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Induction of interferon-stimulated genes by Simian virus 40 T antigens

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ABSTRACT

Simian virus 40 (SV40) large T antigen (TAg) is a multifunctional oncoprotein essential for productive viral infection and for cellular transformation. We have used microarray analysis to examine the global changes in cellular gene expression induced by wild-type T antigen (TAg^{wt}) and TAg-mutants in mouse embryo fibroblasts (MEFs). The expression profile of approximately 800 cellular genes was altered by TAg^{wt} and a truncated TAg (TAg^{N136}), including many genes that influence cell cycle, DNA-replication, transcription, chromatin structure and DNA repair. Unexpectedly, we found a significant number of immune response genes upregulated by TAg^{wt} including many interferon-stimulated genes (ISGs) such as ISG56, OAS, Rsad2, Ifi27 and Mx1. Additionally, we also observed activation of STAT1 by TAg^{wt}. Our genetic studies using several TAg-mutants reveal an unexplored function of TAg and indicate that the LXCXE motif and p53 binding are required for the upregulation of ISGs.

Introduction

Simian virus 40 (SV40) is a small DNA virus that under permissive conditions replicates in cells leading to the propagation of the viral progeny. However, under nonpermissive conditions it is oncogenic in animals and capable of inducing transformation of multiple cell lines in cell culture systems. To perform these functions SV40 encodes two major transforming proteins, the large T antigen (T antigen) and the small t antigen (t antigen). The small t antigen targets the cellular protein phosphatase PP2A and is required for transformation under some conditions. On the other hand, the expression of large T antigen alone is sufficient for SV40's oncogenic potential in most cases (Ahuja et al., 2005).

The large T antigen (TAg) is a 708 amino acid protein that contains several independently folding domains/regions. These domains/regions interact with host cellular proteins and thus provide an excellent tool to understand the different cellular processes affected by viral infection. Three regions of TAg are essential to elicit cellular transformation (Ahuja et al., 2005; Ali and DeCaprio, 2001). The LXCXE motif and the J domain reside in the N-terminus of TAg, and are involved in the inactivation of Rb family functions. The LXCXE motif mediates binding to the Rb family members (pRb, p107 and p130) (DeCaprio et al., 1988). The J domain, via binding to the chaperone hsc70, stimulates the ATPase activity of hsc70, and the resulting energy is used to release E2F from Rb (Sullivan et al., 2001). This release leads to the upregulation of E2F transactivation activity and subsequent progression of cells into S phase. The C-terminus of TAg is essential for inactivation of the tumor suppressor p53 (Kierstead and Tevethia, 1993; Pipas and Levine, 2001), as binding of p53 by TAg prevents its degradation by Mdm2 and results in p53 stabilization. Therefore, high levels of p53 are found in TAg transformed cells (Oren et al., 1981).

Infection of cells by viruses often results in a potent and dramatic shift in the transcriptional activity of host cellular genes. These changes reflect the strategies developed by both host and pathogen to facilitate their own survival. For example, many viruses induce the expression of host cell genes involved in DNA-replication and cell cycle to enhance its own replication. On the other hand, the induction of interferon (IFN) and IFN-stimulated genes (ISGs) is the hallmark of the host response to create an antiviral state. As a countermeasure to this response, many viruses encode proteins that interfere with the IFN induction as well as the products of the ISGs (Randall and Goodbourn, 2008). An understanding of the host-pathogen relationship requires the study of complex biological processes which result in altered global gene expression.

Previously we used a mouse cDNA array consisting of ~8000 genes to compare gene expression induced by wild-type TAg in different systems (Cantalupo et al., 2009). Moreover, we have also reported differential gene expression induced by wild-type TAg and mutants in the mouse intestine (Rathi et al., 2009). These studies demonstrated that wild-type TAg regulates a large number of known E2F-responsive genes in both systems. Our studies in intestine showed that these genes require the LXCXE motif and J domain to be regulated by TAg (Rathi et al., 2009; Rathi et al., 2007). In addition, we found sets of genes that were uniquely regulated by TAg in one system but not in the other. For example, TAg downregulates several

