnight treatment with 1 μM GA induced complete loss of cell surface TrkAI and increased intracellular TrkAI accumulation that closely overlapped the ER marker calnexin (Figure 1b). Immunoprecipitation (IP)–western blots detected gp140TrkAI degradation at GA concentrations $\geqslant$0.05 μM (Figure 1c), with complete gp140TrkAI loss at GA concentrations $\geqslant$0.5 μM within 8 h (Figure 1d), associated with gp110TrkAI accumulation (Figure 1c and d, data displayed for 0.1 μM GA only). Co-treatment with 1 μM GA and 25 μM MG-132 abrogated gp140TrkAI degradation without influencing gp110TrkA accumulation, indicating that GA had induced lysosome/proteosome-mediated gp140TrkAI, but not gp110TrkA degradation (Figure 1d). The clinically relevant GA-analogues 17-AAG and 17-DMAG (Miyata, 2005) also induced gp140TrkAI degradation and promoted gp110TrkAI accumulation in TrkAI SH-SY5Y transfectants at concentrations $\geqslant$0.1 μM (Figure 1e, data displayed for 1 μM 17-AAG and 1 μM 17-DMAG only). Furthermore, GA at concentrations of 0.1 and 1 μM induced degradation of endogenous gp140TrkA, but not gp110TrkA expressed by non-transfected IMR-32 and SH-SY5Y NB cells (Figure 1f), which was reversed by 25 μM MG132 in IMR32 cells (Figure 1f) and also reduced cell surface expression of endogenous TrkAI in IMR32 cells (Figure 1g).

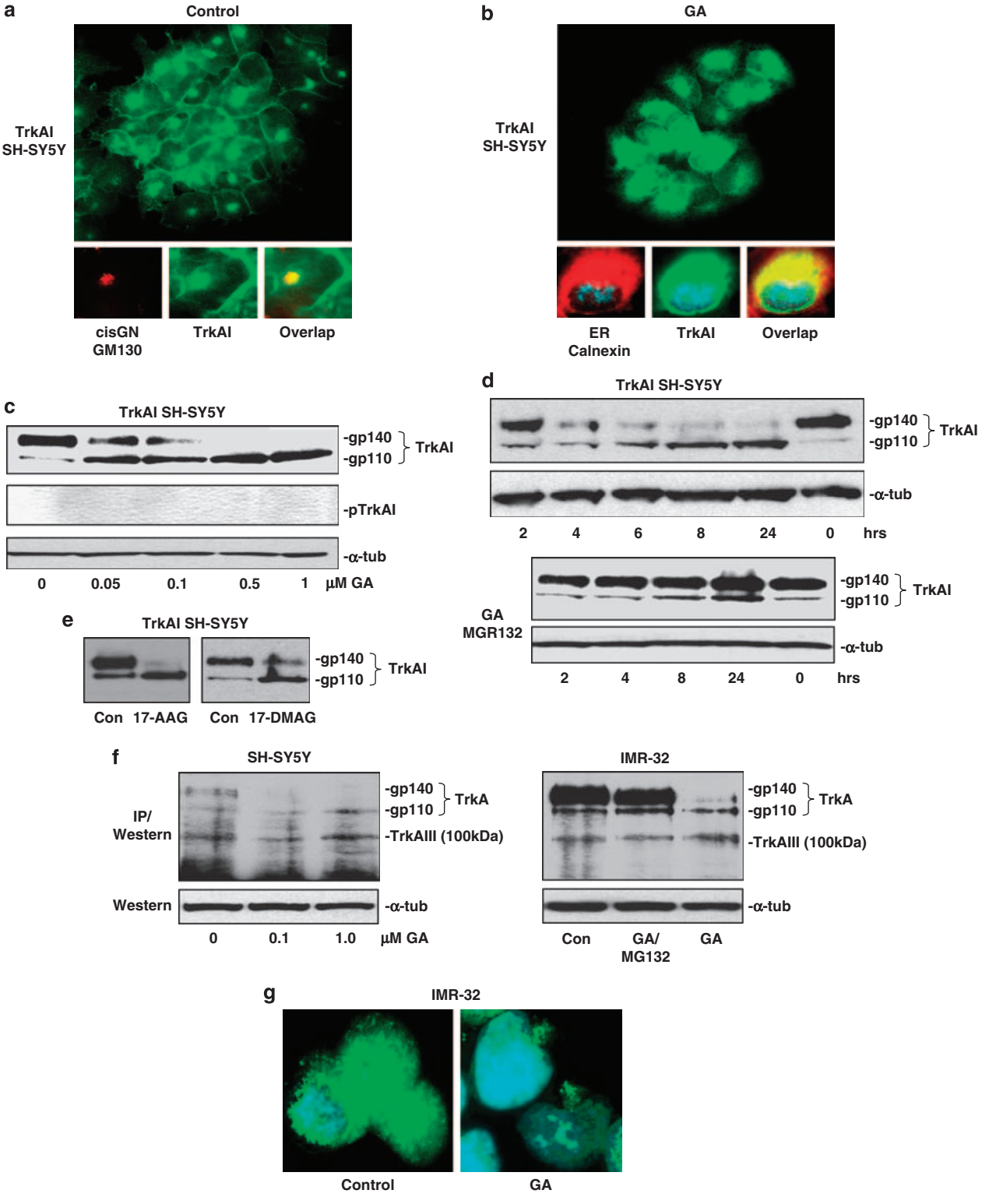
Pre-treatment of TrkAI SH-SY5Y transfectants for 30 min with 1 μM GA, which did not reduce gp140TrkAI levels, attenuated NGF-induced gp140TrkAI tyrosine phosphorylation over a 30 min time course, compared with non-GA pre-treated NGF-activated controls (Figure 2a, different TrkAI levels represent IP artefacts and not differences in expression). Attenuation of NGF-TrkAI activation after short-term GA pre-incubation was not reversed by 25 μM MG132, indicating independence from lysosome/proteosome degradation (Figure 2b). Overnight pre-treatment of TrkAI SH-SY5Y

Figure 1 GA induces degradation of cell surface gp140TrkAI. (**a**) Indirect IF demonstrating cell surface and GN-associated TrkAI immunoreactivity and overlapping expression of intracellular TrkAI and GN-associated GM130 in TrkAI SH-SY5Y cells. (**b**) Indirect IF demonstrating GA-induced loss of cell surface TrkAI associated with intracellular TrkAI accumulation, together with overlapping with ER-associated calnexin, in TrkAI SH-SY5Y transfectants treated overnight with 1 μM GA. (**c**) IP–western blots demonstrating GA-induced loss of gp140TrkAI, associated with gp110TrkAI accumulation in the absence of TrkAI tyrosine phosphorylation, in TrkAI SH-SY5Y transfectants treated overnight with the GA concentrations indicated. (**d**) IP–western blot time course (0–24 h) demonstrating loss of gp140TrkAI and accumulation of gp110TrkAI in TrkAI SH-SY5Y transfectants treated with 1 μM GA in the absence (upper panels) or presence (lower panels) of 25 μM MGR132. Levels of α-tubulin (α-tub) in the whole extracts used for IP are shown. (**e**) IP–western blots demonstrating loss of gp140TrkAI associated with gp110TrkAI accumulation in TrkAI SH-SY5Y transfectants treated overnight with 1 μM 17-AAG or 1 μM 17-DMAG. (**f**) IP–western blots demonstrating GA-induced loss of endogeous gp140TrkAI, but not endogenous gp110TrkAI or TrkAIII in non-transfected SH-SY5Y (left panels) and IMR-32 (right panels) cells treated overnight with 0.1 and 1.0 μM GA for SH-SY5Y cell and 1 μM GA in the presence or absence of 25 μM MG132 for IMR-32 cells. (**g**) Indirect IF demonstrating the difference in TrkA immunoreactivity in IMR-32 cells treated overnight in the presence or absence of 1 μM GA.

transfectants with 1 μM GA, which induced complete gp140TrkAI loss, abrogated TrkAI responsiveness to 100 ng/ml NGF in terms of tyrosine phosphorylation (Figure 2c). GA (1 μM) alone did not induce TrkAI tyrosine phosphorylation (Figure 2d).

GA and GA analogues inhibit TrkAIII tyrosine phosphorylation

TrkAIII SH-SY5Y transfectants treated overnight with 1 μM GA exhibited less-centralized more diffuse intracellular TrkAIII expression (Figure 3a). GA at



concentrations of $\geqslant 0.05\,\mu M$ inhibited TrkAIII tyrosine phosphorylation, with complete abrogation detected within 2 h at GA concentrations $\geqslant 0.1\,\mu M$ (Figure 3b and c), and reduced TrkAIII levels at GA concentrations of $\geqslant 0.1\,\mu M$ (Figure 3b and c). Co-incubation with $1\,\mu M$ GA and $25\,\mu M$ MG-132 reversed GA-induced TrkAIII – loss, but failed to restore TrkAIII tyrosine phosphorylation (Figure 3c). TrkAIII exhibited re-phosphorylation

