Extended Abstract

Genome-wide analysis of enhancer-RNA transcription reveals regulatory mechanisms by an anti-diabetic drug in adipocyte

Hee-Woong Lim[1,2*], Sonia E. Step[1,3,4,5,*], Jill M. Marinis[1,3,4], Andreas Prokesch[1,3,4,6], David J. Steger[1,3,4], Seo-Hee You[1,3,4], and Mitchell A. Lazar[1,2,3,4,5], Kyoung-Jae Won[1,2,†]

[1]The Institute for Diabetes, Obesity, and Metabolism, [2]Department of Genetics, [3]Division of Endocrinology, Diabetes, and Metabolism, [4]Department of Medicine, [5]Department of Pharmacology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA

[6]Institute for Genomics and Bioinformatics, Graz University of Technology, Graz 8010, Austria

SUMMARY

Enhancer RNA (eRNA) is an excellent marker that shows enhancer activities, which strongly correlate with their target gene transcription levels. Therefore, investigating the eRNA levels enables us to interrogate activating/repressive gene regulatory logic. We applied this strategy to investigate gene regulatory mechanisms of an anti-diabetic drug, Rosiglitazone (rosi). Rosi is a powerful insulin sensitizer, but serious toxicities have curtailed its widespread clinical use. Rosi functions as a high-affinity ligand for PPARγ, the adipocyte-predominant nuclear receptor (NR). The classic model, involving binding of ligand to the NR on DNA, explains positive regulation of gene expression, but ligand-dependent repression is not well understood. Here, we addressed this issue using global run-on sequencing (GRO-seq). Rosi-induced gene transcriptional changes occurred within 10 minutes and correlated with steady-state mRNA levels as well as with transcription at nearby enhancers (eRNAs) but with a time delay. Upregulated eRNAs occurred almost exclusively at PPARγ binding sites, to which rosi treatment recruited the coactivator MED1. By contrast, transcriptional repression by rosi involved a loss of MED1 from eRNA sites devoid of PPARγ and enriched for other TFs including AP-1 factors and C/EBPs. Thus, rosi activates and represses transcription by fundamentally different mechanisms that could inform the future development of anti-diabetic drugs.

1 INTRODUCTION

Peroxisome proliferator activated receptor gamma (PPARγ) is a nuclear receptor (NR) that is dramatically induced during adipogenesis and expressed predominantly in adipose tissue (Chawla and Lazar, 1994; Tontonoz et al., 1994a). It is necessary (Rosen et al., 1999) and sufficient (Tontonoz et al., 1994b) for adipogenesis, and also critical for the functions of mature adipocytes, including lipid metabolism, adipokine secretion, and insulin sensitivity (Rangwala and Lazar, 2004). PPARγ binds near most adipogenic genes as a heterodimer with retinoid X receptor (RXR) (Lefterova et al., 2008; Nielsen et al., 2008).

PPARγ, like most NRs, is a ligand-dependent transcription factor (Glass and Rosenfeld, 2000). High affinity ligands for PPARγ include the thiazolidinediones (TZDs) (Lehmann et al., 1995), which are insulin-sensitizing drugs (Nolan et al., 1994). TZDs contribute to insulin sensitization by acting on adipose tissue to regulate gene transcription both positively and negatively. The most potent TZD in the clinic is rosiglitazone (rosi) (Lehmann et al., 1995), which has durable anti-diabetic effects but, unfortunately, has toxicities that limit its widespread use (Ahmadian et al., 2013; Kung and Henry, 2012). Because PPARγ expression in adipose tissue is required for the *in vivo* systemic insulin sensitizing effects of TZDs (Chao et al., 2000; He et al., 2003), it is critical to understand how rosi binding to PPARγ modulates gene expression.

Binding of rosi to PPARγ results in recruitment of coactivators, including SRC-1, CBP, p300, and MED1, that function to induce gene expression (Bugge et al., 2009; Ge et al., 2002; Gelman et al., 1999; Westin et al., 1998). However, the mechanism by which rosi represses transcription is not well understood. Ligand binding to other NRs has been suggested to repress gene expression by recruiting limiting coactivators away from other transcription factors (TFs), thereby repressing those target genes. Support for this coactivator competition model, often referred to as squelching, has been largely based on TF overexpression experiments in transfection systems (Fronsdal et al., 1998; He et al., 2012; Kamei et al., 1996; Kim et al., 2001; Lee et al., 2000; Li et al., 2000; Manna and Stocco, 2007), although a recent study demonstrated coactivator redistribution with endogenous factors and chromatin on a genome-wide scale in the context of E2-treated MCF-7 cells (He et al., 2012).

