Co-activation of Atrial Natriuretic Factor Promoter by Tip60 and Serum Response Factor*

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Tat-interactive protein 60 (Tip60) is a member of the MYST family of histone acetyltransferases (HATs). In addition to its HAT domain, Tip contains a heterochromatin-associated protein 1-like chromodomain and a zinc finger-like domain. Several alternative splice variants of Tip60 have been characterized, including full-length Tip60α, Tip60β (which lacks exon V encoded by the Tip60 gene), and Tip55 (which encodes a novel 103-amino-acid C terminus). We report here that isoproteins recognized by a pan-Tip60 antibody are strongly and transiently expressed between embryonic days 8 and 11 in the embryonic mouse myocardium. A functional role for Tip60 isoproteins in cardiac myocyte differentiation is suggested by immunoprecipitation experiments showing that Tip60α, Tip60β, and Tip55 can bind serum response factor (SRF) and by transient transfection experiments showing that Tip60 and SRF cooperatively activate the atrial natriuretic factor promoter. Although this combinatorial activity is inhibited by histone deacetylase 7, it was unexpectedly enhanced by point mutation of the HAT domain. Ablation of the chromodomain from Tip60 caused derepression. These findings suggest that Tip60 modulates expression of SRF-dependent cardiac genes.

Since its discovery in 1996 (1), numerous findings have established Tat-interactive protein 60 kDa (Tip60) as a member of the “MYST” family, an acronym denoting the founding members MOZ, YBF2, SAS2, and Tip60 of the family. Although Tip60 proteins are essential for embryonic development as determined by very early embryolethality resulting from homozygous ablation of the Tip60 gene,4 the cellular function of its alternatively spliced isoforms as well as of its functional domains is only beginning to be understood. Among possible functions, Tip60 has been implicated in chromatin-remodeling complexes wherein it is required for apoptosis and DNA repair (3), during which it mediates acetylation-dependent exchange of histone H2A.X (4). Several studies utilizing transient transfection analysis have implicated Tip60 in the regulation of gene transcription, although whether it functions as a co-activator or co-repressor appears to be context-dependent. For example, Tip60 co-activates transcription with human immunodeficiency virus-1 Tat (1), androgen receptor (5, 6), and β-amyloid precursor protein-F65 complex (7). By contrast, Tip60 co-represses transcription with cAMP-response element-binding protein (8), zinc finger E box binding protein (9), and STAT3 (10), which repression is potentiated by histone deacetylase 7 (HDAC7).

Several putative functional domains are present in Tip60, all of which are atypical. These include a chromatin organization modifier domain (chromodomain), a zinc finger-like domain, and a histone acetyltransferase (HAT) domain. The HAT domain was recently shown to obligatorily acetylate nucleosomal histones during DNA repair (4); however whether Tip60-mediated histone acetylation is required during transcription is unclear. Little is known about the function of the other domains of Tip60, including its chromo-like domain, which in canonical proteins mediates heterochromatin formation via binding to methylated histones (11). The atypical zinc finger domain, which is highly conserved in the MYST family, was recently shown to bind HDAC7 (10).

We previously reported that expression of Tip60 is developmentally regulated during early chick embryogenesis in a fashion highlighted by the transient enrichment of Tip protein in developing myocardium (12). It was therefore of interest to investigate whether Tip60 interacts with cardiac transcription factors to regulate cardiac gene expression. We report here that Tip60 isoproteins bind the serum response factor (SRF) and that Tip60 and SRF cooperatively activate the atrial natriuretic factor (ANF) promoter. Although HDAC7 inhibited Tip60-mediated activation as expected, point mutation of the HAT domain of Tip60 was surprisingly derepressive. In addition, deletion of the chromodomain of Tip60β increased ANF activation, consistent with a repressive function for this domain.