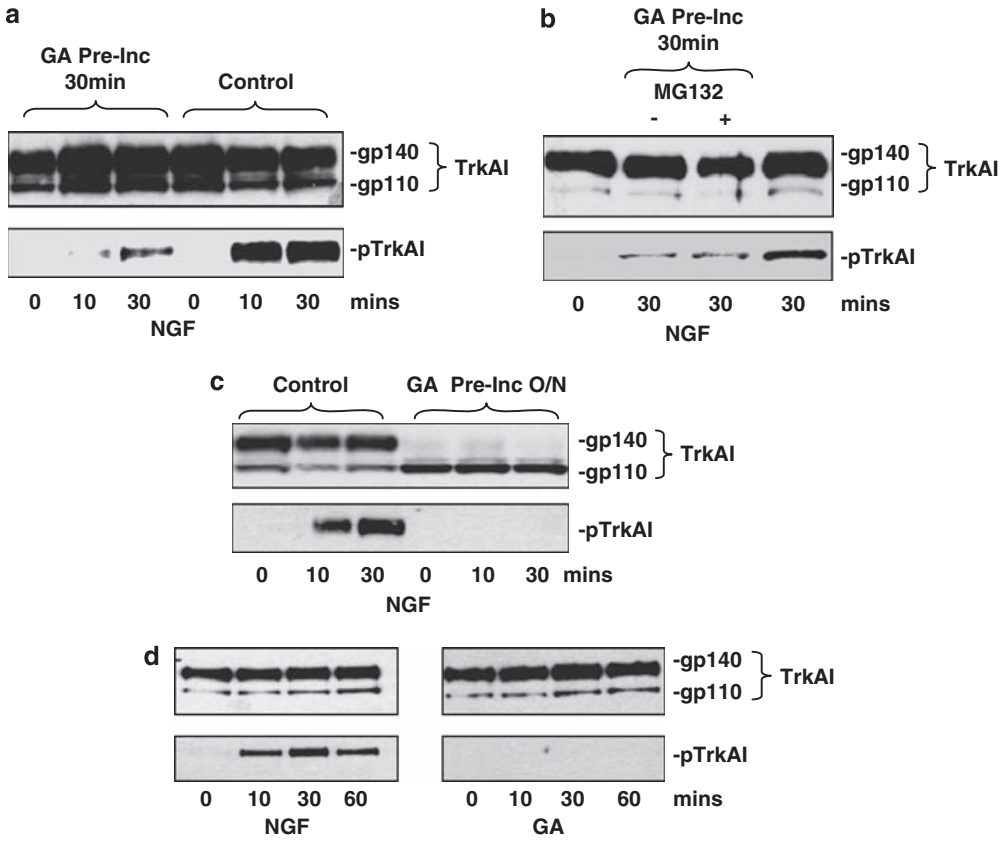
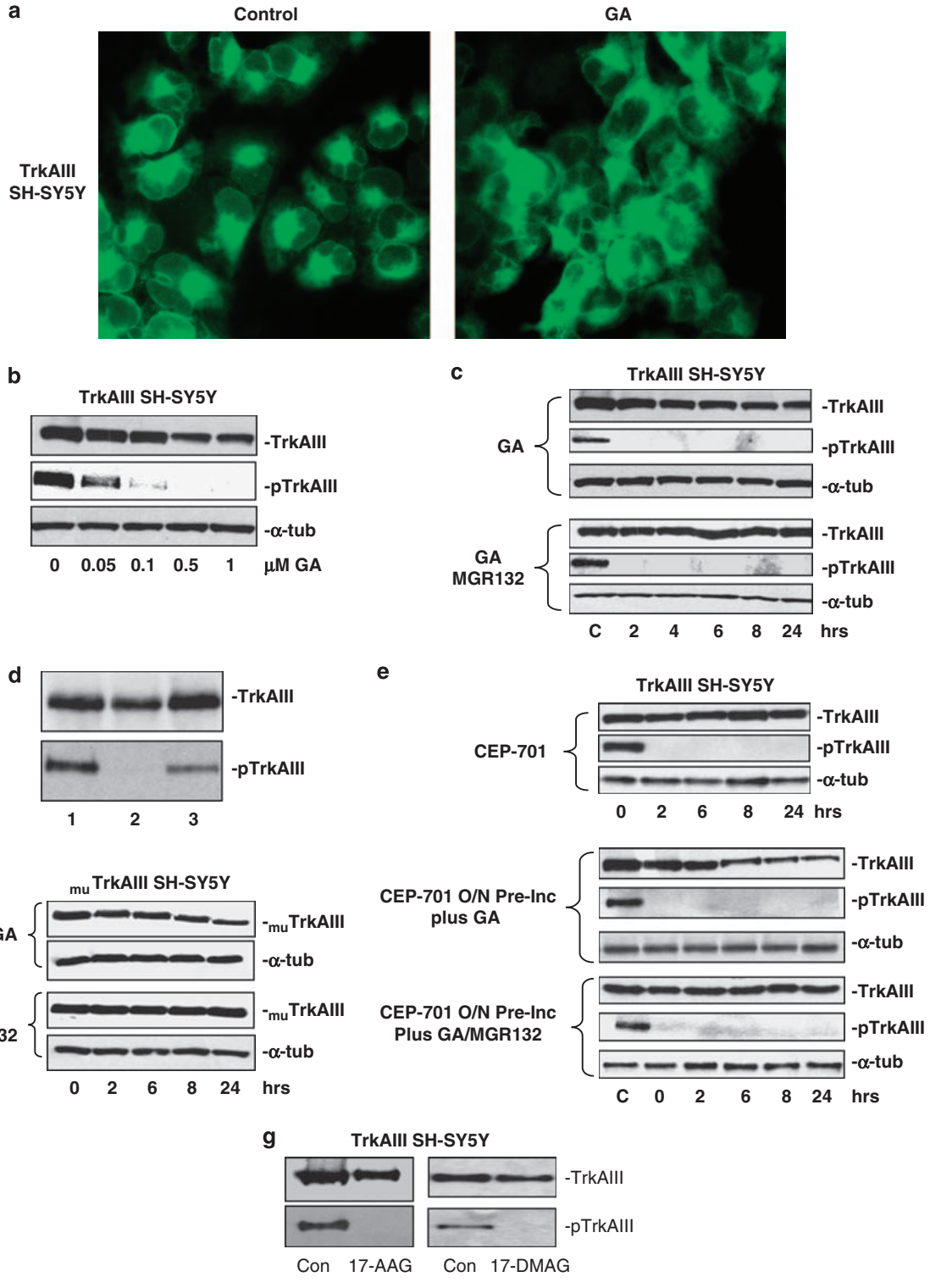


Figure 2 GA inhibits TrkAI NGF responsiveness. (**a**) IP–western blots demonstrating relative attenuation of NGF-induced TrkAI tyrosine phosphorylation (pTrkAI) in the absence of gp140TrkAI loss in TrkAI transfectants pre-treated for 30 min with $1\,\mu M$ GA, followed by 100 ng/ml NGF for 0–30 min, compared with non-GA pre-treated NGF-treated controls. (**b**) IP–western blots demonstrating that MG132 ($25\,\mu M$) does not reverse GA-attenuated NGF-induced TrkAI tyrosine phosphorylation (pTrkAI) in TrkAI transfectants pre-treated for 30 min with $1\,\mu M$ GA, compared with non-GA pre-treated NGF-treated controls. (**c**) IP–western blots demonstrating induction of pg140TrkAI tyrosine phosphorylation (pTrkAI) by 100 ng/ml NGF in non-GA pre-treated TrkAI SH-SY5Y transfectants, but not in TrkAI SH-SY5Y transfectants pre-treated overnight with $1\,\mu M$ GA, before treatment with 100 ng/ml NGF, for the times indicated. (**d**) IP–western blots demonstrating total (TrkAI) and tyrosine phosphorylated TrkAI (pTrkAI) in TrkAI SH-SY5Y transfectants treated with 100 ng/ml NGF (left panels) or $1\,\mu M$ GA (right panels) for the times indicated.

Figure 3 GA induces partial degradation of TrkAIII and inhibits TrkAIII tk activity. (**a**) Indirect IF demonstrating subtle changes in intracellular TrkAIII immunoreactivity in TrkAIII SH-SY5Y transfectants treated overnight with $1\,\mu M$ GA. (**b**) IP–western blots demonstrating GA-induced loss of TrkAIII associated with inhibition of constitutive TrkAIII tyrosine phosphorylation in TrkAIII SH-SY5Y transfectants treated overnight with GA, at the concentrations indicated. (**c**) IP–western blot time course (0–24 h) demonstrating limited loss of TrkAIII and inhibition of TrkAIII tyrosine phosphorylation in TrkAIII SH-SY5Y transfectants treated with $1\,\mu M$ GA, for the times indicated (upper panels) and reversal of TrkAIII loss, but not restoration of TrkAIII tyrosine phosphorylation in TrkAIII SH-SY5Y transfectants co-treated with $1\,\mu M$ GA and $25\,\mu M$ MG-132 (lower panels). (**d**) IP–western blots demonstrating constitutive TrkAIII tyrosine phosphorylation in TrkAIII transfectants (lane 1), loss of TrkAIII tyrosine phosphorylation after overnight treatment with $1\,\mu M$ GA (lane 2) and restoration of TrkAIII tyrosine phosphorylation 8 h after GA removal (lane 3). (**e**) IP–western blot time courses (0–24 h) demonstrating: inhibition of TrkAIII tyrosine phosphorylation without loss of total TrkAIII in TrkAIII SH-SY5Y transfectants treated overnight with 100 nM CEP-701 (upper panels); $1\,\mu M$ GA-induced loss of CEP-701-inhibited non-phosphorylated TrkAIII (middle panels) and reversal of this effect by $25\,\mu M$ MG-132 (lower panels). Constitutive TrkAIII tyrosine phosphorylated in untreated controls are presented for comparison (**c**) and relative α-tubulin levels (α-tub) in the whole extracts used for IP shown. (**f**) IP–western blot time course (0–24 h) demonstrating $1\,\mu M$ GA-induced loss of $_{mu}$TrkAIII in stable SH-SY5Y transfectants in the absence of MG-132 (GA, upper panels) and reversal of this effect by $25\,\mu M$ MG132 (GA/MG-132, lower panels). (**g**) IP–western blots demonstrating limited loss of TrkAIII, associated with the abrogation of constitutive TrkAIII tyrosine phosphorylation in TrkAIII SH-SY5Y transfectants treated overnight with $1\,\mu M$ 17-AAG or $1\,\mu M$ 17-DMAG.

on GA removal (Figure 3d). MG-132 (25 μM) alone did not alter TrkAIII expression or constitutive tyrosine phosphorylation in TrkAIII SH-SY5Y transfectants (data not shown). GA (1 μM) also induced low-level degradation of non-phosphorylated CEP-701-inhibited TrkAIII and $_{mu}$TrkAIII expressed by $_{mu}$TrkAIII stable SH-SY5Y transfectants, which was reversed by 25 μM MG-132 (Figure 3e and f). CEP-701 (100 nM) alone did not induce TrkAIII loss (Figure 3e). GA-analogues 17-AAG and 17-DMAG at concentrations of $\geqslant$1 μM also abrogated TrkAIII tyrosine phosphorylation and reduced TrkAIII levels (Figure 3g). Endogenous TrkAIII



expressed by non-transfected SH-SY5Y and IMR-32 NB cells was not reduced by overnight treatment with 1 μM GA (Figure 1g).

Pulse-chase analysis of GA effects on TrkAI and TrkAIII
Pulse-chase metabolic and biotin labelling of SH-SY5Y transfectants confirmed that gp140TrkAI matures from nascent immature gp110TrkAI within 20 min before cell surface translocation within 1–2 h (Figure 4a). Pre-treatment of TrkAI transfectants for 8 h with 1 μM GA before pulse chase completely inhibited gp140TrkAI maturation and cell surface translocation (Figure 4b). Pulse-chase metabolic and biotin labelling of TrkAIII SH-SY5Y transfectants confirmed that nascent 100 kDa TrkAIII neither matures further in molecular size nor translocates to the cell surface (Figure 4c). Pre-treatment of TrkAIII SH-SY5Y transfectants for 8 h with 1 μM GA before pulse chase resulted in low-level TrkAIII loss at times $\geqslant$1 h, associated appearance of a lower molecular weight potential degradation product (Figure 4d, arrow).

SH-SY5Y transfectants exhibit cell surface Hsp90 expression
Hsp90 interaction with gp140TrkAI at the cell surface was supported by (a) FACS analysis, using a dual-specific anti Hsp90α/β antibody, which detected cell surface Hsp90 (α/β) expression in non-permeabilized pcDNA, TrkAI and TrkAIII transfected SH-SY5Y cells, reduced in TrkAI transfectants by siRNA Hsp90α/β knockdown (Figure 5a); (b) siRNA Hsp90β, but not Grp94 knockdown, which reduced cell surface TrkAI expression in TrkAI SH-SY5Y transfectants (Figure 5d); (c) gp140TrkAI co-IP of Hsp90 from TrkAI SH-SY5Y transfectant membranes (Figure 5b) and (d) overlapping cell surface Hsp90 (α/β) and TrkAI expression in TrkAI SH-SY5Y transfectants (Figure 5c). In contrast, TrkAIII SH-SY5Y transfectants exhibited overlapping intracellular TrkAIII and Hsp90 (α/β) expression (Figure 5c).

GA promotes TrkAI and TrkAIII ER retention and alters interactions with Hsp90 and Grp78/BiP
GA-sensitive interactions between Hsp90 and TrkA variants were confirmed by GA-induced reduction in TrkAI and TrkAIII co-IP of Hsp90, in whole cell extracts, associated with GA-induced TrkAI binding of Grp78/BiP and GA-increased TrkAIII binding of Grp78/BiP (Figures 6a and 7a).