Many studies have used transcriptome analysis to infer the effects of rosi on steady state gene expression in adipocytes (Choi et al., 2010; Li and Lazar, 2002; Rong et al., 2011; Sears et al., 2007). However, steady state mRNAs levels are determined both by their rates of transcription and degradation. Here, for the first time, we have directly measured rates of adipocyte transcription genome-wide, using global run-on followed by sequencing (GRO-seq) (Core et al., 2008).

This is an extended abstract for RegGenSIG2014, and a full paper is currently in press in *Genes and Development*.

*These authors contributed equally to this work.

†To whom correspondence should be addressed. (wonk@mail.med.upenn.edu)

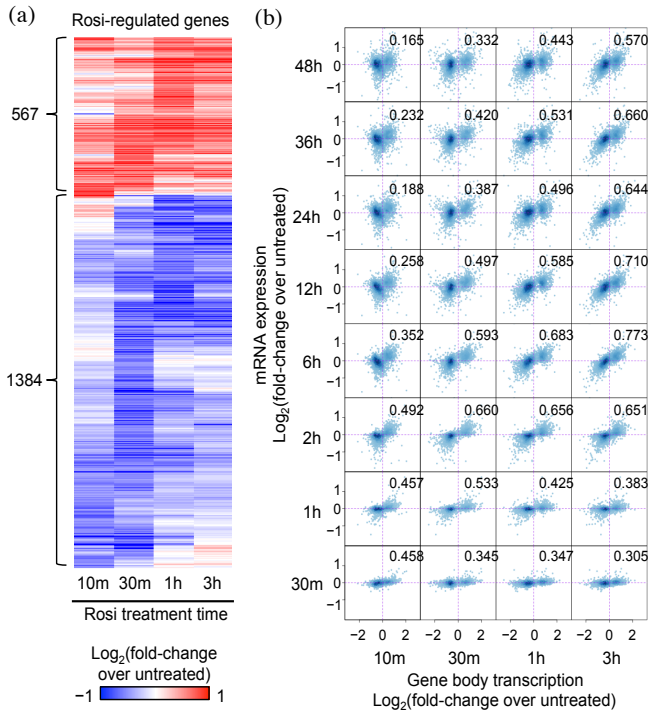

Figure 1. Gene transcriptional regulation upon rosi-treatment and their correlation with steady-state mRNA level. (a) Changes of nascent transcript level in gene body. (b) Correlation between gene transcriptional regulation and steady-state mRNA level changes from microarray in various time points.

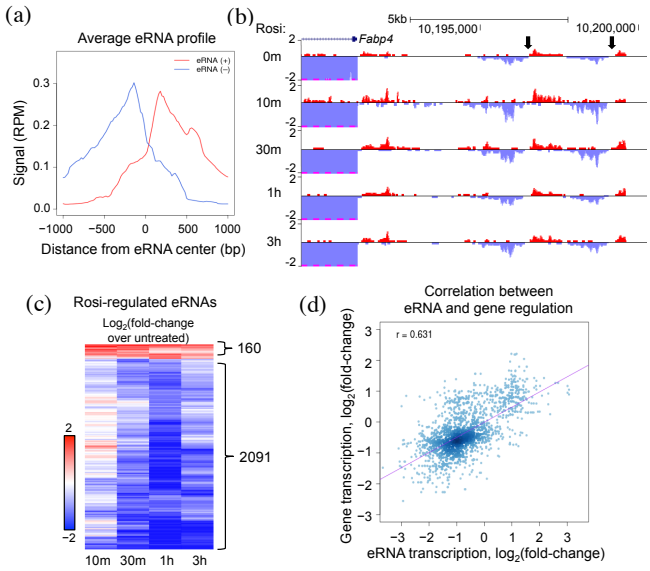

Figure 2. eRNA regulation. (a) Genome-wide average signal of eRNA in adipocytes from the plus and minus strands. (b) Example of two eRNAs in the upstream of Fabp4, which is upregulated upon rosi-treatment. (c) Clustering heatmap of all rosi-regulated eRNAs (Up=160, Down=2091). (d) Strong positive correlation between rosi-regulated eRNAs and the regulation of the nearby genes within 100kb TSS.