**EXPERIMENTAL PROCEDURES**

*Antibodies—An affinity-pure rabbit polyclonal antibody was prepared that recognizes a peptide (DFNKRLDEWTPLERDLQ) in the chromodomain of Tip60 (Research Genetics, Huntsville, AL); this domain is present in all known Tip isoforms and is absent from all other proteins based on data base searches. Anti-FLAG is a monoclonal mouse antibody (Sigma catalog number F3165) that recognizes the peptide encoded in p3xFLAG-CMV (Sigma catalog number E4026). Anti-HA is affinity-purified rabbit polyclonal antibody (Santa Cruz Biotechnology catalog number sc-805) that recognizes HA-tagged fusion proteins. Normal rabbit and mouse IgGs were used as controls (R & D Systems catalog number AB-105-C).

*Immunohistochemistry—Mouse embryos were fixed by cryopreservation and immunostained using the anti-chromodomain antibody (1:50). All procedures were as previously described (12). Controls included substitution of the primary antibody with a 1:50 dilution of normal rabbit IgG or preabsorption of primary antibody with 8 μg of immunogenic peptide. To ensure cardiac specificity of staining, parallel
sections were immunostained for sarcomeric α-actin (1:800; Sigma catalog number A-2172), in which instance the secondary antibody was fluorescein isothiocyanate-conjugated goat anti-mouse IgM (1:200; ICN catalog number 55498).

Immunoprecipitation and Western Blotting—Binding of Tip60 iso-proteins to SRF was demonstrated by transfecting HeLa Cells (American Type Culture Collection catalog number CCL-2) with either pFLAG-Tip60β, pFLAG-Tip55, or pFLAG-Tip60α, each of which encodes a 22-amino-acid 3× FLAG epitope fused in-frame with the N terminus of full-length mouse cDNAs encoding each Tip isoresponse. Each construct was separately co-transfected with pHA-SRF encoding a hemagglutinin motif in-frame with the N terminus of full-length human SRF cDNA cloned in vector pcDNA3. Three micrograms each of the Tip60 and SRF plasmids were transfected by mixing DNA and FuGENE 6 (Roche Applied Science catalog number 1814443) at a 1:3 ratio. Sub-confluent cells in 100-mm dishes were transfected for 36 h after which cells were pelleted and lysed with 800 μl of lysis buffer (150 mM NaCl/50 mM Tris-HCl (pH 7.4)/1 mM EDTA/1% Triton-X-100) containing protease inhibitor mixture (Roche Applied Science catalog number 1863153). Lysates were centrifuged for 10 min at 10,000 rpm after which 50 μl were removed to determine FLAG-Tip and HA-SRF protein levels in lysates; the remainder (750 μl) was incubated 3 h at 4 °C with 20 μl of EZview™ Red anti-FLAG™ M2 affinity gel (Sigma catalog number F2426) beads. Prior to mixing lysate with beads, these were blocked by incubation with 1% bovine serum albumin for 1 h at 4 °C and washed with lysis buffer. Lysates were then applied with constant gentle mixing in the presence of 1% bovine serum albumin for 1 h at 4 °C after which the beads were thoroughly washed (4× 10 min each using lysis buffer at 4 °C). The immune complexes were eluted with 20 μl of SDS-PAGE sample buffer, separated in 7.5% acrylamide/SDS gels and subjected to Western blotting using antibody concentrations for anti-HE at 1 μg/ml and anti-FLAG at 10 μg/ml. Bands were detected by chemiluminescence using ECL Western blotting detection reagent (Amersham Biosciences).

Transient Transfection—HeLa cells were cultured in minimum Eagle’s medium without antibiotics supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids (Invitrogen). In all instances, cultures were plated at a density of 10⁵ cells/22 mm diameter dish. After 24 h, the cells were transfected using plasmid DNA and FuGENE 6 mixed at a 1:3 ratio. In all experiments, the total amount of transfected plasmid DNA was maintained constant by including pcDNA3 empty expression vector, and to normalize for efficiency of transfection, plasmid pRL-TK (Renilla luciferase, Promega) was included at one-tenth the mass of promoter-reporter plasmid. Cells were harvested 40 h after transfection, and normalized luciferase activity was assessed using the Dual-Luciferase reporter assay system (Promega catalog number E1960).

