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Thyroid hormone induces cardiac myocyte hypertrophy in a $TR\alpha_1$-specific manner that requires TAK1 and p38 MAPK.

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Summary

Alterations in thyroid hormone receptor (TR)1 isoform expression have been reported in models of both physiologic and pathologic cardiac hypertrophy as well as in patients with heart failure. In this report, we demonstrate that thyroid hormone (TH) induces hypertrophy as a direct result of binding to the $TR\alpha_1$ isoform and moreover, that over-expression of $TR\alpha_1$ alone is also associated with a hypertrophic phenotype, even in the absence of ligand. The mechanism of TH and $TR\alpha_1$-specific hypertrophy is novel for a nuclear hormone receptor and involves the transforming growth factor beta activated kinase (TAK1) and p38. Mitigating $TR\alpha_1$ effects, both $TR\alpha_2$ and $TR\beta_1$ attenuate $TR\alpha_1$-induced myocardial growth and gene expression by diminishing TAK1 and p38 activities, respectively. These findings refine our previous observations on TR expression in the hypertrophied and failing heart and suggest that manipulation of thyroid hormone signaling in an isoform-specific manner may be a relevant therapeutic target for altering the pathologic myocardial program.

Keywords

nuclear hormone receptor; thyroid hormone receptor; mitogen activated protein kinase; p38MAK; TGFbeta activated kinase; cardiac hypertrophy

Introduction

It is well accepted that alterations in thyroid function occur in patients with heart failure (1–4). Although previously felt to represent the “euthyroid-sick” syndrome rather than frank hypothyroidism, recent data suggests that a primary change in the myocardial response to thyroid hormone might underlie some of the alterations in myocardial form and function seen in the failing heart. In fact, the use of the thyroid hormone (TH) supplementation as a means of increasing cardiac function for patients with heart failure has met with limited success (5–7). This tactic is considered by many to be sub-optimal, however, since thyroid supplementation may be associated with potential adverse effects on heart rate and myocardial oxygen consumption. With increased use of β-blockade in heart failure patients, these side effects may

[1]Abbreviations: TR, thyroid hormone receptor; TH, thyroid hormone; TAK, TGFbeta activated kinase; MHC, myosin heavy chain; SERCA, sarcoplasmic reticulum Ca^{2+}-ATPase; MAPK, mitogen activated protein kinase; Ad, adenoviral; T_3, triiodo-L-thyronine; MKK, MAPK kinase; CA, constitutively activated; DN, dominant negative; WT, wild type; JNK, c-Jun N-terminal kinase; βGal, β-galactosidase; ERK, extracellular-signal-regulated kinase; GST, glutathione S transferase; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; skACT, skeletal α-actin; TRE, thyroid responsive element.

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well be controlled and interest in TH therapy for these patients has been renewed. Further, several TH analogues with limited effects on heart rate have also been developed and, in preliminary clinical trials, have been associated with improved myocardial function (8).

In response to our observation that myocardial TR isoform expression is decreased in patients with heart failure (9), it is possible that these changes may be responsible, at least in part, for certain aspects of the failure phenotype. In the work described here, we have found that TR isoforms have differential effects on the cardiac myocyte phenotype. Specifically, TRα appears to be linked to robust changes in cardiac myocyte growth that are dependent upon the p38MAPK cascade. In contrast, TRβ does not induce a growth program, limits p38 activation, and stimulates the classic thyroid responsive cardiac myocyte genes (namely αMHC and SERCA).

These data support our hypothesis that changes in the expression of TR isoforms and their signaling partners are likely to play a direct role in myocardial growth and gene expression in heart failure. It is tempting to speculate from these findings that manipulation of the TH:TR axis in an isoform-specific manner may represent a new therapeutic approach to CHF that may complement treatment profiles already in use for this devastating syndrome.

Results

Cellular distribution of endogenous and over-expressed TR isoforms.

To better understand the role of individual TR isoforms in the heart, a series of adenoviral vector constructs containing each of the TR isoforms found in the heart ($TR\alpha_1$, $TR\alpha_2$, and $TR\beta_1$) (9) was developed. As indicated by immunostaining (Figure 1a), electrophoretic mobility shift assay (Figure 1b), and Western blot (Figure 1c, upper panel), all three TRs can be successfully over-expressed in cardiac myocytes, with over 90% of myocytes successfully infected at MOIs of ~1–5. Importantly, radioligand binding assays confirm that the Adenoviral over-expression system increases cellular TRs by only ~2–4 fold when compared with control cells (Figure 1c, lower panel-basal binding is ~0.5 fmol/10^6 cells, which increases to ~1.0 fmol/10^6 at 5MOI and ~2 fmol/10^6 at 50MOI). Unexpectedly, distribution of expressed hTRs appears to show some isoform specificity. Specifically, unless over-expressed to very high levels (> 200MOI), $hTR\beta_1$ is localized in the nucleus. In contrast, both $hTR\alpha_1$ and $hTR\alpha_2$ are found in both cytosolic and nuclear fractions. As shown by EMSA, both nuclear and cytosolic TRs are fully competent for binding to a consensus thyroid responsive element (TRE). Notably, T_3 (1–100 nM) did not change the localization pattern of over-expressed TRs (data not shown), as seen by others in different systems (10,11).

Over-expression of $TR\alpha_1$ induces myocyte hypertrophy independent of ligand.

Consistent with reports from our lab and others, T_3 stimulates cardiac myocyte hypertrophy in culture (~75% increase in synthesized protein, $p<0.05$, n=5) with an EC_{50} of ~0.3nM (12–14). As shown in Figure 2a, even in the absence of exogenous hormone, $AdTR\alpha_1$ (but not $TR\beta_1$ or $TR\alpha_2$) also increased protein synthesis and cell surface area (1.88 +/− 0.13-fold over control surface area at an MOI of 50, $p< 0.05$). $AdTR\alpha_1$-induced hypertrophy was enhanced by addition of T_3, but not with the TRβ-selective agonist GC-1 (15) (Figure 2b). Notably, hypertrophy induced by both T_3 and $AdTR\alpha_1$ was inhibited by both $AdTR\alpha_2$ and $AdTR\beta_1$ (Figure 2b and c).

TH and $TR\alpha_1$ activate the p38 signaling cascade and hypertrophy is p38-dependent.