2 RESULTS

2.1 Gene regulation

We found that rosi rapidly up- or downregulates the transcription of thousands of adipocyte genes within 10 minute. Overall, 1,951 annotated RefSeq genes were transcriptionally regulated by rosi at one or more of the time points tested s (**Figure 1a**). Interestingly, 71% of regulated nascent transcripts were repressed whereas only 29% were activated by rosi. To assess the temporal and gene-specific relationship between nascent gene transcription and steady-state mRNA levels, we determined the adipocyte transcriptome using gene expression microarrays after 30 min, 1h, 2h, 6h, 12h, 24h, 36h, and 48h of rosi treatment, with 3 biological replicates at each time point. While the nascent transcription of many genes was regulated as early as 10 and 30 minutes after rosi, very few mRNA transcripts changed during this time period and for at least 2 hours after rosi treatment (**Figure 1**b). This was not surprising, as the time required to reach new steady state levels is related to the rate of degradation rather than the rate of synthesis (Schimke and Doyle, 1970). However, the correlation between nascent transcription and steady-state mRNA levels was high at later time points, with the greatest correlation noted between the transcription regulation at 3h and the mRNA regulation measured 6h after rosi. The lower correlation with later microarray time points suggests that steady-state regulation at these later times may be dependent on secondary transcriptional changes occurring later than 3h of treatment. Importantly, direct transcriptional regulation by rosi in this model was highly similar to recently published regulation of *in vivo* adipose tissue gene expression in rosi-treated mice, suggesting that many of the rosi-dependent changes we describe are physiologically relevant.

2.2 Enhancer regulation

In addition to gene body transcription, GRO-seq revealed robust bidirectional transcripts at enhancers, or eRNAs (Core et al., 2008; Kim et al., 2010), which were identified and quantified in an unbiased, genome-wide analysis (**Figure 2a**). For example, bidirectional eRNAs were identified at enhancers upstream of the *Fabp4* locus, and their transcription was observed to be upregulated by rosi (**Figure 2b**). Indeed, unbiased de novo calling of bidirectional intergenic transcripts confirmed that the transcription of many eRNAs was strongly and rapidly regulated by rosi, and downregulated eRNAs greatly outnumbered upregulated eRNAs (**Figure 2c**). This was similar to the effect of rosi on gene body transcription, and the effect of rosi on eRNA transcription correlated strongly with transcription at the nearby gene bodies (**Figure 2d**). This correlation was confirmed at eRNA/gene body pairs by qPCR, which also demonstrated that eRNA induction often preceded gene induction. The correlation was validated at repressed eRNA/gene pairs as well. Though intragenic eRNAs were excluded from downstream analysis because of difficulties in identifying them reliably, we observed that many follow a similar pattern of correlation with the target.

2.3 Differential TF enrichment

De novo motif analysis at sites of rosi-induced eRNAs revealed strong enrichment for a sequence that is highly simi-

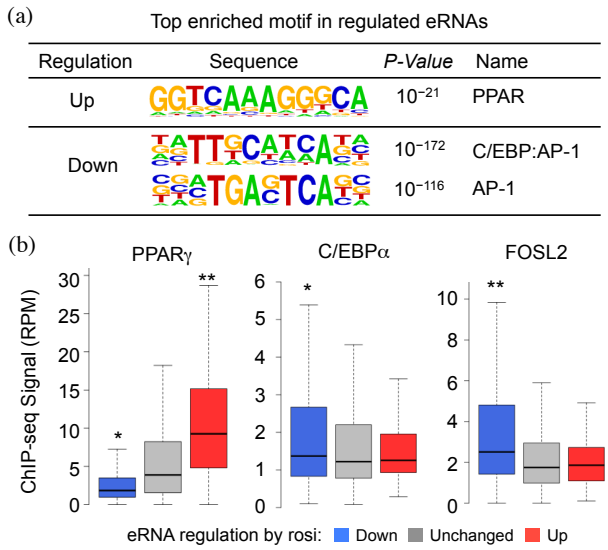

Figure 3. Differential enrichment of TF between rosi-regulated eRNAs. (a) PPAR motif is enriched in upregulated eRNA and CEBP and AP-1 motif are enriched in downregulated ones. (b) ChIP-seq data of PPARγ, CEBPα, and FOSL2 confirms differential TF binding between up/downregulated eRNAs.