Within fractionated intracellular membranes, TrkAI exhibited low levels of predominantly immature gp110TrkAI within calnexin-positive ER membranes and high levels of predominantly mature gp140TrkAI within GM130, TGN46 and COP II positive, calnexin negative, membranes of the ER Golgi-intermediate, GN and COP vesicle compartments (Figure 6b and d). TrkAI within ER membranes constitutively bound Grp94 and calreticulin, but not Hsp90, Grp78/BiP or calnexin, whereas TrkAI in non-ER membranes constitutively bound Hsp90 (Figure 6d). Overnight treatment with 1 μM GA promoted gp110TrkAI accumulation within calnexin-positive ER membranes,

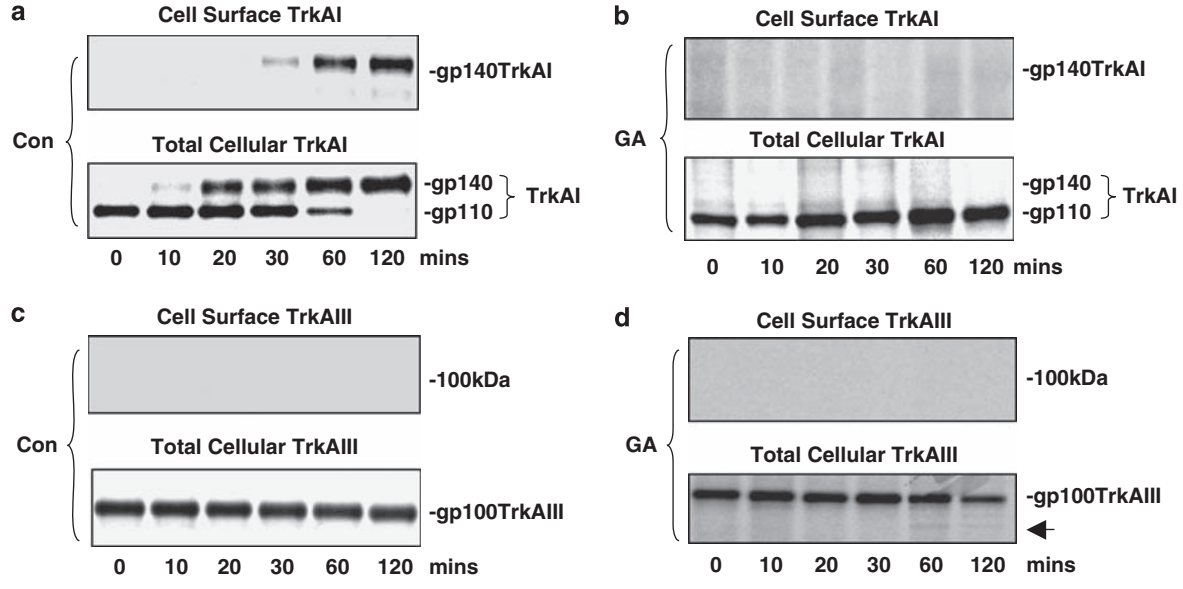


Figure 4 Metabolic and biotin labelling of TrkAI and TrkAIII. Autoradiographs demonstrating (**a**) gp110–gp140 kDa maturation of S^{35} pulse-labelled TrkAI and cell surface translocation of S^{35} and biotin-labelled mature gp140TrkAI in a 120 min pulse-chase experimental time course in TrkAI SH-SY5Y transfectants. (**b**) Inhibition of gp110–gp140 kDa maturation of S^{35} pulse-labelled TrkAI and absence of cell surface TrkAI translocation in TrkAI SH-SY5Y transfectants pre-incubated for 8 h with 1 μM GA before metabolic labelling in a 120 min pulse-chase experimental time course. (**c**) No change in S^{35} pulse-labelled TrkAIII molecular size and absence of cell surface TrkAIII expression in TrkAIII SH-SY5Y transfectants in a 120 min pulse-chase experimental time course. (**d**) Limited loss of gp100TrkAIII in the absence of cell surface TrkAIII translocation in TrkAIII SH-SY5Y transfectants pre-treated for 8 h with 1 μM GA before a 120 min pulse-chase experimental time course (the arrow indicates a potential GA-induced TrkAIII degradation product).

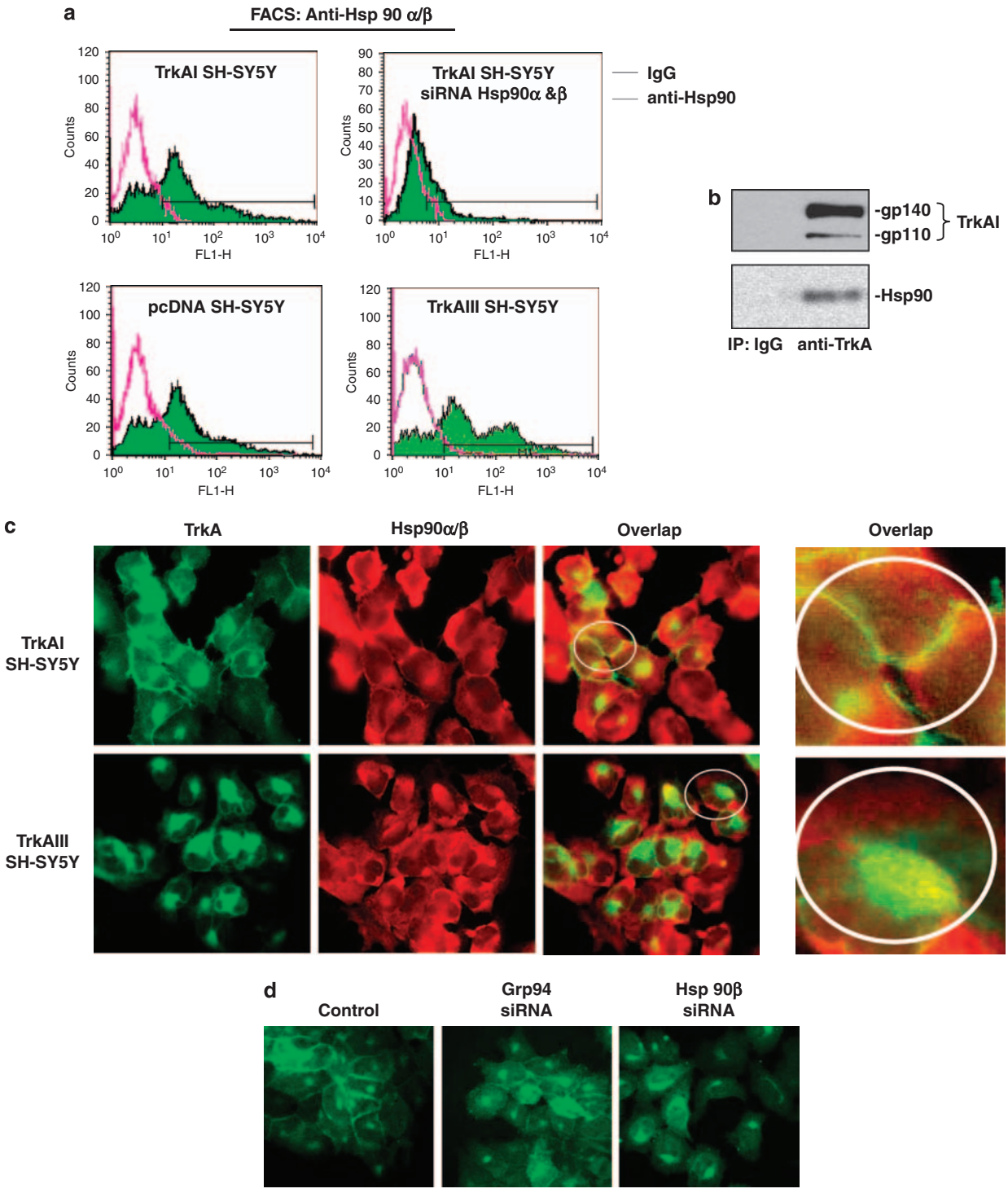


Figure 5 TrkAI binds Hsp90α/β at the cell surface. (**a**) FACS analysis demonstrating cell surface Hsp90α/β expression in non-permeabilized TrkAI SH-SY5Y transfectants (upper left panel); loss of cell surface Hsp90α/β expression in non-permeabilized TrkAI SH-SY5Y transfectants after siRNA knockdown of Hsp90α and β (upper right panel) and cell surface Hsp90α/β expression in non-permeabilized pcDNA (lower left panel) and TrkAIII SH-SY5Y transfectants (lower right panel). (**b**) IP–western blot demonstrating TrkAI co-IP of Hsp90α/β from TrkAI SH-SY5Y cell membranes. (**c**) Indirect IF demonstration of overlapping cell surface Hsp90α/β and TrkAI expression in TrkAI SH-SY5Y transfectants and overlapping intracellular Hsp90α/β and TrkAIII expression in TrkAIII SH-SY5Y transfectants, with circled regions magnified. (**d**) Indirect IF demonstrating loss of cell-surface TrkAI immunoreactivity in TrkAI SH-SY5Y transfectants treated for 6 days with 1 μM Hsp90β siRNA, but not Grp94 siRNA, compared with TrkAI SH-SY5Y transfectants treated with transfection medium alone (control).