Unexpectedly, hypertrophy induced by either T_3 or $AdTR\alpha_1$ was inhibited by pre-incubation with the p38 inhibitor SB201290 (“SB”, Figure 3a). The IC_{50} of SB201290 was ~30 nM, consistent with specific inhibition of p38 (16). Specificity for the p38 family was confirmed

using infection with dominant negative adenoviral vectors for MKK3 or p38α. The failure of either the MEK1/2 inhibitors U0126 and PD98059 (PD98059 not shown), or infection with AdJNK1DN to inhibit hypertrophy provide additional support for a p38-specific pathway (Figure 3b). Although members of the nuclear hormone receptor family have not previously been thought to directly activate the stress kinase (p38 and JNK) family of signaling intermediates, our results with p38 inhibitors suggest that TH/TRα_1 induced hypertrophy requires this arm of the MAPK signaling cascade. Supporting a direct effect for p38, both T_3 and TRα_1 stimulated a rapid increase in the phosphorylated form of p38 and subsequently its kinase activity (Figure 3c). The TRβ_1–specific agonist GC-1 had no effect (data not shown). As shown in the upper panel of Figure 3c, adenoviral over-expression of both TRβ_1 and the dominant negative TRα_2 inhibited p38 activation by T_3.(4-fold induction in AdbGal cells vs a 1.2-fold increase in AdTRα_2 and 1.5-fold increase in AdTRβ_1 cells). Since these effects on myocyte growth pointed to an involvement upstream of p38 itself, we focused on a possible interaction between TRα_1 and the proximate MAPK kinases (MAPKKs, MKKs) and MAPK kinase kinases (MAPKKKs). As indicated in Figures 4a and b, both AdTRα_1 and T_3 stimulated MKK3/6 phosphorylation and the kinase activity of the MAPKKK, TAK1. Notably, although TRα_2 inhibited T_3-induced TAK1 kinase activity, AdTRβ_1 had no apparent effect. Specificity for the p38 arm of the MAPK family was also shown by the inability of T_3 or adenoviral over-expression of any TR to activate either the ERK or JNK cascades in cardiac myocytes (Figure 4c).

TRα_1 and TRα_2 interact with TAK1 in cytosol.

TAK1 is a member of the MAPKKK family activated by various cytokines including the transforming growth factor beta ligands(17). In general, TAK1 forms a complex with other adapter proteins and kinases, ultimately resulting in its own activation and stimulation of downstream kinases including p38. Although multiple partners have been identified for TAK1, an interaction with the nuclear hormone receptor family has not been previously appreciated. When over-expressed in cardiac myocytes, both TRα_1 and TRα_2 isoforms were found to co-localize with endogenous TAK1 (Figure 5a), a finding that also extended to endogenous rat TRα_1 (Figure 5b)As reported by others (18), TAK1 was found only in cytosolic fraction (Figure 5c). The interaction appears to be specific for the TRα isoforms since over-expressed TRβ_1 was never found in complex with TAK1 even under circumstances of nuclear “overflow” with MOIs of > 200 for 48h (data not shown). TRβ_1 did, however, interact with p38α, a finding that did not extend to either TRα_1 or TRα_2 (Figure 5d). Further, in a cell-free system, TRβ_1 reduced both autophosphorylation of p38 and phosphorylation of its substrate ATF2, but did not appear to affect MKK6 phosphorylation of p38 (Figure 5e).

TRs exhibit isoform-specific changes in myocyte gene program.

Given the reported physiologic effects of T_3 on myocyte gene expression (12,13,19,20), we found that AdTRα_1 initiated a gene profile more consistent with a pathologic/fetal myocyte program [increasing βMHC, skACT, ANP, and BNP while decreasing αMHC and SERCA2 expression (Figure 6)]. AdTRα_1 also caused a down-regulation of the myocyte expression of endogenous TRα_1 and TRβ_1. The fetal gene program induced by AdTRα_1 was abrogated somewhat, however, by co-treatment with T_3 and co-infection with AdTRα_2 (data not shown). At relatively low MOIs (10–50), AdTRα_2 also inhibited T_3-induced increases in skACT, ANP, or BNP expression; however, MOIs of >200 were required for attenuation of T_3-induced increased expression of αMHC and SERCA. T_3-induced inhibition of βMHC expression was not affected in AdTRα_2-treated cells, at any MOI. Notably, over-expression of TRβ_1 induced a gene program that was sharp contrast to TRα_1, reflecting a marked TR isoform-specific gene program. The gene expression profile seen with AdTRβ_1 was, in fact, quite similar to that observed with T_3 [increases in αMHC, SERCA2, and endogenous TRβ_1, and repression of βMHC mRNA to nearly undetectable levels (Figure 6)]. Addition of T_3 to AdTRβ_1 further

enhanced the change in αMHC, SERCA, and endogenous $TR\beta_1$ expression. Expression of skACT, ANP, and BNP in $AdTR\beta_1$-infected cells differed from T_3 treatment, exhibiting a substantial inhibition in $AdTR\beta_1$ infected cells (Figure 6).

Discussion

Recent work from both our investigative group and others has renewed the interest in a possible therapeutic role for the TH:TR axis in patients with heart failure. In this regard, we previously reported isoform-specific alterations in TR expression in both human and experimental hypertrophy/failure (9,12). Changes in TR isoforms were believed to play a role in the development and/or maintenance of the pathologic cardiac myocyte gene program and, as such, represented possible therapeutic targets. In the present study, we extend these findings and report previously unappreciated isoform-specific, non-genomic activities for TR isoforms in cardiac myocytes. The major conclusions from these investigations are: (1) T_3-induced cardiac myocyte hypertrophy is p38 dependent and requires $TR\alpha_1$ and activation of the TGFbeta activated kinase, TAK1 and (2) The ultimate effect of TH on myocardial growth/gene expression is the result of the combinatorial effects (both complementary and antagonistic) of the three TR isoforms found in heart, $TR\alpha_1$, $TR\alpha_2$, and $TR\beta_1$.

T_3 and $TR\alpha_1$ activate TAK1 and the p38 cascade.

Several lines of evidence indicate that the effects of TH on the myocyte gene program are characterized by an adult/physiologic phenotype. The work presented here, however, shows that T_3 stimulates p38, the arm of the MAPK family most frequently associated with pathologic hypertrophy (21). In these investigations we examine this seemingly contradictory phenomenon. Our studies indicate that T_3-induced p38 activity and myocyte growth is exclusively due to the action of the $TR\alpha_1$ isoform on the upstream kinase, TAK1. The sequential activation of TAK1 and p38 by $T_3/TR\alpha_1$ is capable of being modulated at two points in the cascade. First, $TR\alpha_2$ can compete with $TR\alpha_1$ for binding to TAK1, and second, $TR\beta_1$ can associate with, and inhibit, the downstream target p38. This $TR\beta_1$-p38 interaction is similar to the interaction of $TR\beta_1$ with ERK2 (20,22,23), and likely occurs in the nucleus where the majority of activated p38 is found (24). Thus, although the $TR\alpha_1$ isoform facilitates T_3-induced P38 activity, the P38 activity (and program of pathological hypertrophy) can be altered by both $TR\alpha_2$ and $TR\beta_1$. Given that both $TR\alpha_1$ and $TR\alpha_2$ interact with TAK1, the interaction domain must reside within the common 5′-half of the TR proteins, a domain known to interact with other transcription factors or signaling molecules such as MEF2 or ERK (22,25–27). Activation of TAK1 does not appear to result from any inherent kinase activity of $TR\alpha_1$ itself, but rather likely results from an adapter function of $TR\alpha_1$, possibly similar to that described for the TAK1-binding protein (TAB1) (28). $TR\alpha_1$, however, was not found to interact with TAB1 (data not shown). Although somewhat unextected by us, the finding that TRs may have differential sub-cellular locations and shuttle between nuclear and cytoplasmic compartments has been reported by a number of investigative groups (10,11,29–31). The mechanism(s) of nucleo-cytoplasmic shuttling have not been identified with certainty, but may involve involve protein partners (10) and possibly post-translational modification of TRs (i.e. phosphorylation, (22). Notably, this has not reliably been altered by ligand (10,30,31). The exact mechanism for either the differential localization or movement from one compartment to the other in the cardiac myocyte context has not been identified in the present work and is certainly worthy of further investigation.