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components of the cytochrome P450 pathway in intestine, but this pathway is not altered by TAg in mouse embryo fibroblasts (MEFs) (Cantalupo et al., 2009; Saenz-Robles et al., 2007b). Similarly, we noted that some immune response genes were regulated by TAg in MEFs, but not in intestine (Cantalupo et al., 2009). However, the limited gene coverage of the array used in these studies coupled with a lack of accompanying genetics prevented us from discerning if those genes were independently regulated or if they were responding to the activation of an immune response pathway. In the current study we examine TAg's effects on gene expression in MEFs, a well characterized cell culture system. In this case, we have used mouse whole genome arrays to examine global changes in cellular gene expression in MEFs induced by the presence of wild-type T antigen (TAg^{wt}) and TAg-mutants (Fig. 1A). The mutants used for this study are TAg^{N136} (expresses the first 136 amino acids of T antigen), TAg^{3213} (mutation in the LXCXE motif) and TAg^{D44N} (mutation in the J domain). The transformation potential of these mutants has been studied in several different systems. In particular, TAg^{N136} is unable to transform MEFs while TAg^{3213} and TAg^{D44N} show reduced efficiency in transformation (Hahn et al., 2002; Markovics et al., 2005; Stubdal et al., 1997; Thompson et al., 1990). The current study reveals an unexplored function of TAg in regulation of immune response genes.

Results

Global patterns of cellular gene expression in MEFs expressing wild-type or mutant T antigens

We analyzed the RNA profile from two day post-confluent MEFs stably expressing wild-type or mutant T antigens. Protein levels of the wild-type or mutant TAgs in MEFs were assessed by western blot analysis (data not shown) and only those clones were selected for the study which were expressing similar levels of protein. The Affymetrix mouse whole genome chip, which consists of 21,635 unique genes, was used for microarray analysis. Genes showing three or more than threefold upregulation or downregulation in comparison to normal MEFs were selected for gross analysis of gene expression (Fig. 1B).