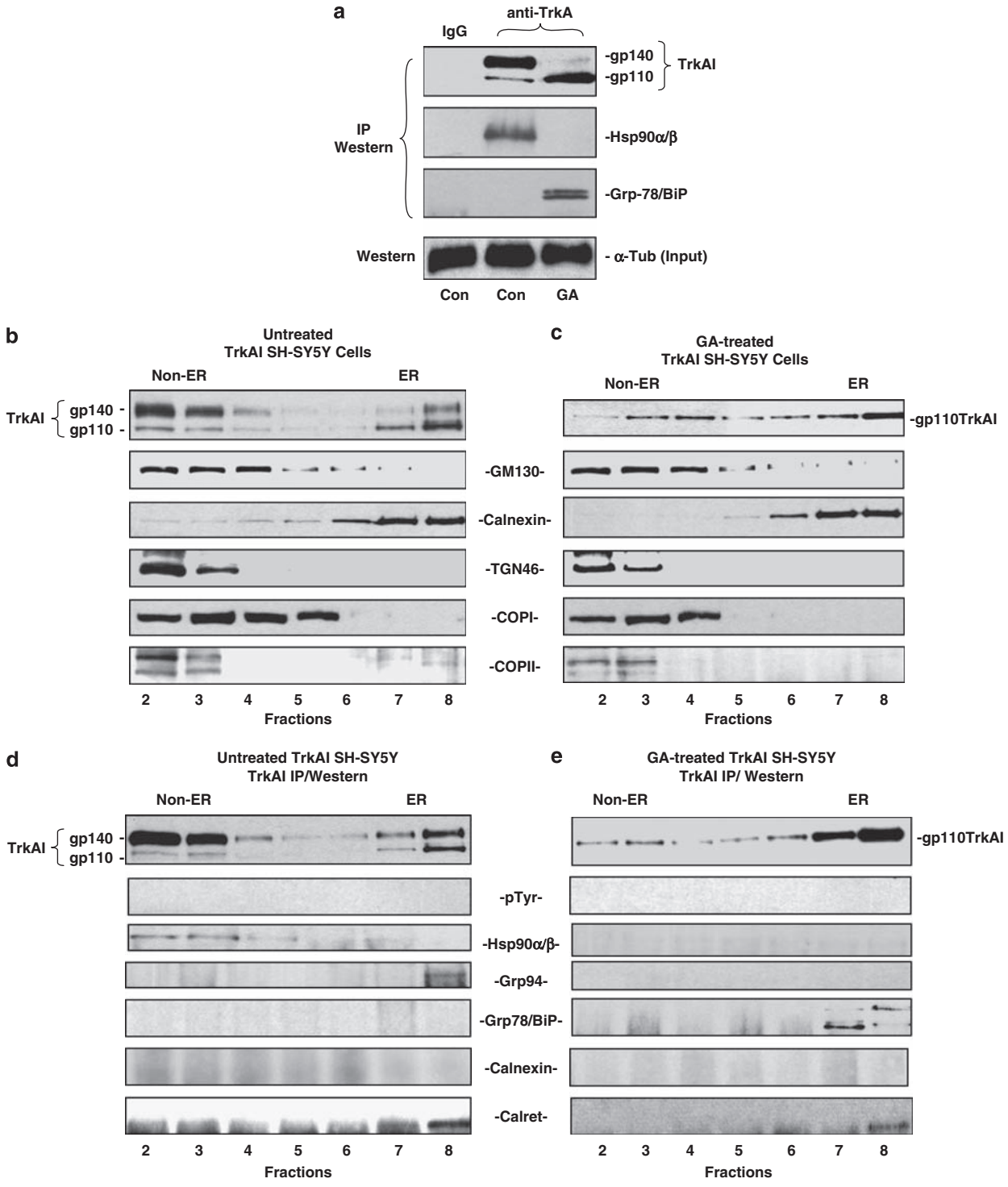


Figure 6 GA induces ER retention of gp110TrkAI. (**a**) IP–western blots demonstrating the effect of GA on gp110 and gp140TrkAI isoform expression and co-IP of Hsp90 and Grp78/BiP in whole cell extracts from untreated (Con) and 1 μM GA overnight-treated (GA) TrkAI SH-SY5Y transfectants. (**b**, **c**) Western blots demonstrating the distribution of gp110 and gp140TrkAI, GM130, TGN46, calnexin, COP I and COP II in Histodense ultracentrifugation-fractionated intracellular membranes purified from (**b**) untreated TrkAI SH-SY5Y transfectants and (**c**) TrkAI SH-SY5Y transfectants treated overnight with 1 μM GA. (**d**, **e**) IP–western blots demonstrating the relative differences in the distribution of non-phosphorylated gp110 and gp140TrkAI and co-IP of Hsp90, Grp94, Grp78/BiP, calnexin and calreticulin (calret) from Histodense ultracentrifugation-fractionated intracellular membranes purified from (**d**) untreated TrkAI SH-SY5Y transfectants and (**e**) TrkAI SH-SY5Y transfectants treated overnight with 1 μM GA.

associated with loss of Grp94 binding, induction of Grp78/BiP binding and loss of TrkAI from non-ER membranes (Figure 6c and e), but did not induce re-distribution of calnexin, GM130, TGN46, COP I or COP II (Figure 6c).

TrkAIII within internal membranes exhibited an approximately equal steady state distribution between calnexin-positive ER membranes and calnexin negative, GM130, TGN46, COP I and COP II-positive ER Golgi-intermediate compartment/GN/COP vesicle membranes, within which TrkAIII also exhibited tyrosine phosphorylation (Figure 7b and d). ER-associated TrkAIII bound Hsp90, Grp78/Bip and calnexin in addition to calreticulin and Grp94, and in non-ER membranes bound Hsp90 and calreticulin (Figure 7d). Overnight treatment with 1 μM GA increased ER retention of non-phosphorylated TrkAIII, loss of Hsp90 and Grp94 binding, and also increased Grp78/BiP binding and TrkAIII loss from non-ER membranes (Figure 7c and e).

SiRNA Hsp90 knockdown mimics GA

To further characterize Hsp90 interactions with TrkAI and TrkAIII, specific Accell SMART Pool siRNAs were used to knockdown Hsp90α, Hsp90β and Grp94 expression (four siRNAs per pool). Each gene-specific siRNA induced maximal-specific knockdown of target within 6 days (Figure 8a and b). In TrkAI transfectants, siRNA Hsp90α and Hsp90β but not Grp-94 knockdown induced gp140TrkAI loss and gp110TrkAI accumulation (Figure 8a). In TrkAIII SH-SY5Y transfectants, siRNA Hsp90β and Hsp90α but not Grp94 knockdown inhibited constitutive TrkAIII tyrosine phosphorylation and reduced TrkAIII levels (Figure 8b). Potential off-target siRNA effects were ruled out by the specificity of knockdown in the absence of co-lateral effects on the other Hsp90 chaperones and α-tubulin, and by comparison with siRNA Grp94 knockdown, which did not influence TrkAI or TrkAIII expression (Figure 8a and b).

TrkAIII tk-loop tyrosines and tk activity regulate intracellular distribution

TrkAIII *tk*-loop tyrosine and *tk*-activity involvement in determining TrkAIII intracellular distribution was supported by comparing intracellular distribution of non-mutated TrkAIII and non-phosphorylated *tk*-loop tyrosine-mutated $_{mu}$TrkAIII (Figure 9a). $_{mu}$TrkAIII exhibited greater ER retention than TrkAIII and bound similar levels of Grp94, calnexin and 82 kDa Grp78/BiP, but lower levels of Hsp90 (Figure 9b and c). Furthermore, overnight treatment of TrkAIII transfectants with 100 nM CEP-701 inhibited TrkAIII tyrosine phosphorylation and augmented ER retention of non-phosphorylated TrkAIII, associated with reduced co-IP of Hsp90 (Figure 9d and e), whereas overnight treatment with 1 mM sodium orthovanadate stimulated TrkAIII tyrosine phosphorylation and promoted TrkAIII re-distribution to non-ER membranes, associated with increased co-IP of Hsp90 (Figure 9d and e). TrkAI intracellular distribution was not associated with receptor tyrosine phosphorylation.

TrkAIII induces a partial ER-stress response

TrkAIII ER retention and constitutive Hsp90, Grp78/Bip and calnexin binding indicates difficulty in overcoming ER quality control and the possibility that TrkAIII may induce ER stress. ER stress was assessed by analysis of ATF6 transcription factor activation and alternative XBP-1 transcription factor splicing (Schroder, 2008). ATF6 transcriptional factor activity, assessed by reporter gene assay using the 5xATF6-GL3 construct (Wang *et al*., 2000), was significantly increased by 6.2-fold in TrkAIII SH-SY5Y transfectants compared with <1-fold in pcDNA and TrkAI transfectants, over luciferase activity from cfos-promoted enhancerless PoFLuc-GL3PGL3 ($P<0.001$ Student' *t*-test) (Figure 10a). Proteolytic ATF6 processing to its 50 kDa nuclear form was detected in TrkAIII, but not pcDNA or TrkAI SH-SY5Y transfectants (Figure 10d, arrow indicates processed ATF-6). TrkAIII transfectants also exhibited higher constitutive expression of the ER-stress chaperone Grp78/Bip, relative to α-tubulin and Hsp90 protein expression, compared with pcDNA and TrkAI transfectants (Figure 10b, d and e). Constitutive alternative XBP-1 splicing was not detected in either pcDNA, TrkAI or TrkAIII SH-SY5Y transfectants (Figure 10c, lanes 0 h treatment with GA).

GA (1 μM) induced transient alternative XBP-1 splicing in pcDNA, TrkAI and TrkAIII transfectants at 3 h, but no later (Figure 10c), reduced transcription activity in 5xATF6PGL3 reporter gene assays and inhibited ATF6 proteolytic processing from 3 h onwards in TrkAIII transfectants (Figure 10a and d), without influencing ATF6 processing in pcDNA or TrkAI transfectants (Figure 10d); increased Grp78/BiP expression in pcDNA and TrkAI transfectants to levels expressed by TrkAIII SH-SY5Y transfectants within 9 h (Figure 10d and e) and induced Bcl-2 expression exclusively in TrkAIII SH-SY5Y transfectants at 9 and 12 h (Figure 10d).

TrkAIII decreases SH-SY5Y GA-sensitivity

GA at concentrations of 0.1 μM (closed squares) and 1 μM (closed triangles) completely inhibited pcDNA and TrkAI, but not TrkAIII SH-SY5Y transfectant proliferation in 1, 2 and 3 days MTS assays (Figure 11a). Transient TrkAIII expression for 4 days in pcDNA and TrkAI SH-SY5Y stable transfectants significantly reduced GA-induced elimination associated with low-level proliferation in 2 and 3 days MTS assays ($P<0.001$ for both transient TrkAIII transfectants at 2 and 3 days versus sham transfected counterparts), but did not significantly alter proliferation in the absence of GA (Figure 11b).

GA significantly decreased 48 h survival of pcDNA and TrkAI SH-SY5Y transfectant at concentrations $\geqslant$0.1 μM, whereas TrkAIII SH-SY5Y transfectants exhibited reduced 48 h survival at GA concentrations of $\geqslant$1.0 μM ($P>0.01$, Student's *t*-test) (Figure 11c). GA