Summary-The myocardial response to TH results from the combinatorial effects of individual TR isoforms (Figure 7).

Our investigations indicate that T_3-induced gene expression in neonatal cardiac myocytes is the result of two parallel signaling cascades. One is the classical, direct (or “genomic”) pathway

in which TH interacts with nuclear TRs, likely bound to characteristic response elements (TREs) on target genes. The second pathway, novel for a nuclear hormone receptor, is a true cascade in which T_3 activates TAK1 through the action of cytoplasmic $TR\alpha_1$. This activation ultimately results in the stimulation of a series of p38-dependent processes that include myocyte protein synthesis (hypertrophy) and the induction of a set of genes whose expression characterizes the pathologic growth program (skACT, ANP, and BNP). This action of the $TR\alpha_1$ isoform is tempered somewhat by opposing effects of both the $TR\alpha_2$ and $TR\beta_1$ receptor isoforms which prevent the tonic activation of the p38 arm at two points in the cascade, while maintaining expression of TRE-dependent genes. This latter effect may be largely due to the T_3-dependent up-regulation of the $TR\beta_1$ isoform, which appears to be a potent stimulus for αMHC and SERCA expression. As such, T_3-responsive genes can be divided into three categories. The first group consists of TRE-containing genes that are regulated by both $TR\alpha_1$ and $TR\beta_1$, a category that includes αMHC and SERCA. Observations on $TR\alpha_1$-deficient mice (32,33) and data from our transfection study (12) also suggest significant roles of $TR\alpha_1$ on these genes. The second group is made-up of TRE-containing genes that are mostly regulated by $TR\beta_1$. This category consists of β MHC and endogenous $TR\beta_1$, and is supported by our previous promoter assays suggesting a $TR\beta_1$-specific role in the regulation of the latter (12). For both groups, TRE-mediated effects appear to be dominant in the final response to TH. The third category includes skACT, ANP, BNP, and endogenous $TR\alpha_1$, genes that do not contain TREs in their promoter regions, and are only modestly responsive to TH. For these genes, it appears that the $TR\alpha_1$ stimulation of the p38 pathway is dominant in the final response to TH. Consistent with these findings, IL-1β, which is a strong p38 activator (34,35), has synergistic effects with T_3 for increases in ANP and BNP expression as well as myocyte protein synthesis (unpublished observations). We have reported that the failing human heart exhibits fetal gene expression (36–38). Since human heart failure is often associated with enhanced p38 activity (39–41), a TRβ-specific agonist could result in a decrease in p38 activation and an alteration in the fetal gene program, and possibly the growth program. In contrast, thyroid hormone supplementation could theoretically lead to excessive p38 activation and additional myocyte injury, particularly if there were an imbalance in TR expression toward $TR\alpha_1$ as seen in some cases of pathologic hypertrophy (12). Although there is a currently available TRβ agonist, GC-1, it is probably not as highly TRβ-specific as would be necessary (15), and its bioavailability has been questioned (42). Additionally, relative to $TR\alpha_1$, $TR\beta_1$ gene expression in the human left ventricle is quite low (9). Therefore, the utility of a $TR\beta_1$ agonist in the treatment of heart failure will need to await the development of a more highly selective $TR\beta_1$ ligand.

Materials and Methods

Cell culture.

Ventricular myocytes from one-day-old rats were cultured as described (9). Vehicle for triiodo-L-thyronine (T_3) (Sigma) was NaOH. GC-1 (a gift from G. Chiellini and T. S. Scanlan, University of California San Francisco) and U0126 (Cell Signaling) were dissolved in DMSO. Effects of SB202190 (Calbiochem) were always compared with those of inactive SB202474. All animal experimentation described was approved by the University of Colorado IACUC and was conducted in accord with accepted standards of humane animal care as out lined in the NIH guide for the Care and Use of Laboratory Animals. As described previously, cells are kept in 5% serum containing medium (which has not been previously “stripped” of TH) for 24 hours followed by washing and change to serum-free medium whose residual T_3 content has been measured at ~0.1nM (12).

Adenoviral constructs.

Adenoviral (Ad) constructs for β-galactosidase (AdβGal), HA-tagged, constitutively activated MAPK kinase (MKK-6, AdMKK6CA) or dominant negative (DN) MKK-3 (AdMKK3DN), dominant negative c-Jun N-terminal kinase (AdJNK1DN) and Flag-tagged Adp38αDN or wild type p38α (Adp38αWT) were provided by J. Han (Scripps Institute), L. Heasley (University of Colorado), and K.A. Heidenreich (Denver Veterans Affairs Medical Center, Denver, Colorado), respectively. By X-Gal staining, ~95% of cells expressed β-Gal protein after exposure to AdβGal for 72h at a multiplicity of infection (MOI) of≥1. Expression of other constructs was confirmed by Western blot with epitope-specific antibodies (Roche).

Preparation of adenoviral constructs for TRs and over-expression in myocytes.

cDNAs for human TR/c-erbA isoforms (α_1, α_2, and β_1) were provided by R.C.J. Ribeiro (University of Brasilia) and J.D. Baxter (University of California, San Francisco). The full-length cDNAs were subcloned into adenovirus shuttle vector (pAC-CMV), and transfected together with adenoviral arm in 293 cells (43). Plaques negative for X-Gal staining were selected, and ones positive for TR cDNA by PCR were purified.

Western blot analysis and T_3-binding assay.

Total or fractionated cell extracts from equal number of cells were subjected to Western blot analysis (12) or [^{125}I]T_3-binding assay (44). Antibodies used included those for phopho-p38α/β, p38α, phospho-MKK3/6, phospho-ERK1/2, ERK1/2, phospho-JNK1/2 from Cell Signaling (all polyclonal, used at 1:1000 dilution); for MKK3 (I-20), p38α/β(A-12), TAK1 (C-9, M-579), JNK2/1 (D-2), TRβ_1 (J51), TRα/β (C-1) from Santa-Cruz Biotechnology (used at 0.2–2μg/mL); and for TRα_1 (PA1-211A) from Affinity BioReagents (1:200 dilution). Recombinant TR proteins were synthesized using expression vectors for human TRs (gifts from R.C.J. Ribeiro and J.D. Baxter) and rabbit reticulocyte lysate (TNT T7 Quick Coupled System, Promega).

Immune complex kinase assay.