Plasmids—cDNAs for full-length Tip60α, Tip60β, and Tip55 plus ~250 bp of each 3′-UTR were subcloned into the EcoRI and BamHI sites of expression vector p3xFLAG-CMV-7.1 (Sigma catalog number E4026), creating N-terminal FLAG-Tip fusion proteins for use in immunoprecipitation and transient transfection experiments. Tip60β cDNA was from I.M.A.G.E. Consortium clone number 3154297 (GenBank™ accession number AW762337). Tip 55 is a splice variant that utilizes previously unrecognized exons in intron 11 of the Tip60 gene (20). We discovered Tip55 by characterizing I.M.A.G.E. Consortium clone number 4011689 (GenBank™ accession number AY759980), and to normalize for efficiency of transfection, plasmid pRL-TK (Renilla luciferase, Promega) was included at one-tenth the mass of promoter-reporter plasmid. Cells were harvested 40 h after transfection, and normalized luciferase activity was assessed using the Dual-Luciferase reporter assay system (Promega catalog number E1960).
RNA using Moloney murine leukemia virus-reverse transcriptase Pfu Turbo polymerase (Stratagene). The expression plasmid encoding SRF was pcDNA3-HA-SRF, which encodes a hemagglutinin motif in-frame with the N terminus of human SRF. The expression plasmid encoding mouse HDAC7 (pCMX-mHDAC7-HA) was provided by Dr. Ronald Evans of the Salk Institute.

Tip60 mutations were introduced using the Stratagene QuikChange site-directed mutagenesis kit followed by cloning into the EcoRI and BamHI sites of p3xFLAG-CMV-7.1. The HAT motif in pTip60 was inactivated by introducing two point mutations, Q377E and G380E, into the acetyl CoA binding domain using the primer pair (forward) 5'-CTCTGCCTCCCTACGAGCGGAGTATGGCAAGCTGC-3' and (reverse) 5'-GCAGCTTGCCATACTCGGGCGCTCGTAGGGAGGCAGAG-3'; these mutations were previously shown to abolish the HAT activity of Tip60 (3). The entire chromodomain in pTip60 was deleted by removing base pairs encoding amino acids 32–69 (LSV...KIQ) of Tip60 using the primer pair (forward) 5'-GCCCCTGGCTGAGATCTTTCCCAAGAAAGGGCGCAAGACATGC-3' and (reverse) 5'-GTAGGTGTCTTGGCTTCTTTCTGGGAAAGATCTCAGCCAGGGC-3'. The zinc finger-like domain was deleted by removing base pairs encoding amino acids 260–285 (LYL...CDL) using the primer pair (forward) 5'-CCACGCTACCCGTCCGCGCACCCTCCAGGC-3' and (reverse) 5'-CATTGCGCTGGAGGTGCCGAGGGGTAGCGTGCG-3'. For all clones, DNA sequencing was performed in both directions to verify mutations and retention of coding frames at insertion and mutated sites. Expression of transfected constructs was verified by the detection of proteins in Western blots. The ANF promoter construct was pGL2-ANF, which contains base pairs 639 to 65 of the rat ANF promoter (13), including the highly conserved proximal SRE required for full activation, cloned into the BglII site of pGL2 (Promega catalog number E1641).

RESULTS

Immunolocalization of Tip60 in the Early Embryonic Heart—The immunostains in Fig. 1 demonstrate the presence of Tip60 proteins during early embryonic heart development in the mouse. Identity of the specific Tip60 isoproteins present at these early stages is unknown, because the antibody used in this determination recognizes the chromodomain in Tip60α, Tip60β, and Tip55. Note that Tip60 proteins were detected in the cytosol and nucleus of myocardial cells (Fig. 1L) as early as embryonic day 8, after which specificity and robustness of expression became pronounced through embryonic day 11 (Fig. 1C). Although the robust expression of Tip60 was selectively detected in the
heart, its expression in all other tissues was above the background. After embryonic day 11, expression in the heart diminished to levels present in other tissues by embryonic day 13.5 (Fig. 1).