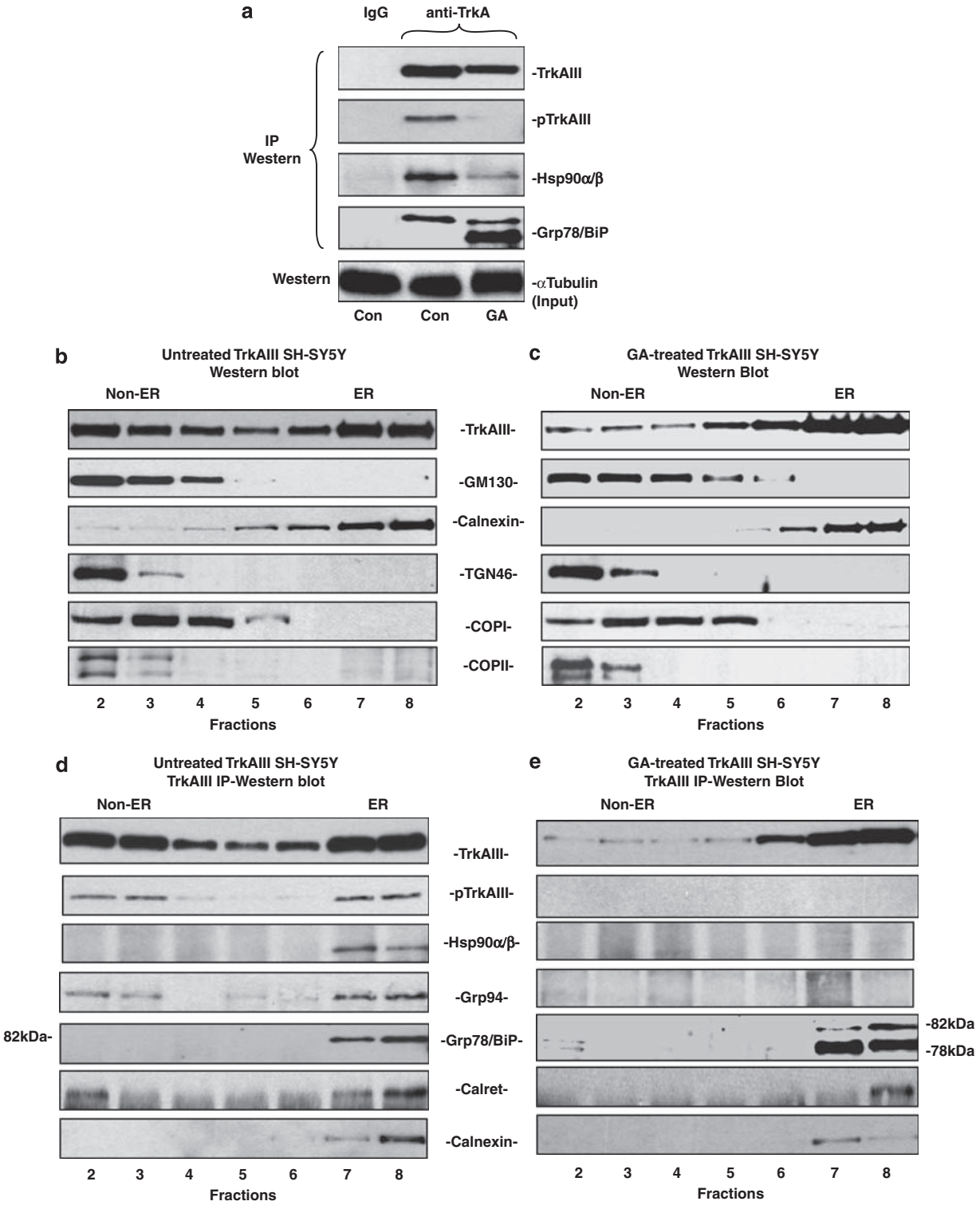


Figure 7 GA augments TrkAIII ER retention. (**a**) IP–western blots demonstrating relative changes in total and tyrosine phosphorylated levels of TrkAIII (pTrkAIII) and TrkAIII co-IP of Hsp90 and Grp78/BiP in whole cell extracts from untreated TrkAIII SH-SY5Y transfectants (Con) and TrkAIII SH-SY5Y transfectants treated overnight with 1 μM GA. (**b**, **c**) Western blots demonstrating the distribution of gp100TrkAIII, GM130, calnexin, TGN46, COP I and COP II in Histodense ultracentrifugation-fractionated intracellular membranes purified from (**b**) untreated TrkAIII SH-SY5Y transfectants and (**c**) TrkAIII SH-SY5Y transfectants treated overnight with 1 μM GA. (**d**, **e**) IP–western blots demonstrating relative changes in gp100TrkAIII levels, tyrosine phosphorylated TrkAIII (pTrkAIII) and TrkAIII co-IP of Grp94, Hsp90α/β, Grp78/BiP, calreticulin (calret) and calnexin in Histodense ultracentrifugation-fractionated intracellular membranes purified from (**d**) untreated TrkAIII SH-SY5Y transfectants and (**e**) TrkAIII SH-SY5Y transfectants treated overnight with 1 μM GA.

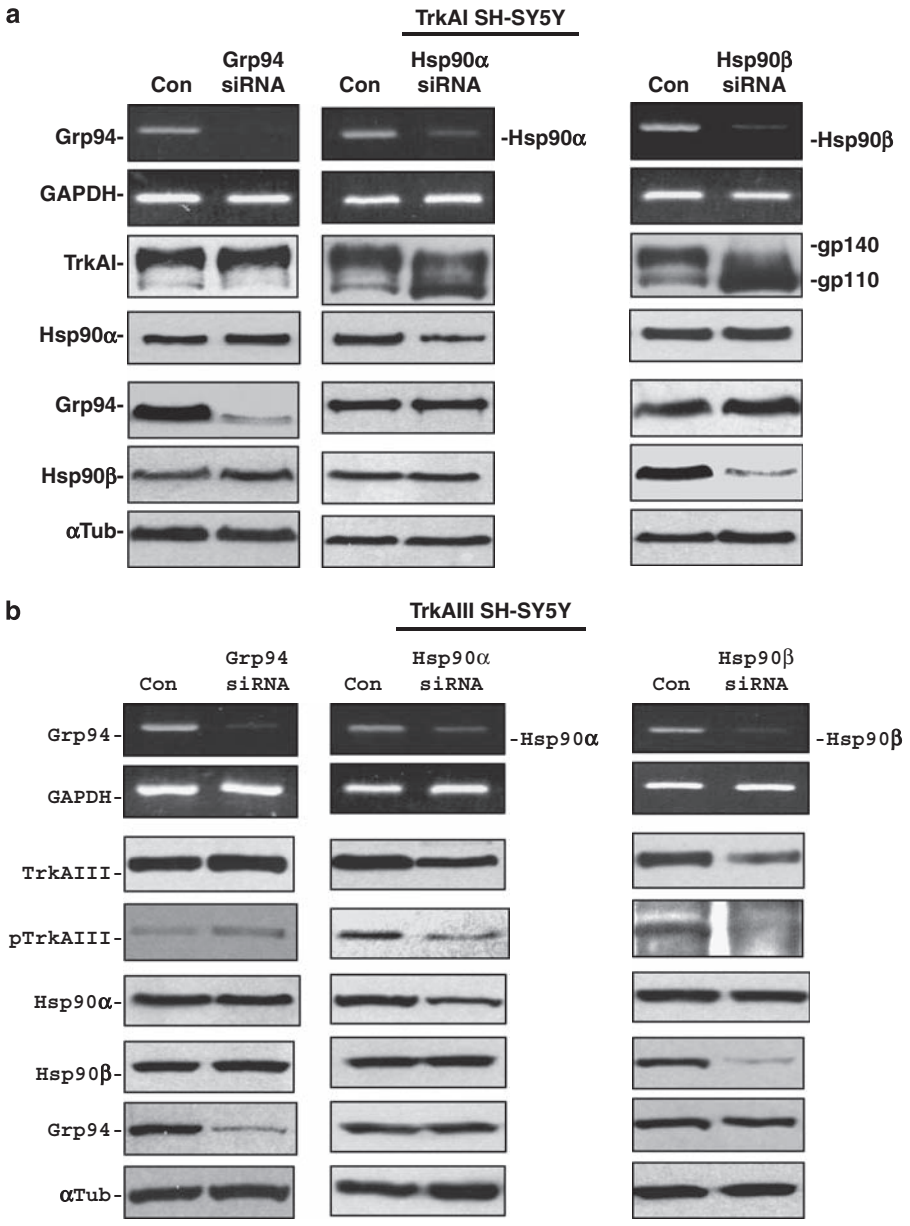


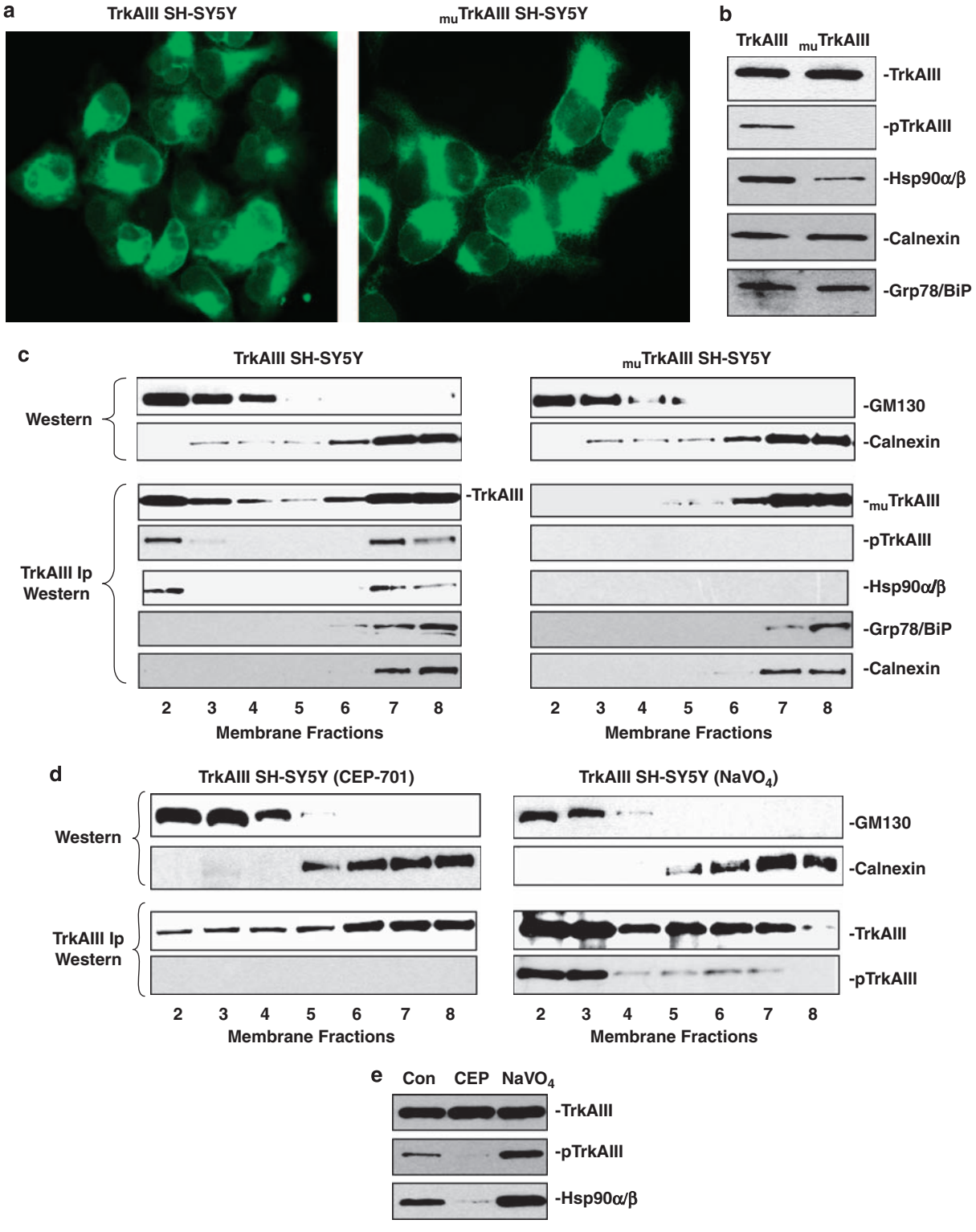
Figure 8 SiRNA knockdown of Hsp90 mimics the effect of GA on TrkAI and TrkAIII. RT–PCR demonstrating the effect of 3 days incubation of (**a**) TrkAI SH-SY5Y transfectants and (**b**) TrkAIII SH-SY5Y transfectants with transfection medium alone (Con) or 1 μM siRNAs specific for Grp94, Hsp90α and Hsp90β on mRNA expression of Grp94, Hsp90α, Hsp90β and GAPDH (upper 6 panels), plus western blots (lower panels) demonstrating the effect of 6 days incubation of (**a**) TrkAI SH-SY5Y transfectants and (**b**) TrkAIII SH-SY5Y transfectants with transfection medium (Con) or 1 μM siRNAs specific for Grp94, Hsp90α or Hsp90β on Hsp90α, Hsp90β, Grp94 and α-tubulin protein levels and expression of gp110 and gp140TrkAI in TrkAI transfectants (**a**) and total and tyrosine phosphorylated TrkAIII (pTrkAIII) in TrkAIII transfectants (**b**).

(0.1 μM) induced significant apoptosis in pcDNA and TrkAI (>50%, $P>0.001$, Student's *t*-test), but not TrkAIII transfectants (Figure 11d). Reduced GA sensitivity of TrkAIII SH-SY5Y transfectant was not associated with expression of the neuronal differentiation markers NF-M and Neuro-D (data not displayed) or morphological neuronal differentiation (this study; Tacconelli *et al*., 2004). Specific knockdown of TrkAIII expression by 7 days incubation of TrkAIII SH-SY5Y transfectants with 10 μM TrkAIII-PNA, which did not knockdown TrkAI expression in TrkAI transfectants (Figure 11e), increased the sensitivity of TrkAIII transfectants to GA-induced growth inhibition at GA concentrations $\geqslant 0.1$ μM ($P<0.001$, Student's *t*-test) (Figure 11f).

In equal ratio TrkAI–TrkAIII SH-SY5Y transfectant co-cultures (Figure 12a, left panel), 1 μM GA significantly reduced proliferation in 3 days MTS assays ($P<0.001$, Student's *t*-test) (Figure 12b), associated with the elimination of TrkAI expressing cells (Figure 12a, right panel), confirmed by complete loss of TrkAI mRNA and protein expression (Figure 12c and d).