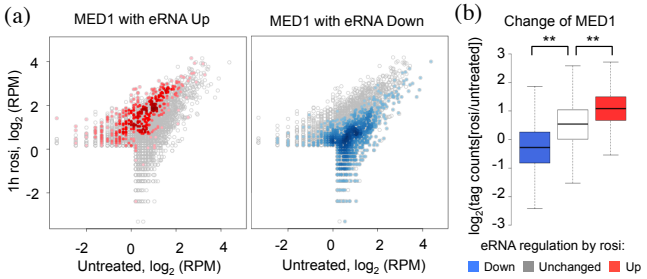

Figure 4. eRNA regulation by MED1 squelching. (a) MED1 ChIP-seq data before and after rosi-treatment shows redistribution of MED1 binding from downregulated eRNAs toward upregulated ones. (b) Quantitative comparison of MED1 occupancy changes among up, unchanged, and downregulated eRNAs.

lar to the canonical PPARγ/RXR binding (**Figure 4a**), and indeed PPARγ ChIP-seq showed strong at upregulated eRNAs but not at downregulated ones (**Figure 4b**).

In contrast to the upregulation, the PPARγ motif were not enriched at repressed eRNAs. Instead, *de novo* motif analysis revealed two highly enriched motifs that most resembled a C/EBP:AP-1 hybrid motif and the canonical AP-1 motif (**Figure 4a**). ChIP-seq data showed higher enrichment of C/EBP and AP-1 at downregulated eRNAs (**Figure 4b**), which has been shown to play a role in adipocyte gene expression Other factors tested, including C/EBPβ, ATF2 and JUND, also showed enrichment at downregulated eRNAs, and the strength of this binding did not change upon rosi treatment (Data not shown). The finding that both C/EBP factors and all three AP-1 factors are enriched at downregulated sites suggests that each of these factors, potentially along with other TFs but notably not PPARγ, is located at eRNAs in *cis* with gene bodies whose transcription is negatively regulated by rosi.

2.4 Coactivator squelching

The majority of eRNA upregulation occurred by 10 minutes after addition of rosi, whereas the number of downregulated eRNAs was markedly fewer at that time, and peaked at 1h. Given the direct nature of rosi regulation of the upregulated eRNAs via PPARγ, we hypothesized that the delayed downregulation could be related to coactivator redistribution to the rosi-bound PPARγ. ChIP-seq for the general coactivator MED1 was performed in the presence and absence of rosi. We then determined the sites of MED1 occupancy that contained eRNAs, and whether these eRNAs were up- or downregulated by rosi. As expected, MED1 recruitment to sites of PPARγ binding at upregulated eRNAs was increased by rosi (**Figure 4a**). Remarkably, 1h of rosi treatment decreased MED1 recruitment at sites of downregulated eRNA transcription despite the general absence of PPARγ at these sites (**Figure 4a**). These results were confirmed at several representative sites of up- and downregulated eRNAs by MED1 ChIP-qPCR (Data not shown). On average, upon rosi treatment MED1 recruitment significantly decreased at downregulated eRNAs and increased at upregulated eRNAs relative to unregulated eRNAs (**Figure 4b**). Since PPARγ was markedly enriched at sites of upregulated eRNAs, these data together suggest that rosi binding to PPARγ redistributed MED1 binding from the sites of downregulated eRNAs to the sites of upregulated eRNAs. Thus, binding of rosi to PPARγ directly activated eRNA transcription at upregulated sites, and the resultant redistribution of coactivator led to downregulation of eRNA transcription mediated by other TFs at sites not bound by PPARγ.

3 CONCLUSION

Our study of nascent transcription has revealed enhancers and up- and down-regulated by rosi. We identified PPARγ is responsible for up-regulation. More importantly, systematic analysis using eRNAs identified squelching is a major reason for down-regulation of eRNA and subsequent gene transcription. The changes in MED1 occupancy further supported identified that redistribution of mediator (MED1) can be the cause of squelching. We believe this is the first genome-wide study to reveal the squelching mechanism associated with rosi. Our systematic analysis using GRO-seq provided key regulatory mechanisms of rosi by showing up- and down-regulated eRNAs and the associated TFs and co-factors. Our study is greatly useful in developing efficacious but low-risk drugs for diabetes.