Total cell extract was immunoprecipitated with p38α/β (A-12) or TAK1 (C-9) antibody (4μg/mL). The immune complexes were used for in vitro kinase assay (45) with ^{32}P-γ ATP and 2μg of recombinant Glutathione S-transferase (GST)-ATF2 (Santa-Cruz Biotechnology) or inactive MalE-MKK6 (Upstate Biotechnology) as substrates at 30°C for 20 min. Phosphorylated products were analyzed by SDS-PAGE. Aliquots of immune complexes were also blotted for total p38α (Cell Signaling) or TAK1 (M-579).

Electrophoretic mobility shift assay.

Both nuclear and cytosolic fractions were resuspended in equivalent volumes for comparison of relative TR expression in electrophoretic mobility shift assay (45). For super-shift assay, antibodies for TRα_1 (PA1-211A, 1:10 dilution), TRβ_1 (J51), or retinoid X receptor (α: D-20, β: C-20, γ : Y-20, Santa-Cruz Biotechnology) (used at 20μg/mL) were added to samples for 30 min at 4°C prior to incubation with ^{32}P-labeled direct repeat 4 (DR4) oligonucleotide (Santa-Cruz Biotechnology). Unlabeled DR4 was used as competitor at 100-fold molar excess.

Immunostaining.

Myocyte expression of exogenous human TR or endogenous TAK1 was visualized by immunostaining (35) using TR (C1) or TAK1 (C-9) antibody (4μg/mL) and FITC-labeled anti-IgG_1 antibody (Santa-Cruz Biotechnology). Cardiac myocytes were identified with sarcomeric α-actin antibody (1:50 dilution, 5C5, Sigma) and rhodamine-conjugated anti-IgM antibody (Santa-Cruz Biotechnology).

Immunoprecipitation.

Total cell extract immunoprecipitated by TAK1 (C-9), p38α/β (A-12), or TRα$_1$ antibody (FL-408, Santa-Cruz Biotechnology) was subjected to Western blot analysis for TR (C1) or TAK1 (C-9). Aliquots of immune complexes were also blotted for total p38α (Cell Signaling) or TAK1 (M-579).

Kinase assay.

Human TR protein (approximately 10ng) synthesized in rabbit reticulocyte lysate was mixed with 10ng of active MalE-MKK6 (~81kDa, Upstate Biotechnology, Waltham, MA) or active GST-p38α (~68kDa, Calbiochem), and incubated with 2μg of inactive GST-p38α (~64kDa, Upstate Biotechnology) or GST-ATF2 as substrates, respectively. Auto-phosphorylation of p38α was also assessed with 1μg of the active GST-p38α. Kinase reaction was carried out at 30°C for 20 min. and products analyzed by SDS-PAGE (45).

Growth assay.

After treatment, myocytes were incubated in fresh media containing ^{14}C-phenylalanine for 24h. The incorporated [^{14}C]-phenylalanine into synthesized protein allowed for quantification of hypertrophy at the steady state by radio-labeled protein assay (12).

RNase protection assay.

Total RNA (5 μg) was used in RNase protection assay (9,12) with rat probes for α/β-MHC, SERCA2, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), skeletal α-actin (skACT), TRα$_1$/TRα$_2$, TRβ$_1$, and GAPDH as an internal control.

Data analyses and statistics.

Specific signals obtained from Western, kinase assay, or RNase protection assay were quantified by densitometry. All values were normalized to the appropriate controls. Data were compared by one-way analysis of variance and the Newman-Keuls test. Mean ± standard error is shown.

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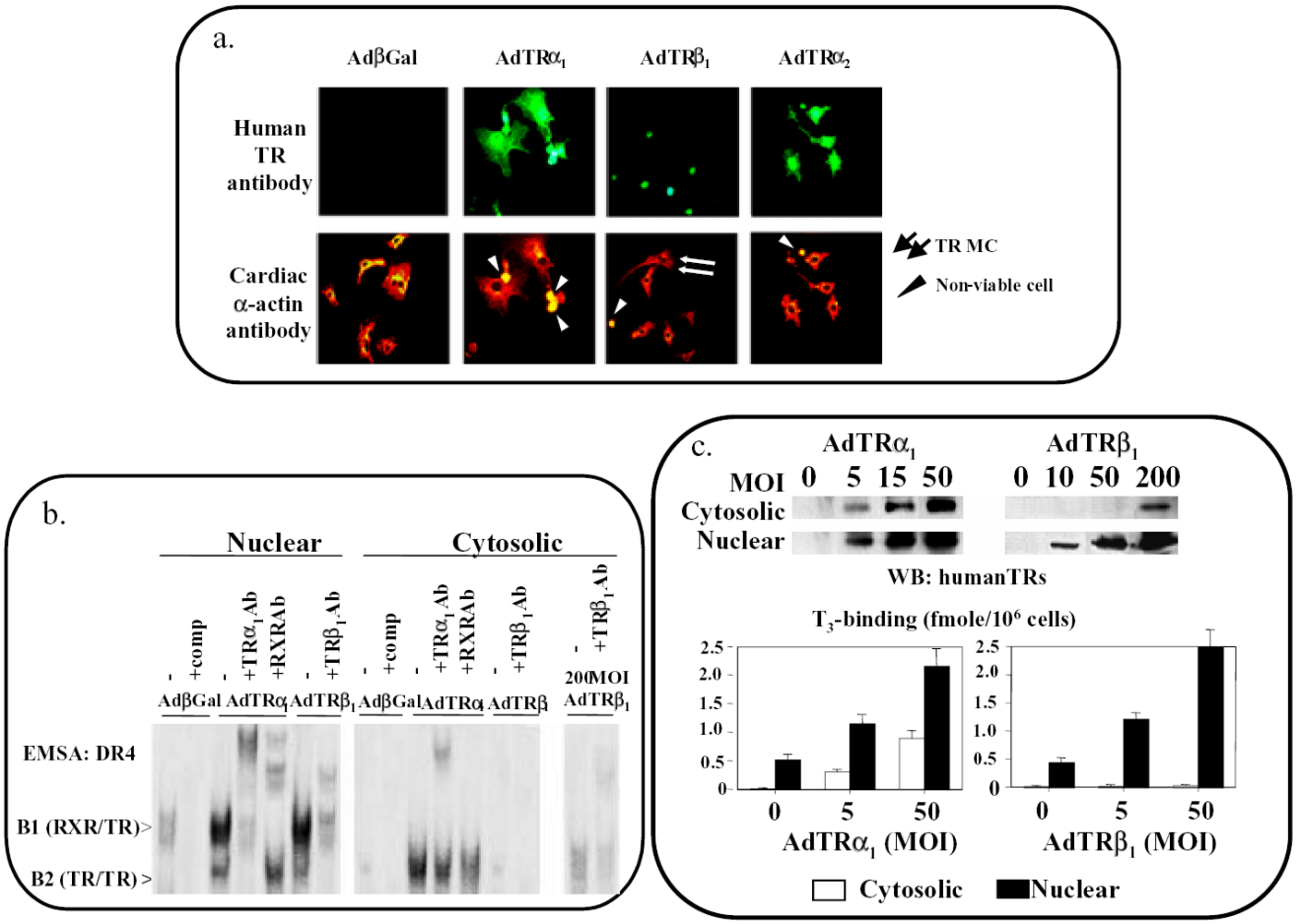