Discussion

In this study, we report important observations concerning the interaction between Hsp90 chaperones and effects of GA on the NB tumour-suppressor TrkAI and its oncogenic alternative TrkAIII splice variant in human NB cells. For the first time, we characterize TrkAI and TrkAIII as novel Hsp90 clients, report that TrkAI is involved in GA-sensitive interactions with Hsp90α and Hsp90β required for immature receptor ER –export, maturation, cell surface translocation, stability and ligand-dependent activation and that TrkAIII exhibits GA-sensitive interactions with Hsp90α and Hsp90β required for maintaining spontaneous intracellular



activity and to a lesser extent stability. We show that TrkAIII reduces NB cell sensitivity to GA-induced growth inhibition and elimination, and we characterize a novel TrkAIII-specific PNA inhibitor that counteracts this effect. Our data implicate GA-sensitive interactions with Hsp90 as potentially critical for TrkAI tumour suppressor and TrkAIII oncogenic function in NB.

Hsp90 stabilization of cell surface TrkAI, suggested by GA-induced loss of cell surface TrkAI immunoreactivity and gp140TrkAI protein expression, was confirmed by siRNA Hsp90α and Hsp90β knockdown, which also induced gp140TrkAI loss and siRNA Hsp90β knockdown, which induced loss of cell surface TrkAI immunoreactivity. GA effect on gp140TrkAI was reversed by MG-132 (Marques *et al*., 2004), indicating lysosome/proteosome-mediated gp140TrkAI degradation. This presumably results from GA-induced internalization of inactive cell surface TrkAI, as described earlier for degradation of internalized ligand-activated TrkA (Geetha and Wooten, 2008). The differences in time taken for GA and siRNAs to influence TrkA reflect relatively immediate GA inhibition of Hsp90 catalytic activity, but relatively slow siRNA knockdown of Hsp90 expression in the absence of catalytic inhibition. The clinically relevant GA-analogues 17-AAG and 17-DMAG (Miyata, 2005) also induced lysosomal/proteosome-mediated gp140TrkAI degradation in stable TrkAI transfectants, and GA also induced lysosome/proteosome-mediated degradation of endogenous gp140TrkA expressed by non-transfected SH-SY5Y and IMR32 NB cells, confirming an effect not restricted to transfected TrkA.

TrkAI interaction with Hsp90 at the cell surface was supported by overlapping cell surface TrkAI and Hsp90α/β expression, TrkAI co-IP of Hsp90 from purified membranes and reduced cell surface TrkAI expression after siRNA Hsp90 knockdown, in TrkAI transfectants. However, because of lack of adequate mono-specific anti-Hsp90α and Hsp90β antibodies for FACS and IF, we are unable to state whether Hsp90α, in addition to Hsp90β, is also expressed at the SH-SY5Y cell surface. Cell surface Hsp90 (α/β) expression was also detected in pcDNA and TrkAIII transfectants, adding SH-SY5Y cells to other tumour and normal cell types that express cell surface Hsp90 (α/β) (Becker *et al*., 2004; Sidera *et al*., 2004, 2008) and TrkAI to other cell surface receptors that depend on interaction with Hsp90 for stability (Vega and De Maio, 2003; Peng *et al*., 2005).

Mature cell surface gp140TrkA is synthesized initially within the ER as the immature 110-kDa form gp110TrkA, which contains a 30-kDa *N*-linked sugar moiety (Martin-Zanca *et al*., 1989; Watson *et al*., 1999) that is subsequently matured to gp140TrkA by carbohydrate moiety modification before cell surface translocation (this study; Jullien *et al*., 2002). GA and GA analogues induced accumulation of intracellular gp110TrkAI. This was not impaired by MG-132 and was, therefore, independent of gp140TrkAI degradation, indicating that gp110TrkAI, unlike pg140TrkAI, is not stabilized by a GA-sensitive interaction with Hsp90. GA also increased gp110TrkAI ER retention, implicating Hsp90 in gp110TrkAI ER export, as a pre-requisite for maturation and cell surface translocation (this study; Jullien *et al*., 2002). In untreated TrkAI transfectants, ER-associated gp110TrkAI bound Grp94, but not Hsp90, suggesting that Grp94 rather than Hsp90 regulates ER export. However, this was not supported by siRNA Grp-94 knockdown, which in contrast to Hsp90α and Hsp90β knockdown did not influence TrkAI expression. Hsp90 (α/β) may, therefore, regulate TrkAI ER export with binding missed either by the relatively low level of ER-associated TrkAI and/or preferential interaction with gp140TrkAI. In any case, GA disruption of TrkAI suggests that Hsp90 interacts with this receptor at an early stage required for receptor maturation and sorting, which is of relevance to the pharmacological use of Hsp90 inhibitors.

Short-term pre-incubation with GA also attenuated NGF-induced TrkAI tyrosine phosphorylation without reducing gp140TrkAI. This was not reversed by MG132, indicating independence from lysosome/proteosome-mediated degradation, suggesting that Hsp90 facilitates ligand-mediated gp140TrkAI activation. This adds to reports that Hsp90 interacts with the *tk* domains of several kinases to regulate activity (Marcu *et al*., 2002) and original characterization of GA as a *tk* inhibitor (Marczin *et al*., 1993). The observation that *tk*-loop tyrosine mutated $_{mu}$TrkAIII exhibited reduced Hsp90 binding suggests that Hsp90 interacts with TrkA *tk*-domain tyrosines required for TrkA *tk* activity, as reported earlier for Hsp90 interaction with the ErbB2 *tk* domain (Xu *et al*., 2001). We do not rule out, however,

Figure 9 TrkAIII tk activity regulates intracellular trafficking. (**a**) Indirect IF comparison of intracellular TrkAIII and $_{mu}$TrkAIII expression in respective stable transfected SH-SY5Y cells. (**b**) IP–western blots comparing total (TrkAIII), tyrosine phosphorylated TrkAIII (pTrkAIII) and TrkAIII co-IP of Hsp90, calnexin and Grp78/BiP in whole cell extracts from TrkAIII and $_{mu}$TrkAIII SH-SY5Y transfectants. (**c**) Western blots comparing the distribution of calnexin and GM130 in purified Histodense ultracentrifugation-fractionated internal membranes purified from TrkAIII (upper left panels) and $_{mu}$TrkAIII stable SH-SY5Y transfectants (upper right panels), plus IP–western blots (TrkAIII IP/western blot) demonstrating differences in the relative distribution of total (TrkAIII), tyrosine phosphorylated TrkAIII (pTrkAIII) and differences in TrkAIII and $_{mu}$TrkAIII co-IP of Hsp90, Grp78/BiP and Calnexin in Histodense ultracentrifugation-fractionated internal membranes from respective stable SH-SY5Y transfectants. (**d**) Comparative western and IP–western blots demonstrating the distribution of GM130 and calnexin in Histodense ultracentrifugation-fractionated internal membranes and differences in the distribution of non-phosphorylated (TrkAIII) and tyrosine phosphorylated TrkAIII (pTrkAIII) in Histodense ultracentrifugation-fractionated internal membranes purified from TrkAIII SH-SY5Y transfectants treated overnight with 100 nM CEP-701 (left panels) or with 1 mM sodium orthovanadate ($NaVO_4$) (right panels). (**e**) IP–western blots demonstrating total (TrkAIII) and tyrosine phosphorylated TrkAIII (pTrkAIII) and the difference in TrkAIII co-IP of Hsp90 from whole cell extracts from untreated TrkAIII SH-SY5Y transfectants (Con), TrkAIII SH-SY5Y transfectants treated overnight with 100 nM CEP-701 or 1 mM sodium orthovanadate-treated ($NaVO_4$).

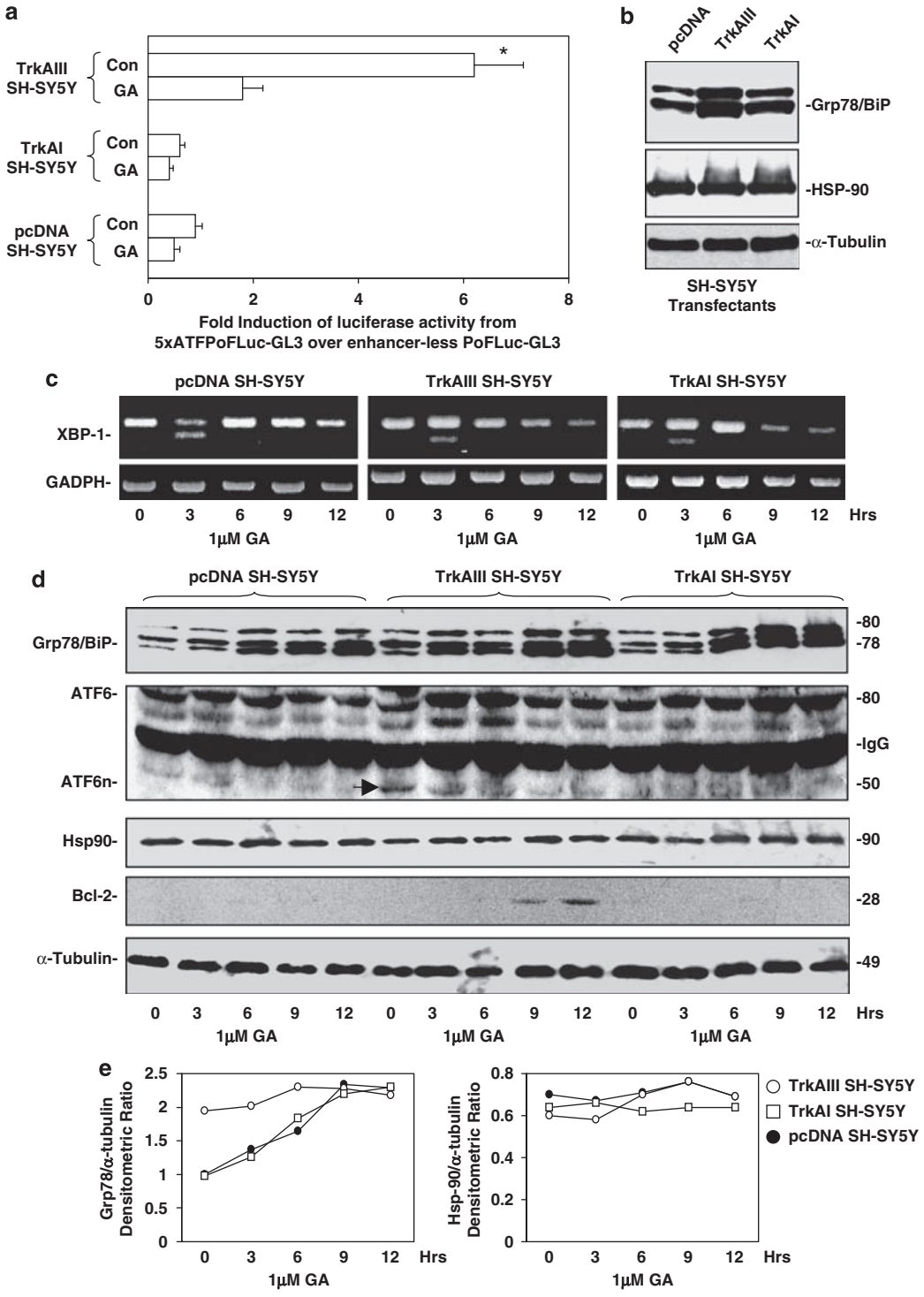


Figure 10 TrkAIII induces partial activation of the ER-stress response. (**a**) Reporter gene assays demonstrating significantly elevated (*) levels of constitutive transcriptional activity from the 5xATF6PoFLuc-GL3 reporter gene in TrkAIII SH-SY5Y transfectants compared with pcDNA and TrkAI transfectants (Con), normalized for each cell line to background luciferase activity from the cfos-promoted enhancer-less PoFLuc-GL3, plus the effect of 12 h incubation of SH-SY5Y transfectants with 1 μM GA on ATF6 transcriptional activity (GA). (**b**) IP–western blots demonstrating increased constitutive expression of Grp78/BiP to Hsp90 and α-tubulin proteins in TrkAIII compared with pcDNA and TrkAI SH-SY5Y transfectants. (**c**) RT–PCR demonstration of lack of constitutive alternative XBP-1 splicing in pcDNA, TrkAI and TrkAIII SH-SY5Y transfectants (0 lane for each transfectant) with transient induction alternative XBP-1 splicing in pcDNA, TrkAI and TrkAIII SH-SY5Y transfectants after treatment with 1 μM GA for 3 h, but not at later times, relative to GAPDH mRNA expression. (**d**) Western and IP–western blots demonstrating the effect of treating pcDNA, TrkAIII and TrkAI SH-SY5Y transfectants over a 12 h time course with 1 μM GA on Grp78/BiP, ATF6 (IP–western), Hsp90, Bcl-2 and α-tubulin protein expressed. (**e**) Graphic representation of the above western blots demonstrating changes in Grp78/BiP to α-tubulin densitometric ratios (left) and Hsp90 to α-tubulin densitometric ratios (right) after treatment of pcDNA, TrkAI and TrkAIII SH-SY5Y transfectants with 1 μM GA.

that GA may interfere with NGF-TrkA ligation or promote rapid internalization of non-degraded receptor to account for this effect. Nevertheless, these data indicate that Hsp90 is required for TrkAI cell surface stability and ligand-dependent activation, identifying Hsp90 as a critical component of TrkAI tumour-suppressor activity in NB (Nakagawara *et al*., 1992; Nakagawara, 2001).