ACKNOWLEDGEMENTS

A.P. was supported by a GEN-AU mobility grant from the Austrian Ministry of Science and Research. This work was also supported by National Institutes of Health grants R01 DK49780 (to M.A.L.), R21DK098769 (to K.-J.W.), and 5T32GM008076 (to S.E.S.) and the JPB Foundation.

REFERENCES

Ahmadian, M., Suh, J.M., Hah, N., Liddle, C., Atkins, A.R., Downes, M., and Evans, R.M. (2013). PPARγ signaling and metabolism: the good, the bad and the future. Nat Med *19*, 557-566.

Bugge, A., Grontved, L., Aagaard, M.M., Borup, R., and Mandrup, S. (2009). The PPARgamma2 A/B-domain plays a gene-specific role in transactivation and cofactor recruitment. Molecular endocrinology *23*, 794-808.

Chao, L., Marcus-Samuels, B., Mason, M.M., Moitra, J., Vinson, C., Arioglu, E., Gavrilova, O., and Reitman, M.L. (2000). Adipose tissue is required for the antidiabetic, but not for the hypolipidemic, effect of thiazolidinediones. The Journal of clinical investigation *106*, 1221-1228.

Chawla, A., and Lazar, M.A. (1994). Peroxisome proliferator and retinoid signaling pathways co-regulate preadipocyte phenotype and survival. Proceedings of the National Academy of Sciences of the United States of America *91*, 1786-1790.

Choi, J.H., Banks, A.S., Estall, J.L., Kajimura, S., Bostrom, P., Laznik, D., Ruas, J.L., Chalmers, M.J., Kamenecka, T.M., Bluher, M.*, et al.* (2010). Anti-diabetic drugs inhibit obesity-linked phosphorylation of PPARgamma by Cdk5. Nature *466*, 451-456.

Core, L.J., Waterfall, J.J., and Lis, J.T. (2008). Nascent RNA sequencing reveals widespread pausing and divergent initiation at human promoters. Science *322*, 1845-1848.

Fronsdal, K., Engedal, N., Slagsvold, T., and Saatcioglu, F. (1998). CREB binding protein is a coactivator for the androgen receptor and mediates cross-talk with AP-1. The Journal of biological chemistry *273*, 31853-31859.

Ge, K., Guermah, M., Yuan, C.X., Ito, M., Wallberg, A.E., Spiegelman, B.M., and Roeder, R.G. (2002). Transcription coactivator TRAP220 is required for PPAR gamma 2-stimulated adipogenesis. Nature *417*, 563-567.

Gelman, L., Zhou, G., Fajas, L., Raspe, E., Fruchart, J.C., and Auwerx, J. (1999). p300 interacts with the N- and C-terminal part of PPARgamma2 in a ligand-independent and -dependent manner, respectively. The Journal of biological chemistry *274*, 7681-7688.

Glass, C.K., and Rosenfeld, M.G. (2000). The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev *14*, 121-141.

He, H.H., Meyer, C.A., Chen, M.W., Jordan, V.C., Brown, M., and Liu, X.S. (2012). Differential DNase I hypersensitivity reveals factor-dependent chromatin dynamics. Genome research *22*, 1015-1025.

He, W., Barak, Y., Hevener, A., Olson, P., Liao, D., Le, J., Nelson, M., Ong, E., Olefsky, J.M., and Evans, R.M. (2003). Adipose-specific peroxisome proliferator-activated receptor gamma knockout causes insulin resistance in fat and liver but not in muscle. Proceedings of the National Academy of Sciences of the United States of America *100*, 15712-15717.

Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S.C., Heyman, R.A., Rose, D.W., Glass, C.K.*, et al.* (1996). A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. Cell *85*, 403-414.

Kim, S.W., Kim, H.J., Jung, D.J., Lee, S.K., Kim, Y.S., Kim, J.H., Kim, T.S., and Lee, J.W. (2001). Retinoid-dependent antagonism of serum response factor transactivation mediated by transcriptional coactivator proteins. Oncogene *20*, 6638-6642.

Kim, T.K., Hemberg, M., Gray, J.M., Costa, A.M., Bear, D.M., Wu, J., Harmin, D.A., Laptewicz, M., Barbara-Haley, K., Kuersten, S.*, et al.* (2010). Widespread transcription at neuronal activity-regulated enhancers. Nature *465*, 182-187.