Figure 1. Cardiac myocyte expression of human TRs.

a. Immunostaining. Neonatal rat cardiac myocytes (MCs) were exposed to adenovirus at 100 MOI for 72 h. Upper panels are immunofluoresence pictures of cells infected with the indicated AdTRs incubated with the C1 antibody that only recognizes human TR isoforms (α and β). Bottom panels were the same cells co-incubated with antibody to sarcomeric α-actin which identifies cardiac myocytes. Less than 5% of cells were sarcomeric actin-negative (nonmyocytes, NMCs). A myocyte without expression of human $TR\beta_1$ (↑↑) is identified. Note the restriction of $hTR\beta_1$ expression to the nucleus of these cells while both $hTR\alpha_1$ and $hTR\alpha_2$ appear to be distributed in both nuclear and cytoplasmic compartments. **b.** Electrophoretic mobility shift assay for the DR4 (direct repeat-4) TRE. Cells were exposed to adenovirus at 50 MOI for 48 h. For supershift assays, the same human TR-specific antibodies used in Figure 1c were used, and are denoted as "+" isoform-specific Ab. B1 and B2 consists of heterodimers of retinoid X receptor (RXRα, β, or γ) and TR (1 molecule of each), and homodimers of TRs (2 TR molecules), respectively. No monomer binding was observed. Competitor lanes were with unlabeled oligonucleotide. The 200MOI lane for hTRβ was included since this was the only condition where cytosolic hTRβ was found **c.** Quantification and sub-cellular location of human TR over-expression in neonatal rat cardiac myocytes. Myocytes were infected with the individual AdTRs at the indicated MOIs for 48 hours. Fractionated cell extracts were prepared and subjected to Western blotting with human-specific TR antibodies in the upper panels (hence no rat TR is detected in un-infected lanes). In the binding experiments, cell extracts from equal numbers of cells were subjected to [^{125}I]

T_3-binding assay as described previously (44). Notably, expression of $TR\alpha_1$ was readily found in both nuclear and cytoplasmic fractions while $AdTR\beta_1$ expression was generally limited to the nucleus.

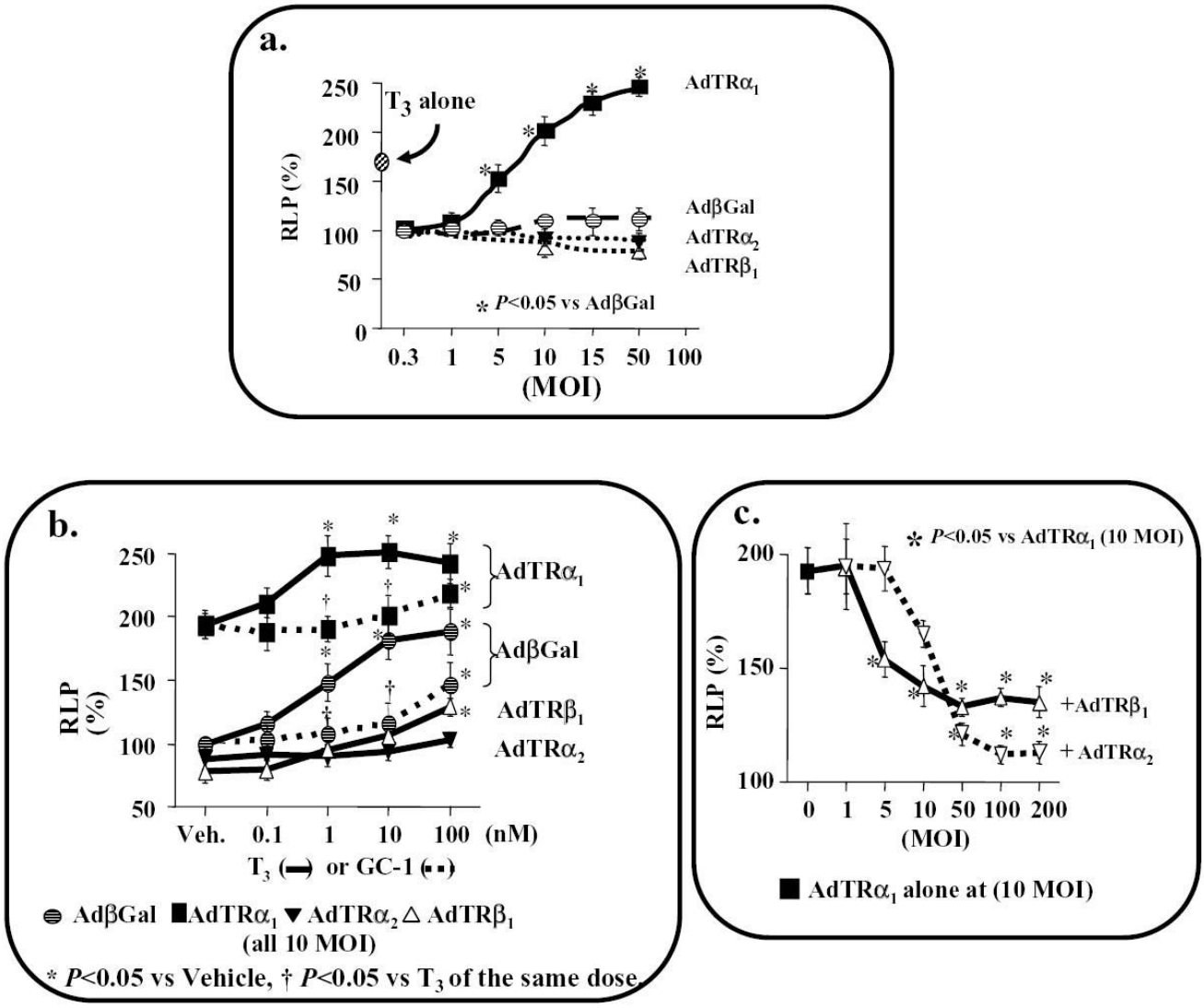

Figure 2. Over-expression of TRα_1 induces myocyte hypertrophy

a. MOI-dependent effects on protein synthesis by AdTRs. Cells were infected with AdβGal, AdTRα_1, AdTRα_2, or AdTRβ_1 for 48h at the designated MOI. Radiolabeled protein content (RLP) was normalized to AdβGal at identical MOIs and at the 0.3MOI level for the subsequent increases in AdβGal itself. For comparison, the RLP seen with 100nM T_3 alone is shown. **b.** Effects of T_3 or GC-1 on protein synthesis. Cells were infected with AdβGal, AdTRα_1, AdTRα_2, or AdTRβ_1 for 48h at 10MOI with various concentrations of T_3 or GC-1. Values were normalized to vehicle + AdβGal at 10MOI. **c.** Effects of AdTRα_2 or AdTRβ_1 on AdTRα_1-induced hypertrophy. Cells were treated with AdTRα_1 at 10MOI (□) with the addition of AdTRα_2 or AdTRβ_1 at the indicated MOIs for 48h.