In contrast to TrkAI, TrkAIII was not expressed at the cell surface or as two distinct forms, indicating that TrkAIII does not undergo carbohydrate moiety modification and implicating extracellular-domain *N*-linked carbohydrates, omitted from TrkAIII, in gp140TrkAI maturation and cell surface translocation (this study; Watson *et al*., 1999; Jullien *et al*., 2002). The complete absence of TrkAIII biotinylation in pulse-chase experiments confirms that intracellular TrkAIII accumulation does not result from increased cell surface receptor internalization or instability. Furthermore, cell surface Hsp90α/β expression by all SH-SY5Y transfectants indicates that intracellular TrkAIII accumulation does not depend on lack of cell surface Hsp90. We are investigating the possibility that cell surface Hsp90 may exhibit a unique interaction with TrkAI extracellular-domain sequence deleted from TrkAIII.

A requirement for GA-sensitive interaction with Hsp90 for spontaneous intracellular TrkAIII activity was suggested by GA and GA-analogue (17-AAG and 17-DMAG) inhibition of TrkAIII tyrosine phosphorylation and reduction of TrkAIII levels and confirmed by siRNA Hsp90β and Hsp90α, but not Grp-94 knockdown, which also inhibited TrkAIII tyrosine phosphorylation. However, in contrast to gp140TrkAI, GA induced only low-level TrkAIII loss, indicating that TrkAIII similar to gp110TrkAI is less dependent on Hsp90 for stability, which bears similarity to GA inhibition of Src *tk* activity in the absence of degradation (Bijlmakers and Marsh, 2000; Crevecoeur *et al*., 2008). Limited GA-induced TrkAIII loss was reversed by MG132 and was, therefore, lysosome/proteosome mediated and was not restricted to phosphorylated receptor, but also detected with non-phosphorylated CEP-701-inhibited TrkAIII and $_{mu}$TrkAIII. In contrast to transfected TrkAIII, however, GA did not reduce endogenous TrkAIII levels in non-transfected SH-SY5Y or IMR32 cells, indicating that Hsp90 stabilizes non-phosphorylated transfected, but not endogenous TrkAIII, which may relate to relative expression levels. MG-132 alone did not influence TrkAIII expression or tyrosine phosphorylation, indicating that spontaneously active intracellular TrkAIII is not subject to the same proteosome/lysosome-mediated degradation pathway as internalized ligand-activated cell surface TrkAI (Geetha and Wooten, 2008).

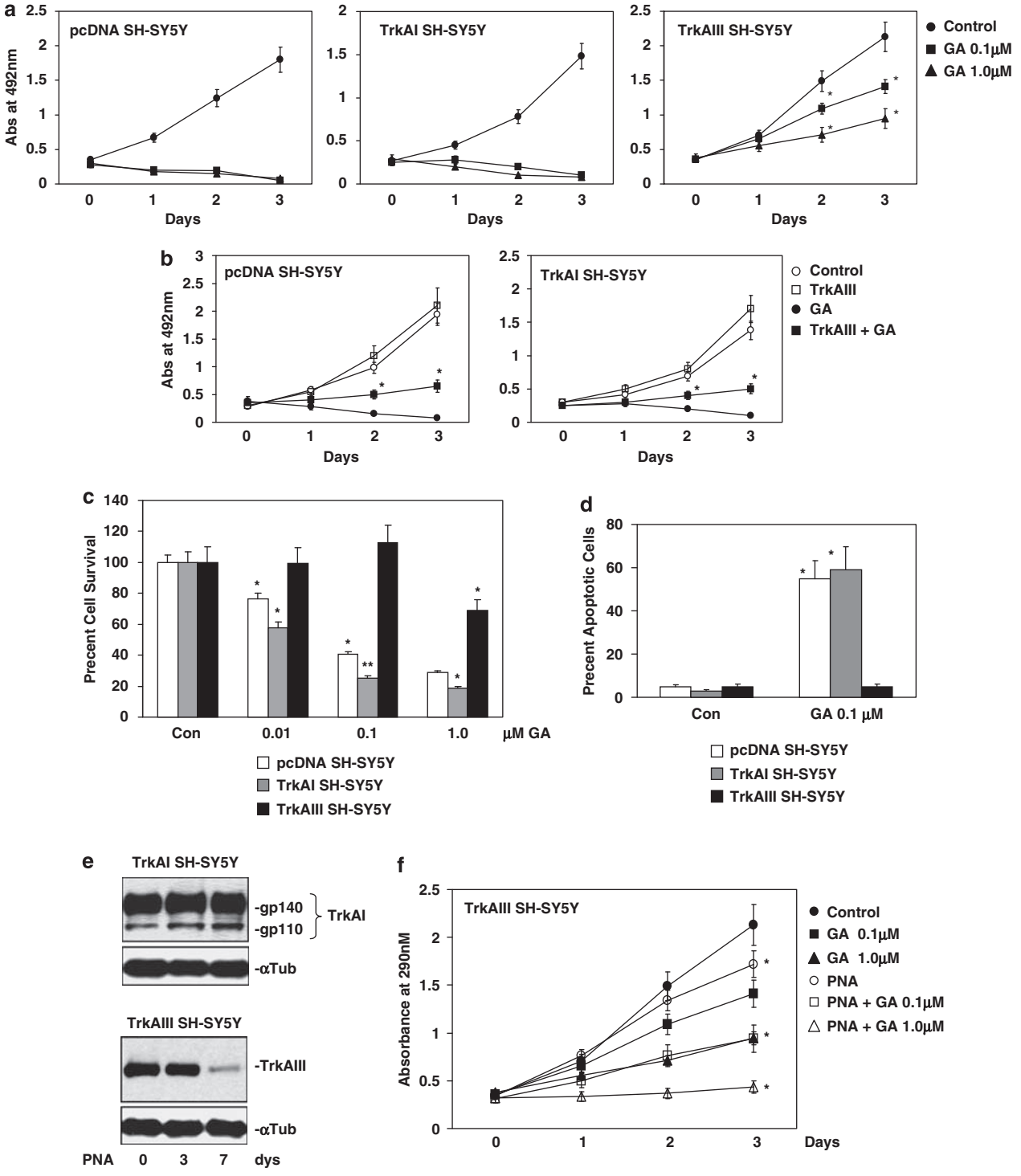
Potential involvement of phosphorylated *tk*-loop tyrosines in Hsp90 binding, suggested by relatively high level Hsp90 binding by tyrosine phosphorylated TrkAIII compared with non-phosphorylated CEP-701-inhibited TrkAIII and $_{mu}$TrkAIII, is supported by reports that Hsp90 binds the ErbB2 receptor *tk* domain (Xu *et al*., 2001). The possibility that this interaction not only stabilizes TrkAIII *tk* –activity but also determines TrkAIII intracellular distribution is supported by observations that (a) TrkAIII exhibited tyrosine phosphorylation and Hsp90 binding in ER and non-ER membranes; (b) GA and CEP-701 inhibited TrkAIII tyrosine phosphorylation and promoted ER retention; (c) non-phosphorylated $_{mu}$TrkAIII exhibited predominant ER retention and (d) sodium orthovanadate stimulated TrkAIII tyrosine phosphorylation and translocation to non-ER membranes. This differs from Hsp90 involvement in TrkAI ER export, maturation and cell surface translocation, which occurs in the absence of receptor tyrosine phosphorylation. This implicates extracellular IG-C1 and mature extracellular-domain carbohydrates in regulating TrkAI ER export and sorting, and preventing ligand-independent activation, consistent with earlier reports (Watson *et al*., 1999; Arevalo *et al*., 2000), with their omission from TrkAIII altering intracellular distribution by promoting intracellular accumulation, spontaneous activation and ER quality control recognition. TrkAIII, $_{mu}$TrkAIII and CEP-701-inhibited TrkAIII also exhibited low-level GA-insensitive Hsp90 binding, indicating passive interaction with Hsp90 similar to that reported for citrate synthase and MyoD (Shankovich *et al*., 1992; Jakob *et al*., 1995; Scheibel *et al*., 1998).

ER quality control recognition of TrkAIII, confirmed by high level ER retention and constitutive Grp78/Bip, Hsp90 and calnexin binding, suggests that TrkAIII may induce ER stress. This was confirmed by constitutive ATF-6 transcription factor activity, ATF6 processing and elevated ER-stress chaperone Grp78/Bip expression, which characterize the ER-stress response (Schroder, 2008), in TrkAIII, but not pcDNA or TrkAI transfectants. TrkAIII transfectants did not, however, exhibit alternative XBP-1 splicing, indicating that ER perturbation is below the threshold required for full ER-stress response activation (Schroder, 2008). Nevertheless, this provides a novel mechanism, in addition to constitutive PI3K/Akt/NF-*k*B activation (Tacconelli *et al*., 2004), through which TrkAIII may increase NB cell stress resistance.

TrkAIII SH-SY5Y transfectants exhibit increased stress resistance (Tacconelli *et al*., 2004) and also reduced sensitivity to GA-induced inhibition of proliferation and survival, compared with pcDNA and TrkAIII transfectants. This was confirmed as TrkAIII mediated by PNA inhibition of TrkAIII expression, which significantly restored TrkAIII transfectant sensitivity to GA-induced growth inhibition. Potential clonal differences in GA sensitivity were also ruled out by transient TrkAIII expression, which significantly increased survival of pcDNA and TrkAI stable transfectant in the presence of GA. The reduced GA sensitivity of TrkAIII transfectants was not, however, associated with neuronal differentiation, earlier reported to reduce GA sensitivity of SH-SY5Y cells (Shen *et al*., 2007).