Tip60 Isoproteins Bind SRF—Results from Fig. 1 (revealing the robust presence of Tip60 in developing myocardium, plus our results (not shown), as well as those reported by others (5, 10) showing that GALA-tethered Tip60 has little if any intrinsic activity when bound to a heterologous promoter) raised the possibility that Tip60 regulates cardiac gene transcription in cooperation with binding partners. Based on immunoprecipitation experiments to identify candidate cardiac transcription factors that bind endogenous Tip60, it was revealed that Tip60 binds SRF (data not shown). This finding was verified by glutathione S-transferase pull-downs, as recently reported (14), as well as by the determinations shown in Fig. 2 in which HeLa cells were co-transfected with cDNAs encoding HA-SRF and/or pFLAG-Tip60 plasmids as shown plus 25 ng of pRL-TK, 250 ng of pANFLuc, and sufficient pcDNA3 to attain an equivalent mass of transfected plasmid in each dish. As shown in A, the cells were harvested 40 h post-transfection and processed to determine normalized luciferase activity (fold activation). Each bar denotes the mean ± S.E. of nine determinations combined from three separate experiments. Asterisks indicate statistical significance between cells transfected with pHA-SRF alone and cells transfected with SRF plus pFLAG-Tip (*, p < 0.05; **, p < 0.01; ***, p < 0.0001). B shows Western blots using anti-HA or anti-FLAG antibodies to detect protein manufactured by pHA-SRF or pFLAG-Tip, respectively.

ANF Induction by Tip60 and SRF—To investigate whether Tip60 and SRF cooperatively regulate cardiac gene transcription, the cardiac-neutral environment of HeLa cells was selected for transient transfection experiments. The ANF promoter, which is cardiac-selective, was chosen for these experiments, because this promoter is SRF-dependent (15) and is strongly expressed at the earliest stages of heart development (16). As shown in Fig. 3, transfection with SRF plasmid alone activated the ANF promoter nearly 4-fold, consistent with previous findings (15). Co-transfection of SRF with Tip60α (60 kDa), Tip60β (53 kDa), or Tip55 (55 kDa) resulted in co-activation that, although modest for the latter two isoproteins, was more than doubled by Tip60α in dose-dependent fashion. In accord with the inability of Tip60 to bind DNA, none of the Tip60 isoproteins was able to activate ANF when present alone or when co-transfected with mutated SRF (ΔMADS box) that cannot bind DNA (not shown).

HDAC7 Inhibits Tip60α+SRF-induced Co-activation—HDAC7, a transcriptional inhibitor specifically expressed in lung and heart (17), was recently shown to bind the zinc finger-like domain of Tip60 (10), a domain shared by all three Tip60 isoproteins. To assess whether the SRF+Tip60α-induced co-activation of ANF was affected by HDAC7, the determinations shown in Fig. 4 were performed. When HDAC7 at any level was co-transfected with SRF and Tip60α, the increase in ANF activity was reduced to the level induced by SRF alone. Similar experiments in which Tip60α was replaced by Tip60β yielded essentially identical results; in addition, HDAC7 did not affect the activation induced by SRF alone (not shown).

Mutation of the HAT Domain of Tip60 Increases Co-activation—Experiments were performed to address the effect of introducing point mutations in the HAT domains of Tip60α and Tip60β that abrogate the histone acetylase activity of these proteins (3), hypothesizing that this would inhibit their ability to co-activate the ANF promoter with SRF.