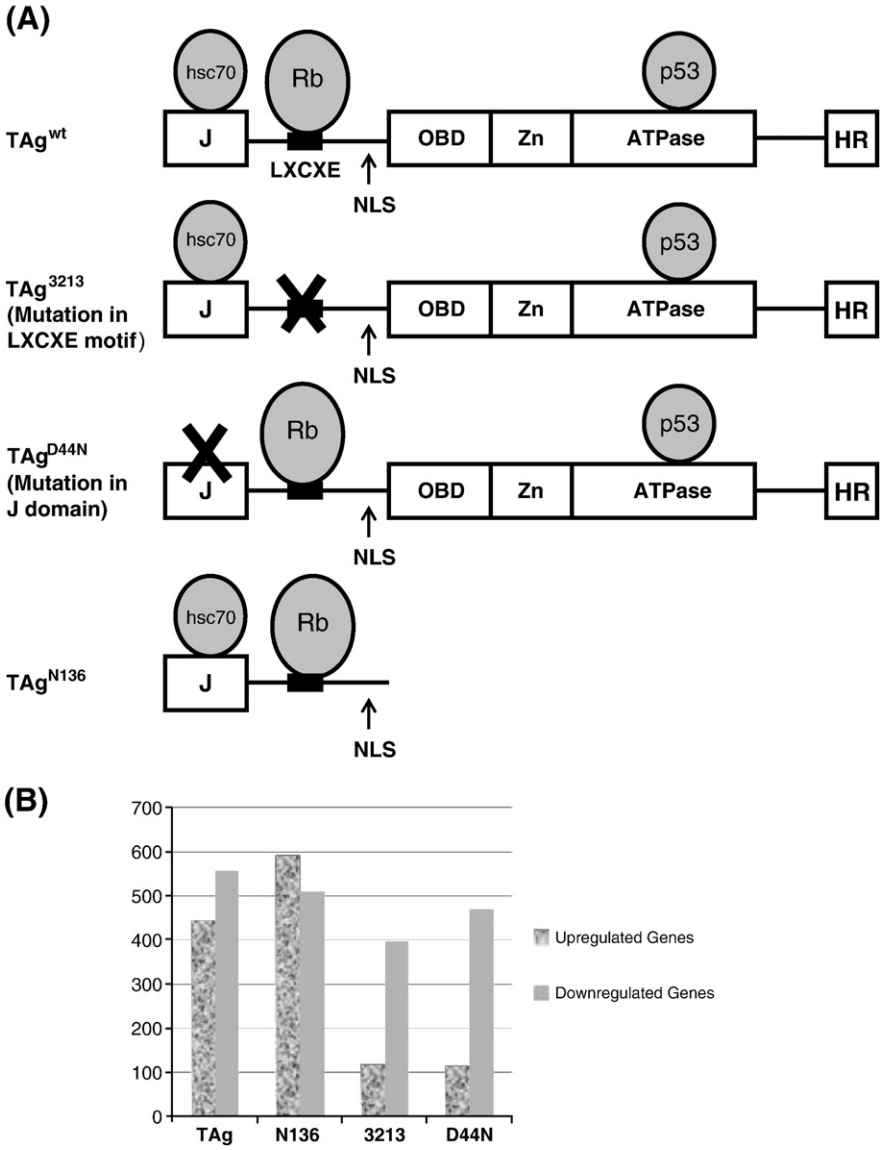

Fig. 1. (A) Domain maps of SV40 TAg^{wt} and mutants. J domain (J), Rb-protein (pRb, p130 and p107) binding motif (LXCXE), nuclear localization signal (NLS), origin binding domain (OBD), Zn domain (Zn), ATPase domain (ATPase), and host range domain (HR) are labeled on the linear representation of TAg. Grey circle represents cellular proteins interacting with TAg or mutants. (B) Global patterns of cellular gene expression in MEFs expressing wild-type or mutant T antigens. Genes 3 fold up or down with present call were selected for this analysis (see Materials and methods for details).

Using these criteria, we found that MEFs expressing TAg^{wt} upregulates 446 genes and downregulates 558 genes in comparison to normal MEFs. MEFs expressing a truncated TAg (TAg^{N136}) upregulated 593 genes and downregulated 512 genes. Noticeably, TAg^{N136} upregulated more genes than wild-type TAg. Mutation of the LXCXE motif (TAg^{3213}) or the J domain (TAg^{D44N}) resulted in a significant reduction in the number of genes upregulated by TAg^{wt} and TAg^{N136}, suggesting the importance of these domains in gene regulation. MEFs expressing TAg^{3213} upregulated 119 genes and downregulated 399 genes, while MEFs expressing TAg^{D44N} upregulated 117 genes and downregulated 470 genes. Thus, the number of genes downregulated by TAg was not affected significantly by these mutations. These results differ from our previous studies in the intestine, where nearly all gene regulation required the TAg J domain and LXCXE motif (Rathi et al., 2009).

TAg regulates E2F-dependent gene expression and blocks p53-dependent targets

The LXCXE motif and J domain are required for the complete inactivation of Rb family members and thus for the induction of E2F target genes (Srinivasan et al., 1997; Stubdal et al., 1997; Zalvide et al., 1998). Our previous study has shown that mice expressing TAg^{N136} in the villus enterocytes regulated the same set of E2F-target genes as TAg^{wt} while TAg^{3213} and TAg^{D44N} did not regulate a significant number of genes in this system (Rathi et al., 2009). Consistent with our studies in enterocytes (Rathi et al., 2009; Saenz-Robles et al., 2007a) we found a profound upregulation of E2F target genes in MEFs expressing TAg^{wt} and TAg^{N136}. This regulation was significantly reduced in MEFs expressing TAg^{3213} and TAg^{D44N} (Fig. 2). We confirmed the expression levels of several E2F-target genes (BRCA1, RRM2, DHFR, B-myb, TS and Cyclin E) by semiquantitative RT-PCR analysis (data not shown).

TAg^{wt} binds to the tumor suppressor p53 and blocks p53 dependent transcription (Jiang et al., 1993; Lane and Crawford, 1979; Linzer and Levine, 1979). Consistent with the microarray data p53 target genes were upregulated in cell lines expressing TAg^{N136} while these genes were not altered in MEFs expressing TAg^{3213}, TAg^{D44N}, or TAg^{wt} (Fig. 3). We confirmed the expression levels of some of the p53-target genes (Lrdd, p21, NOXA, Mdm2, Cyclin G1, Fas, PERP, Pten and Rprm) by semiquantitative RT-PCR analysis (data not shown). Interestingly, we also found a number of growth factors upregulated only by TAg^{N136} (Fig. 4) and confirmed the expression levels of some of these genes (GD15, LIF, BMP8a, PDGF-α, amphiregulin and proliferin) by semiquantitative RT-PCR analysis (data not shown). To our knowledge, these genes are not known to be regulated by p53. The basis for their regulation by TAg^{N136} and the biological significance of their expression is under investigation.