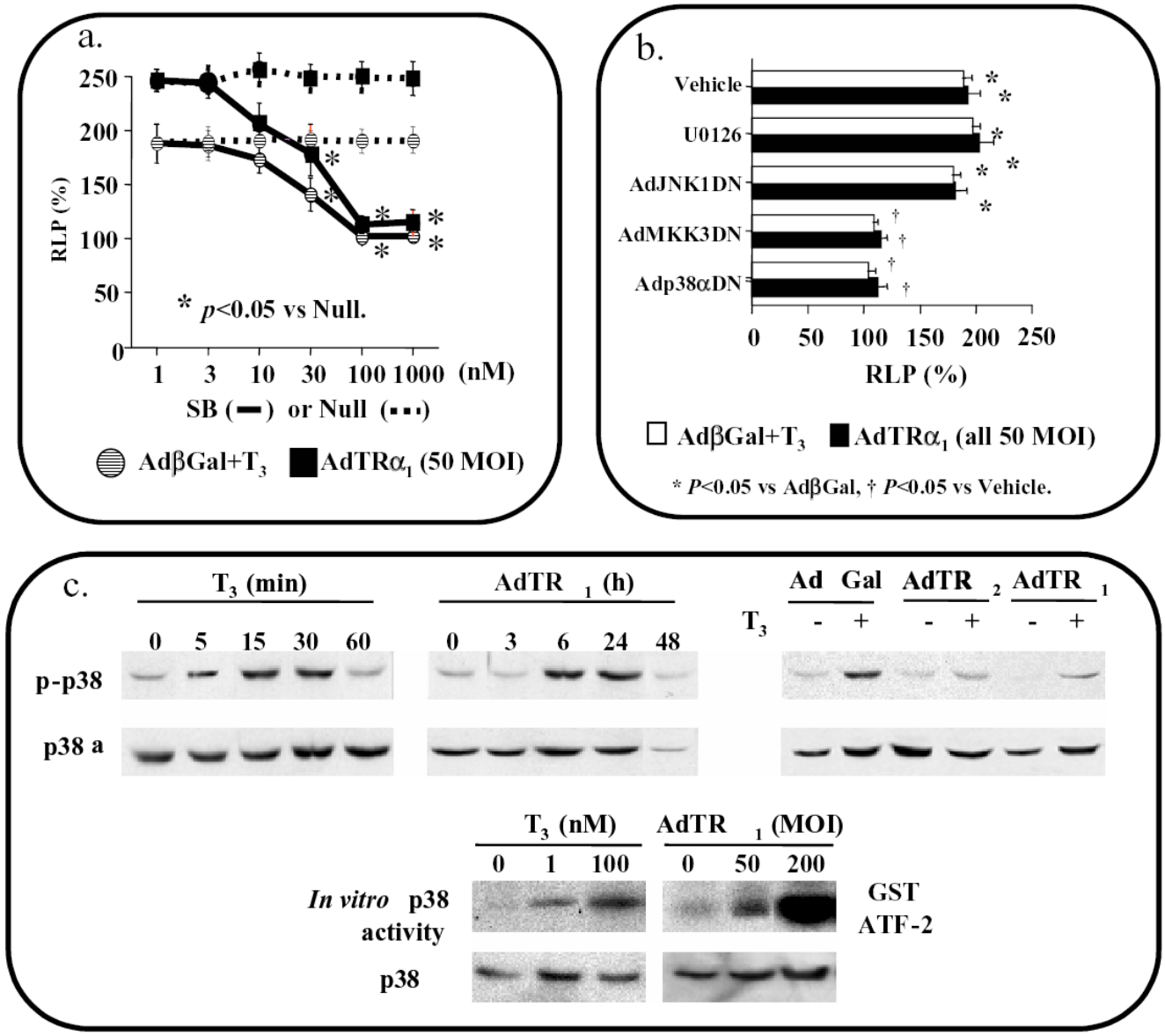

Figure 3. TH and TRα1 hypertrophy is p38-dependent.

a. Dose-dependent effects of SB202190 on T_3 and AdTRα$_1$-induced myocyte growth. Cells were pretreated with the indicated dose of SB202190 ("SB") or null SB202474 ("Null") for 30 min, followed by the addition of AdβGal (50MOI, not shown), AdβGal+T_3 (100 nM) or AdTRα$_1$ for 48h. Values were normalized to that of AdβGal + vehicle. **b.** Cells were pretreated with vehicle (DMSO) or U0126 (1 μM) for 30 min or AdJNK1DN, AdMKK3DN, or Adp38αDN for 24 h. Cells were subsequently infected with AdβGal (50MOI, not shown), and treated with T_3 (100 nM, AdβGal+ T_3), or AdTRα$_1$ for 48 h. Values were normalized to that of AdβGal + vehicle. **c.** T_3/TRα stimulation of p38MAPK. Cells were treated with T_3 (100 nM) or AdTRα$_1$ (50MOI) for the designated times (left and middle panels) or infected with AdβGal or AdTRs (50MOI) for 24h and T_3 (100nM) added for an additional 15 minutes (right panel). Phospho-p38 was then determined by Western blotting. Both T_3 and AdTRα$_1$ activate p38 (bottom panel). AdβGal cells were treated with T_3 (15 min) or AdTRα$_1$ (24h) at indicated doses. In vitro p38 activity was measured by immune complex kinase assay with GST-ATF2.

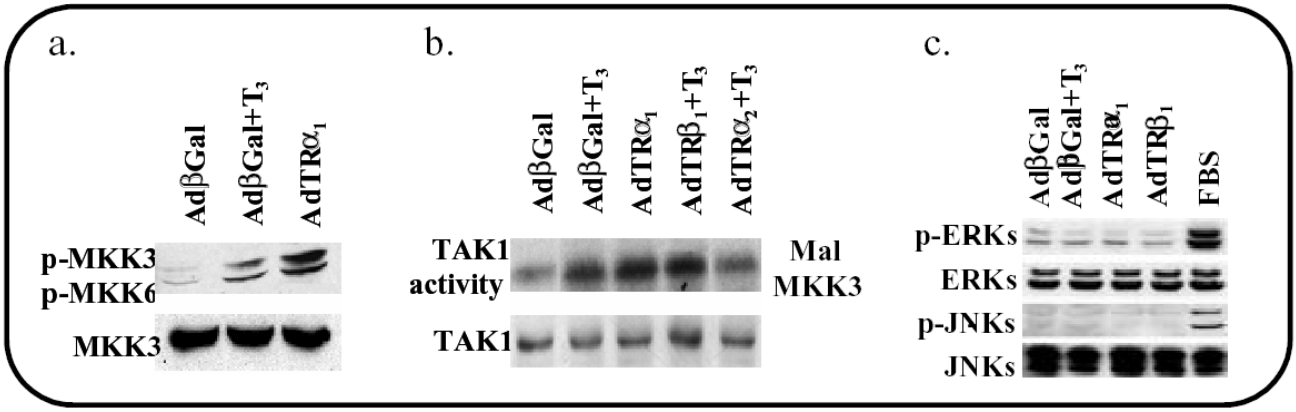

Figure 4. TH and TRα_1 activate MKK3/6 and TAK1, but not ERK or JNK.

a. Activated (phosphorylated) MKK3 (upper band) and MKK6 (lower band) expression increase in T_3 and AdTRα_1 treated cells. **b.** In vitro TAK1 activity was measured by immune complex kinase assay using MalMKK3 in cells treated with T_3 alone (15 min) or in the presence of the indicated AdTRs (48h infection). **c.** Phosphorylated and total ERK1/2 and JNK1/2 expression were also examined in similarly treated T_3 and TR infected cells. As a positive control, cells were treated with 20% of fetal bovine serum (FBS) for 30 min.