Kung, J., and Henry, R.R. (2012). Thiazolidinedione safety. Expert Opin Drug Saf *11*, 565-579.

Lee, Y.K., Dell, H., Dowhan, D.H., Hadzopoulou-Cladaras, M., and Moore, D.D. (2000). The orphan nuclear receptor SHP inhibits hepatocyte nuclear factor 4 and retinoid X receptor transactivation: two mechanisms for repression. Molecular and cellular biology *20*, 187-195.

Lefterova, M.I., Zhang, Y., Steger, D.J., Schupp, M., Schug, J., Cristancho, A., Feng, D., Zhuo, D., Stoeckert, C.J., Jr., Liu, X.S.*, et al.* (2008). PPARgamma and C/EBP factors orchestrate adipocyte biology via adjacent binding on a genome-wide scale. Genes & development *22*, 2941-2952.

Lehmann, J.M., Moore, L.B., Smith-Oliver, T.A., Wilkison, W.O., Willson, T.M., and Kliewer, S.A. (1995). An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). The Journal of biological chemistry *270*, 12953-12956.

Li, M., Pascual, G., and Glass, C.K. (2000). Peroxisome proliferator-activated receptor gamma-dependent repression of the inducible nitric oxide synthase gene. Molecular and cellular biology *20*, 4699-4707.

Li, Y., and Lazar, M.A. (2002). Differential gene regulation by PPARgamma agonist and constitutively active PPARgamma2. Molecular endocrinology *16*, 1040-1048.

Manna, P.R., and Stocco, D.M. (2007). Crosstalk of CREB and Fos/Jun on a single cis-element: transcriptional repression of the steroidogenic acute regulatory protein gene. Journal of molecular endocrinology *39*, 261-277.

Nielsen, R., Pedersen, T.A., Hagenbeek, D., Moulos, P., Siersbaek, R., Megens, E., Denissov, S., Borgesen, M., Francoijs, K.J., Mandrup, S.*, et al.* (2008). Genome-wide profiling of PPARgamma:RXR and RNA polymerase II occupancy reveals temporal activation of distinct metabolic pathways and changes in RXR dimer composition during adipogenesis. Genes & development *22*, 2953-2967.

Nolan, J.J., Ludvik, B., Beerdsen, P., Joyce, M., and Olefsky, J. (1994). Improvement in glucose tolerance and insulin resistance in obese subjects treated with troglitazone. The New England journal of medicine *331*, 1188-1193.

Rangwala, S.M., and Lazar, M.A. (2004). Peroxisome proliferator-activated receptor gamma in diabetes and metabolism. Trends in pharmacological sciences *25*, 331-336.

Rong, J.X., Klein, J.L., Qiu, Y., Xie, M., Johnson, J.H., Waters, K.M., Zhang, V., Kashatus, J.A., Remlinger, K.S., Bing, N.*, et al.* (2011). Rosiglitazone Induces Mitochondrial Biogenesis in Differentiated Murine 3T3-L1 and C3H/10T1/2 Adipocytes. PPAR research *2011*, 179454.

Rosen, E.D., Sarraf, P., Troy, A.E., Bradwin, G., Moore, K., Milstone, D.S., Spiegelman, B.M., and Mortensen, R.M. (1999). PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. Molecular cell *4*, 611-617.

Schimke, R.T., and Doyle, D. (1970). Control of enzyme levels in animal tissues. Annual review of biochemistry *39*, 929-976.

Sears, D.D., Hsiao, A., Ofrecio, J.M., Chapman, J., He, W., and Olefsky, J.M. (2007). Selective modulation of promoter recruitment and transcriptional activity of PPARgamma. Biochemical and biophysical research communications *364*, 515-521.

Tontonoz, P., Hu, E., Graves, R.A., Budavari, A.I., and Spiegelman, B.M. (1994a). mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. Genes & development *8*, 1224-1234.

Tontonoz, P., Hu, E., and Spiegelman, B.M. (1994b). Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. Cell *79*, 1147-1156.

Westin, S., Kurokawa, R., Nolte, R.T., Wisely, G.B., McInerney, E.M., Rose, D.W., Milburn, M.V., Rosenfeld, M.G., and Glass, C.K. (1998). Interactions controlling the assembly of nuclear-receptor heterodimers and co-activators. Nature *395*, 199-202.