FIGURE 3. Augmentation of SRF-induced ANF activity by Tip isoproteins. HeLa cells were transfected 24 h after plating with pHA-SRF and/or pFLAG-Tip plasmids as shown plus 25 ng of pRL-TK, 250 ng of pANFLuc, and sufficient pcDNA3 to attain an equivalent mass of transfected plasmid in each dish. As shown in A, the cells were harvested 40 h post-transfection and processed to determine normalized luciferase activity (fold activation). Each bar denotes the mean ± S.E. of nine determinations combined from three separate experiments. Asterisks indicate statistical significance between cells transfected with pHA-SRF alone and cells transfected with SRF plus pFLAG-Tip (*, p < 0.05; **, p < 0.01; ***, p < 0.0001). B shows Western blots using anti-HA or anti-FLAG antibodies to detect protein manufactured by pHA-SRF or pFLAG-Tip, respectively.
However, as shown in Fig. 5, increasing amounts of either the Tip60/H9251 or Tip60/H9252 HAT or Tip60/H9252/H9004 HAT protein (Fig. 5C) induced significant increases in co-activation in comparison with the effect the respective wild-type counterpart of each protein (Fig. 5A and B).

Removal of the Chromodomain from Tip60/H9252, but Not from Tip60/H9251, Increases Co-activation—Deletion of the 38 amino acids comprising the putatively repressive chromodomain of Tip60/H9252 markedly increased co-activation of the ANF promoter, especially at higher levels of transfected plasmid (Fig. 6A). However, the derepressing effect of chromodomain ablation was specific to Tip60/H9252, because removal of the chromodomain from Tip60/H9251, which unlike Tip60/H9252 contains the 52-amino-acid peptide encoded by exon 5, had no effect (Fig. 6B). These results were observed during independent evaluations of Tip60αΔc and Tip60βΔc and in experiments to verify this phenomenon in which the effects of Tip60αΔc and Tip60βΔc were directly compared.

DISCUSSION

Tip60 Immunolocalization—Results in Fig. 1 indicate that Tip60 protein expression is relatively strong at the earliest stages of heart development, confirming previous results in the chick embryo (12). Although the paucity of protein in hearts at these stages precluded Western blotting, our ongoing immunohistochemical and Western blotting determinations indicate that Tip60 protein is also relatively enriched in adult myocardium. Examination of immunostained embryonic myocardium at high magnification (Fig. 1L) revealed that the strong immunofluorescent signal, which was absent in endocardial cells, was uniformly distributed throughout the nucleus and cytoplasm. In this regard, although previous studies have localized Tip60 proteins to the nucleus (7, 18–20), others have shown that Tip60 may translocate from cytoplasm to nucleus in response to environmental conditions (21, 22). Although a nuclear role for Tip60 is indicated by its transcriptional function, participation in extranuclear signal transduction is suggested by the binding of Tip60 to cell membrane receptors (23), including endothelin receptor A (24) that functions in ANF gene activation, and most recently to amyloid precursor protein (25), which translocates Tip60 to the nucleus. In the former (24), because receptor activation causes migration of the endothelin receptor toward, and a complex containing Tip60 and HDAC7 from, the nucleus, one mechanism by which HDAC7 might regulate Tip60 activity is suggested.
Combinatorial Activity of SRF and Tip60; Tip60 is a Modest Regulator of ANF Transcription

Previously documented Tip60 binding partners range from cell membrane receptors (23–25) to transcriptional regulators including the androgen receptor (5, 6), Fe65 (7), STAT3 and HDAC7 (10), and PITX2 (26). Because all three Tip60 isoproteins we have investigated bind SRF (Fig. 2), we assessed their effects on expression of an SRF-dependent promoter, for which purpose the ANF promoter was selected because of its early expression during cardiogenesis (16). These experiments demonstrated that Tip60, Tip55, and Tip60ΔHAT all significantly augment the activational effect of SRF, the effect of Tip60αΔHAT being the most pronounced. That functional SRF is required for co-activation was evidenced by experiments that SRF, from which the MADS box had been ablated and which prevents binding to DNA as well as to Tip60 (14), could not activate ANF (not shown).