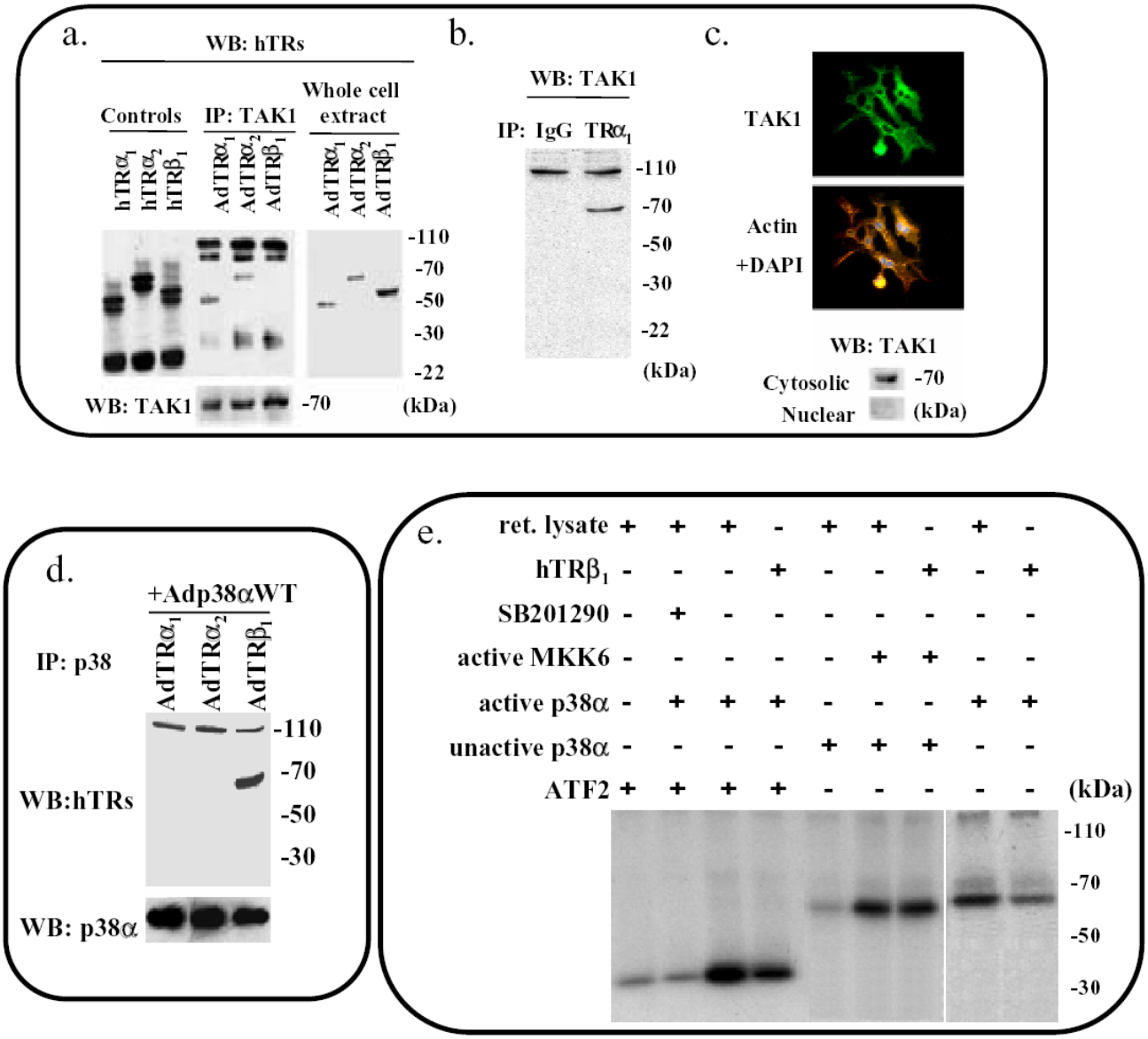

Figure 5. Cytosolic TRα$_1$ interacts with TAK1.

a. TRα$_1$ and TRα$_2$, (but not TRβ$_1$) interact with TAK1. Lanes 1–3: Human-specific TR antibody was validated for Western blotting with control human TRs synthesized in rabbit reticulocyte lysate [TRα$_1$ (~48kDa), TRα$_2$ (~58kDa), and TRβ$_1$ (~52kDa)]. Doublets represent lysate-specific in vitro processing and are not seen in AdTR-infected cells. Lanes 4–6: Myocytes were infected with AdTRs at 50 MOI for 24h followed by immunoprecipitation of endogenous TAK1. This was subjected to Western for TR. Lanes 7–9: Expression of human TRs in each sample was confirmed using the same antibody**. b.** Whole cell extract from un-infected cells was immunoprecipitated with rabbit IgG or rat-specific TRα$_1$ antibody, and subjected to Western blotting for TAK1**. c.** Western blotting and immunofluorescence microscopy for endogenous cardiac myocyte TAK1 expression**. d and e.** TRβ$_1$ (but not TRα$_1$ and TRα$_2$) interacts with p38 and diminishes its kinase activity. Cells were infected with AdTRs and Adp38αWT for 24h. Total p38 was immunoprecipitated, and subjected to Western for humanTR (C1). **e.** In vitro synthesized human TRβ$_1$ or control rabbit reticulocyte lysate

was mixed with active MKK6 or active p38α (~68kDa), and their activities measured on unactive recombinant GST-p38α (~64kDa) or GST-ATF2 (~40kDa), respectively. SB202190 was used at 10nM.