Differential TrkAI and TrkAIII transfectant sensitivity to GA resulted in GA selection of TrkAIII transfectants from co-culture with TrkAI transfectants, confirming TrkAIII negative impact on GA-induced NB

cell eradication and adding TrkAIII to other oncogenes, including ERGFR, which decrease tumour cell GA sensitivity (Theodoraki *et al*., 2007; Puyo *et al*., 2008). This, combined with TrkAIII re-phosphorylation after GA removal, provides a potential mechanism for post GA therapeutic relapse. Potential mechanisms through which TrkAIII reduces GA sensitivity, despite GA inhibition of TrkAIII activity include (a) constitutive activation of the anti-apoptotic PI3K/Akt/NF-κB pathway (Tacconelli *et al*., 2004), which may offer initial protection before GA inhibition of NF-κB activation (Crevecoeur *et al*., 2008); (b) partial ER-stress response activation and increased Grp78/BiP expression, both of which protect against stress-induced apoptosis (Rao *et al*., 2002; Schroder, 2008) and (c) GA induction of Bcl-2 expression exclusively in TrkAIII transfectants,



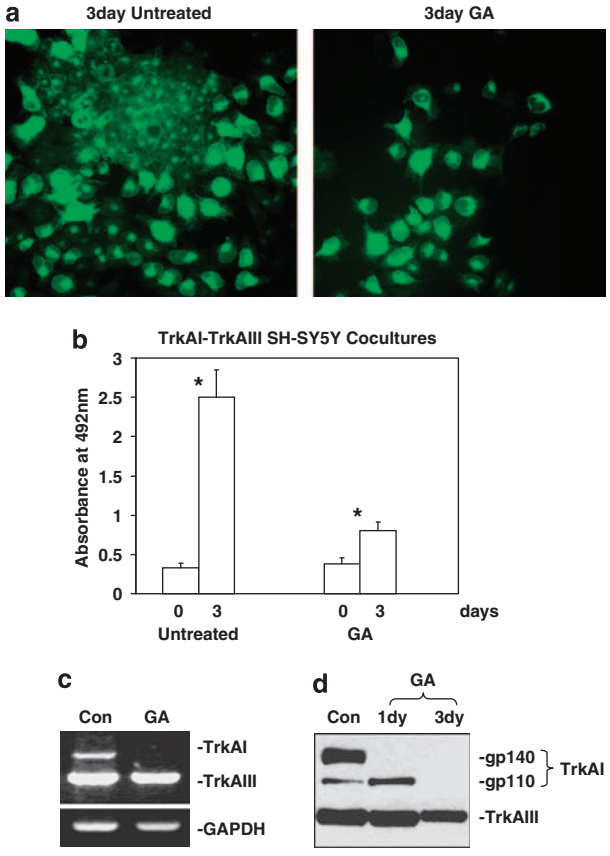


Figure 12 GA-induces selection of TrkAIII transfectants from co-culture with TrkAI transfectants. (**a**) Indirect IF demonstrating differences in the pattern of TrkA I and TrkAIII immunoreactivity in 3 days untreated co-cultures of TrkAI and TrkAIII SH-SY5Y transfectants (3 days untreated) or co-cultures grown in the presence of 1 μM GA (3 days GA) (* refers to a signiicant difference). (**b**) Histogram demonstrating 3 days MTS assays of TrkAI and TrkAIII SH-SY5Y transfectant co-cultures grown for 0 and 3 days in the absence (untreated) or presence of 1 μM GA (GA). (**c**) RT–PCR (left panels) demonstrating relative TrkAI and TrkAIII mRNA expression in TrkAI and TrkAIII SH-SY5Y co-cultures grown for 3 days in the absence of GA (Con) or presence of 1 μM GA (GA) and (**d**) IP/western blots (right panels) demonstrating relative levels of gp140TrkAI, pg110TrkAI and TrkAIII in TrkAI and TrkAIII SH-SY5Y transfectant co-cultures grown in the absence of GA (Con) or in the presence of 1 μM GA for 1 and 3 days.

which may promote long-term survival in the presence of GA, as Bcl-2 inhibits both GA and GA-analogue-induced apoptosis (Nimmanapalli *et al*., 2003). We are further investigating the possibility that TrkAIII may modify the ER-stress response to promote survival.

In conclusion, we classify TrkAI and TrkAIII as novel Hsp90α and Hsp90β clients involved in GA-sensitive interactions critical for maintaining TrkAI tumour suppressor and TrkAIII oncogenic function in NB cells. We propose that TrkAIII reduces GA sensitivity through partial activation and modification of the ER-stress response and that TrkAIII negative impact on GA-induced eradication and re-activation on GA removal provides a potential mechanism for post GA-therapeutic relapse that can be counteracted by TrkAIII-specific PNA. Finally, we caution that GA destabilization of cell surface TrkAI may have unforeseen consequences for normal TrkA-dependent cellular functions.

Materials and methods

Cell lines, reagents and antibodies

Cell lines, reagents and antibodies are detailed in Supplementary Materials.

TrkAIII adenoviral and the TrkAIII Y670/674/675F mutant expression vectors

TrkAIII adenoviral expression vector was constructed by sub-cloning TrkAIII cDNA complete with polyadenylation signal (Tacconelli *et al*., 2004), through the Gateway entry vector into the pAd/CMV/v5-DEST adenoviral expression system, as directed by the manufacturer (Invitrogen, Carlsbad, CA, USA). The pcDNA3.1 TrkAIII Y670/674/675F mutated expression vector was constructed by PCR from TrkAIII cDNA and sub-cloned into pcDNA3.1 expression vector, as detailed in Supplementary Materials.

Metabolic labelling of TrkAI and TrkAIII

Pulse-chase metabolic TrkAI and TrkAIII labelling was performed as described earlier (Jullien *et al*., 2002) and detailed in Supplementary Materials.

Figure 11 TrkAIII reduces SH-SY5Y sensitivity to GA-induced growth inhibition. (**a**) Graphic representation of comparative 3 days MTS proliferation assays of control (pcDNA), TrkAI and TrkAIII SH-SY5Y transfectants grown in the absence of GA (control) or presence of 0.1 μM GA or 1.0 μM GA in duplicated assays performed using six cultures per point. (**b**) Graphic representation of 3 days MTS proliferation assays of pcDNA transfectants (left graph) and TrkAI transfectants (right graph) either sham transiently transfected and grown in the absence of GA (control), sham transiently transfected and grown in the presence 1 μM GA (GA), transiently transfected with TrkAIII expression vector and grown in the absence of GA (TrkAIII) or transiently transfected with TrkAIII expression vector and grown in the presence of 1 μM GA (TrkAIII + GA) in duplicated assays performed using six cultures per point. (**c**) Histogram of MTS survival assays demonstrating the percentage 48 h survival of pcDNA, TrkAI and TrkAIII SH-SY5Y transfectants treated with GA treatment at the concentrations indicated in duplicated assays performed using six cultures per point. (**d**) Histogram demonstrating comparative percentage levels of apoptosis in pcDNA, TrkAI and TrkAIII SH-SY5Y cultures grown for 48 h in the absence (Con) or presence of 0.1 μM GA in triplicate cultures per cell line. (**e**) IP–western blots demonstrating the effect of treating TrkAI and TrkAIII SH-SY5Y transfectants with 10 μM TrkAIII-PNA for 0, 3 and 7 days on TrkAI, TrkAIII and α-tubulin (α-tub) expression. (**f**) Graphic representation of 3 days MTS proliferation assays comparing proliferation of untreated TrkAIII SH-SY5Y transfectants (control), TrkAIII SH-SY5Y transfectants in the presence of 0.1 μM GA (GA 0.1 μM) and 1.0 μM GA (GA 1.0 μM) compared with TrkAIII SH-SY5Y transfectants pre-treated for 7 days with 10 μM TrkAIII-PNA, before MTS assay in the absence of GA (PNA), in the presence of 0.1 μM GA plus 10 μM PNA (PNA + GA 0.1 μM) or 1.0 μM GA plus 10 μM PNA (PNA + GA 1.0 μM). Duplicate assays were performed using six independent cultures per point. Asterisks refer to significant differences.

RT–PCR conditions and primers
Reverse transcription was performed using mRNA (1 μg) and a Moloney Murine Leukaemia virus reverse transcriptase kit in a final volume of 20 μl for 45 min at 42 °C, according to the manufacturer (Life Technologies, Paisley, UK). RT reaction aliquots (1 μl) were subjected to 35 cycles of PCR (1 min at 94 °C, 1 min at optimized annealing temperature and 2 min at 72 °C) using specific primers for TrkA I/II, TrkAIII, Hsp90α, Hsp90β, Grp94, NF-M, Neuro-D, XBP-1 and GAPDH, as detailed in Supplementary Materials.

IP and western blots
IP and western blotting was performed using standard techniques, as described in Supplementary Materials.

Histodense ultracentrifugation fractionation of internal cell membranes
Histodense ultracentrifugation fractionation of intracellular membranes was performed as described earlier (Yoshimura *et al*., 2001) and detailed in Supplementary Materials.

siRNA Hsp90 knockdown
Accell SMART pool siRNAs were used to knockdown Hsp90α, Hsp90β and Grp94 expression in stable TrkAI and TrkAIII SH-SY5Y transfectants, as outlined by the manufacturer (Thermo Scientific/Dharmacon, Lafayette, CO, USA) and described in Supplementary Materials.

MTS proliferation and survival assays
MTS proliferation and survival assays were performed, as outlined by the manufacturer (Promega, Milan, Itlay) and detailed in Supplementary Materials.

Apoptosis
Apoptosis was assayed by fluorescence microscopic identification of condensed and/or fragmented Heochst 33258-stained nuclei as a percentage of total nuclei. Briefly, SH-SY5Y transfectants (50 000 cells/ml) were plated onto Costar culture slides and incubated for 48 h in either complete medium or medium containing 0.1 μM GA. After incubation, cells were fixed overnight in 70% ethanol at 4 °C and stained with Heochst 33258. Apoptotic cells exhibited nuclear condensation and/or fragmentation associated with membrane blebbing.

FACS analysis
For non-permeabilized cells analysis, SH-SY5Y transfectants detached in PBS/EDTA were resuspended in PBS, dispersed into 12×75 mm tubes (0.5×10^6 cells per tube), centrifuged and incubated with 0.5 μg of anti Hsp90α/β antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or 0.5 μg of normal mouse IgG, in 100 μl PBS at 4 °C for 1 h. After washing with PBS, cells were incubated with FITC-conjugated goat anti mouse IgG (Sigma, St Louis, MO, USA, diluted 1:128 in PBS) at 4 °C for 45 min, washed in PBS and resuspended in 500 μl PBS for analysis in an FACSCAN analyser (Becton Dickinson, San Jose, CA, USA).

TrkAIII PNA synthesis
Fmoc (Bhoc) synthesis and HPLC purification of the PNA KKAA-TrkAIII $(KKAA)_4$-GGCCGGGACACA are detailed in Supplementary Materials.

Luciferase reporter assay
Reporter gene assays were performed in stable SH-SY5Y transfectants transfected with either the 5xATF-PoFluc-GL3 reporter gene construct (Wang *et al*., 2000) or minimal cfos-promoter enhancer-less PoFLuc-GL3, as detailed in Supplementary Materials.

Statistical analysis
The results were compared using Student's *t*-test and considered statistically significant at *P*-values of <0.05.

Conflict of interest

The authors declare no conflict of interests.

Acknowledgements

We thank Dr Pyrwes (University of Michigan) for providing the 5xATF6GL3 reporter gene construct, Drs B Cinque, N Di Ianni and M Zani and post-graduate students Paolo Ciufici, Stefania Merolle and Marzia Ragone for technical assistance. This work was supported by AIRC, Progetto Speciale Ministero Della Sanità, MURST-Cofin, MIUR-CNR-Oncology project and Center of Excellence BEMM. We dedicate this work to all the students of L'Aquila University who lost their lives in the earthquake of L'Aquila, 6 April 2009.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)