It is well established that SRF co-activates cardiac genes including ANF, cardiac α-actin, and Sm22α with transcription factors, including myocardin, GATA4–6, or Nkx2.5. Activation induced by myocardin (27) is extraordinarily dramatic in comparison with the modest activation induced by the other transcription factors (28, 29). The extent of activation induced by Tip60 isoproteins is similar to that of GATA4–6 and Nkx2.5, especially when the chromodomain is deleted from Tip60αΔHAT. Moreover the induction of ANF activation by SRF/Tip60 is similar to the extent of induction or repression of other promoters by Tip60 when partner-bound to human immunodeficiency virus-Tat (1), androgen receptor (5, 6), cAMP-response element-binding protein (8), zinc-finger E box binding protein (9), or STAT3 (10).

Chromodomain Mutation—The chromodomain (chromatin organization modifier domain) is a conserved motif originally described in Drosophila.
ila protein polycomb and heterochromatin-associated protein 1 (HP1). Proteins containing chromodomains are associated with complexes that mediate heterochromatin formation and function as transcriptional repressors (11). Repression induced by polycomb or HP1 is mediated via binding of the chromodomains of these proteins to specific methylated lysines in histone H3. Alignment of the HP1 and Tip60 chromodomains predicts that the aromatic residues (Trp-26, Tyr-47, Phe-50) of Tip60 should interact with methylated lysine 9 in histone H3 to induce repression (30). Such a prediction is consistent with the activation (i.e. derepressive) effect resulting from ablation of the chromodomain of Tip60β (Fig. 6A). However, the finding that chromodomain deletion did not affect Tip60α (Fig. 6B) is difficult to explain. It is speculated that derepression is prevented by the 52-amino-acid peptide encoded by exon 5, the only currently known function of which is to mediate Tip60α binding to a cell membrane receptor (23) via recruitment of inhibitory factors that counteract the derepressive effect of chromodomain deletion.
HAT Domain Mutation—Acetylation of histones via HAT-containing proteins, such as GCN5, p300/CREB-binding protein is usually associated with transcriptional activation. Although the finding in Fig. 5 that mutation of the Tip60 HAT domain activated rather than repressed ANF promoter activity was unexpected, similar point mutation of the HAT domain of p300 has been observed to not inhibit (and perhaps increase) its ability to coactivate the siamois promoter with β-catenin (31). This result suggests that the atypical HAT domain of Tip60 may not regulate transcription via histone modification. For example, it is possible that Tip60 acetylates non-histone proteins to regulate transcription, as recently shown by its obligatory acetylation of the androgen receptor during activation of the prostate-specific antigen promoter (6). The findings of Fig. 5 are interpreted to mean that acetylation of an unknown non-histone protein(s) by Tip60α/β (perhaps Tip60 itself) causes repression of ANF transcription. Such a possibility is consistent with the finding that transcriptional activity of activator of thyroid and retinoic acid receptor (ACTR) is inhibited by autoacetylation of lysine residues adjacent to its LKRRLL motif (32). Tip60 can also acetylate itself (2), although neither the identity of the target residues nor effects on transcription were described. Whether or not Tip60 regulates transcriptional activity via autoacetylation (perhaps of residues within or adjacent to its LKRRLL motif) is under investigation. Because the results reported here indicate that the Tip60 HAT domain functions as a repressor rather than an activator in this context, the task of identifying the activating domain of Tip60 is also being addressed.

Inhibition of Co-activation by HDAC7—The ability of HDAC7 to repress SRF+Tip60-induced co-activation of the ANF promoter shown in Fig. 4 is nearly identical to its previously reported ability to inhibit Tip60-augmented activation of a STAT3-inducible promoter (10). HDAC7, which is expressed in heart-selective fashion (17), binds Tip60 via the zinc finger domain of the latter (10). Our preliminary observation that HDAC7 inhibition is significantly reduced when co-transfected with a mutant form of Tip60α without the zinc-finger domain is in accord with the latter findings (10). Moreover, in other experiments, we have observed that the concentration of nuclear Tip60α is reduced as increasing levels of HDAC7 are transfected into the cell, suggesting a mechanism of HDAC7 regulation in accord with the findings described above (24). Taken together with the finding that HDAC7 does not inhibit ANF activation attributed to SRF alone, it is concluded that the inhibitory effect of HDAC7 is Tip60-specific.