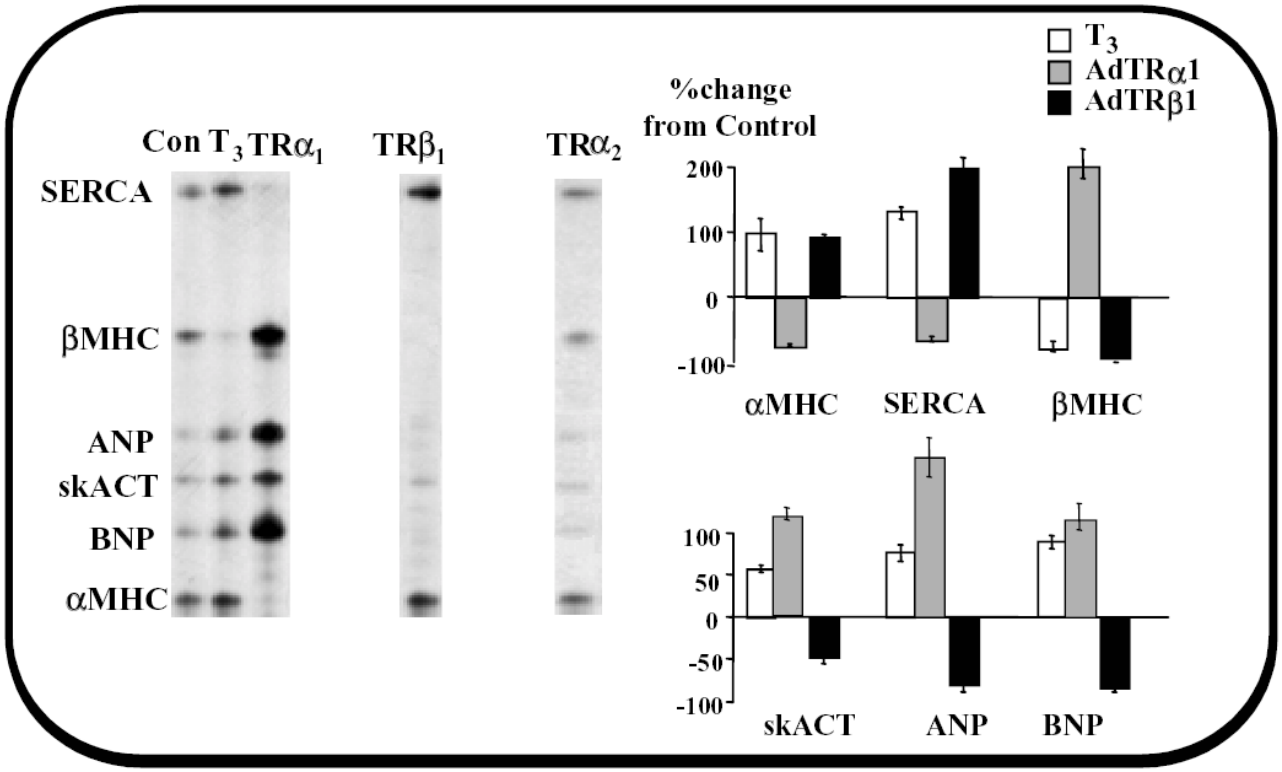

Figure 6. TR isoform-specific changes in the cardiac myocyte gene program.

Cells were treated with AdβGal at 50 MOI with or without T_3 (100nM) for 72h and compared with cells infected with AdTRα_1 or AdTRα_2, or AdTRβ_1 at 50 MOI. Values of the corresponding Adβ Gal group were set at 100%, and data is presented as % change from 100%, n=3–4. As such, a value of 0% equals no change from AdβGal infected cells and 100% represents a doubling of signal. All signals were corrected for RNA loading using an internal GAPDH signal.

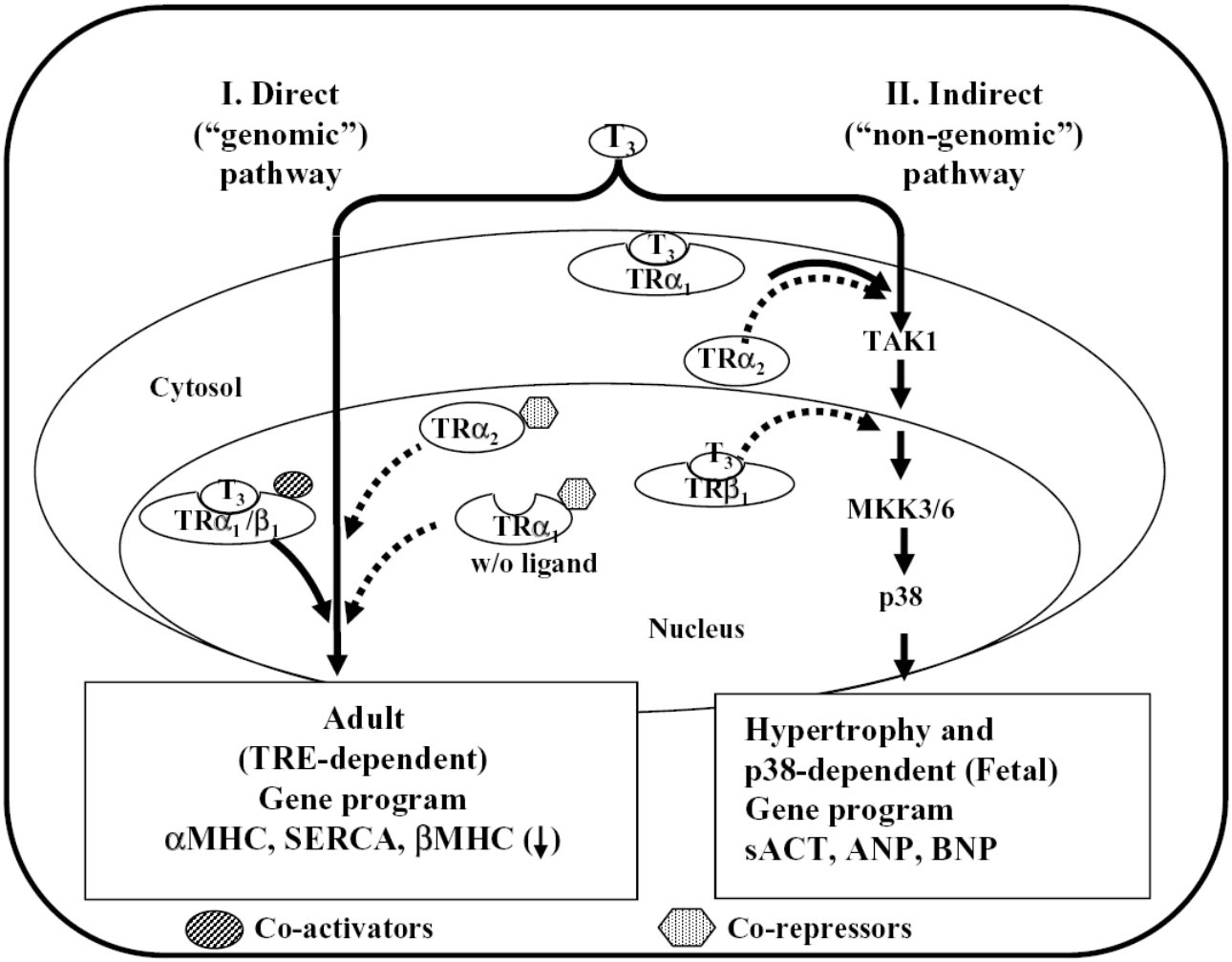

Figure 7. Proposed schema of T_3/TR isoform-specific action on cardiac myocyte MAPK signaling and gene program.
See text for details.