The repressive effect of HDAC7 may be mediated via the histone deacetylase domain in its C terminus and/or via repressor domains in its N terminus (17). Presuming that transcription of the transiently transfected ANF promoter occurs in a non-nucleosomal environment, the favored interpretation is that the N-terminal repressor domains of HDAC7 inhibit ANF transcription. Such an interpretation is compatible with the ability of the N-terminal domain HDAC7 to inhibit transcription induced by another cardiac MADS box transcription factor, MEF2c (30).

All experiments performed to date to elucidate the function of Tip60, including those reported here, have utilized transient transfection of transformed cell lines, indicating that its activity is either co-activational (1, 5–7, this paper) or co-repressive (8–10), depending on context. It is interesting to consider that HDAC7, which is selectively expressed in adult tissues (17), regulates the activational/repressive activity of Tip60 in a tissue-dependent fashion. In this regard, our current experiments addressing Tip60 regulation of the endogenous ANF promoter function in endothelin-induced primary neonatal cardiac myocytes (which express HDAC7) should be revealing.

REFERENCES

**Protein kinase G regulates potassium chloride cotransporter-4 expression in primary cultures of rat vascular smooth muscle cells.**

Mauricio Di Fulvio, Thomas M. Lincoln, Peter K. Lauf, and Norma C. Adragna

The oligonucleotides used in this paper for KCC3 mRNA amplification in vascular smooth muscle cells (VSMCs) were designed based on sequences reported in Mount et al. (1). Because the KCC3 sequence described by Mount et al. in fact corresponds to KCC4 (see Ref. 1 below), the title of our paper (shown corrected above) as well as figure labels and their legends making reference to KCC3 mRNA expression or regulation in VSMCs should be changed to refer to KCC4 (potassium chloride cotransporter-4). Since, independently, we have shown the presence of KCC3a and KCC3b isoforms and their regulation by the NO/sGC/PKG/cGMP signaling cascade (2), this correction does not change the overall interpretation of the data, except that we now conclude that KCC3a, KCC3b, and KCC4 mRNAs are subjected to regulation by the signal transduction pathways originally proposed by us (2, 3).

**REFERENCES**


**The fasting-induced adipose factor/angiopoietin-like protein 4 is physically associated with lipoproteins and governs plasma lipid levels and adiposity.**

Stéphane Mandard, Fokko Zandbergen, Esther van Straten, Walter Wahl, Folkert Kuipers, Michael Müller, and Sander Kersten

PAGE 937:

In Fig. 4D, the labels above the lanes were inadvertently switched. The left lanes are the wild-type mice, and the lanes on the right are FIAF-Tg mice.

**Triptolide, an inhibitor of the human heat shock response that enhances stress-induced cell death.**

Sandy D. Westerheide, Tiara L. A. Kawahara, Kai Orton, and Richard I. Morimoto

PAGE 9620:

The labeling at the top of Fig. 5 was inadvertently duplicated from Fig. 4. The correctly labeled Fig. 5 is shown below.
Nonvisual arrestin oligomerization and cellular localization are regulated by inositol hexakisphosphate binding.
Shawn K. Milano, You-Me Kim, Frank P. Stefano, Jeffrey L. Benovic, and Charles Brenner

In the legend to Fig. 1, the latter part of the second to last sentence should read: “... where the negative and positive potential were set to $-5$ and $+5kT$ (where $k$ is the Boltzmann constant and $T$ is the absolute temperature).”

Co-activation of atrial natriuretic factor promoter by Tip60 and serum response factor.
Min-Su Kim, Xanthi Merlo, Catherine Wilson, and John Lough

Fig. 6C: The proteins recognized by the anti-FLAG antibody in the upper part of panel C were mislabeled. The left side should read “Tip60$\beta c$,” and the right side should read “Tip60$\gamma c$.”