TAg regulates expression of immune response genes

Many immune response genes were upregulated in MEFs stably expressing TAg^{wt} as well as in cell abortively infected with SV40 virions (Table 1 and Fig. 5). Many of these genes are known interferon-stimulated genes (ISGs). The products of ISGs are induced in response to virus infection and act on infected as well as uninfected cells to activate a global antiviral state (Randall and Goodbourn, 2008; Sarkar and Sen, 2004). These ISGs are upregulated both in MEFs infected with SV40 virus as well as MEFs stably expressing TAg. We confirmed the expression levels of some of the ISGs such as Ifi44, Ifi27, Oas2, GTPase2, Rsad2, Mx1, Oasl2, and IRF7 by semiquantitative RT-PCR (Fig. 5A). We found upregulation of these genes by MEFs expressing TAg^{wt} and TAg^{D44N} (lanes 3–4 and 9–10) but not by TAg^{N136} and TAg^{3213} (lanes 5–6 and 7–8). We also determined the protein levels of ISG54 in MEFs abortively infected with SV40 and MEFs stably expressing TAg or its mutants (Fig. 5B). We found that the basal level of expression of ISG54 in normal MEFs (lane 1) was increased in MEFs infected with SV40 (lane 2) and MEFs stably expressing TAg^{wt} or TAg^{D44N} (lanes 3 and 6). In order to confirm that induction of these immune response genes by TAg is not restricted to MEFs, we checked the protein levels of ISG56 and IRF9 in human fibroblast (BJ cells) expressing a vector control or large TAg (BJ-LT) (Fig. 5C). The levels of ISG56 and IRF9 were significantly increased in BJ-LT cells relative to BJ cells (Fig. 5C).

Interferons are not made by MEFs expressing TAg

While many IFN-stimulated genes were upregulated in TAg expressing MEFs, the levels of IFN-α and -β mRNAs were not altered (Fig. 6A). Next, we tested the expression levels of IFN-α2, IFN-α4 and IFN-β in TAg expressing MEFs by semiquantitative RT-PCR analysis (Fig. 6B). MEFs infected with Sendai virus were used as a positive control and these cells showed a robust increase in IFN-α and -β mRNA levels. However, there was no increase in IFN mRNA levels in TAg expressing cells. This suggests that ISG induction is not due to the production of IFNs.

Activation of STAT1 by SV40 TAg

Stat1 plays a major role in both IFN-α/β and IFN-γ induced signaling pathways. Upon exposure to IFNs, Stat1 is activated by tyrosine phosphorylation by tyrosine kinases such as JAK1 (Darnell et al., 1994; Goodbourn et al., 2000). STAT1 is also an ISG whose transcription is upregulated by IFNs. Stat1 mRNA levels were increased in MEFs expressing TAg (see Table 1). To test for Stat1 activation, we analyzed the phosphorylation status of Stat1 by western blot analysis using whole-cell extracts from MEFs stably

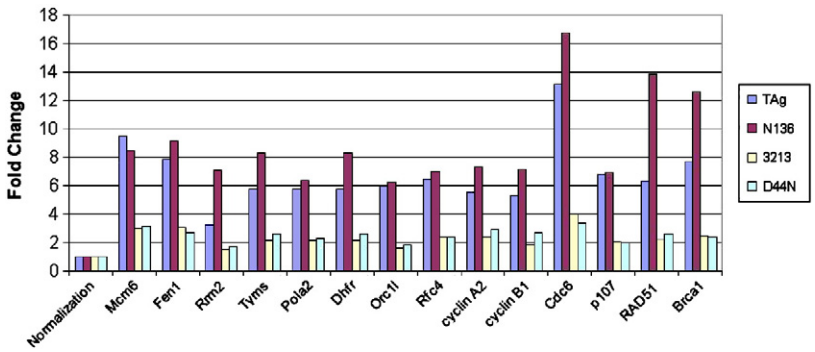

Fig. 2. TAg regulates gene expression by inactivating RB family proteins. TAg^{wt} and TAg^{N136} show significant upregulation while TAg^{3213} and TAg^{D44N} show intermediate upregulation of E2F target genes. Fold change of the specified genes were determined by microarray analysis for the respective category: TAg^{wt} (TAg), TAg^{N136} (N136), TAg^{3213} (3213) and TAg^{D44N} (D44N). "Normalization" represents fold change of 1 (not regulated) in each category.

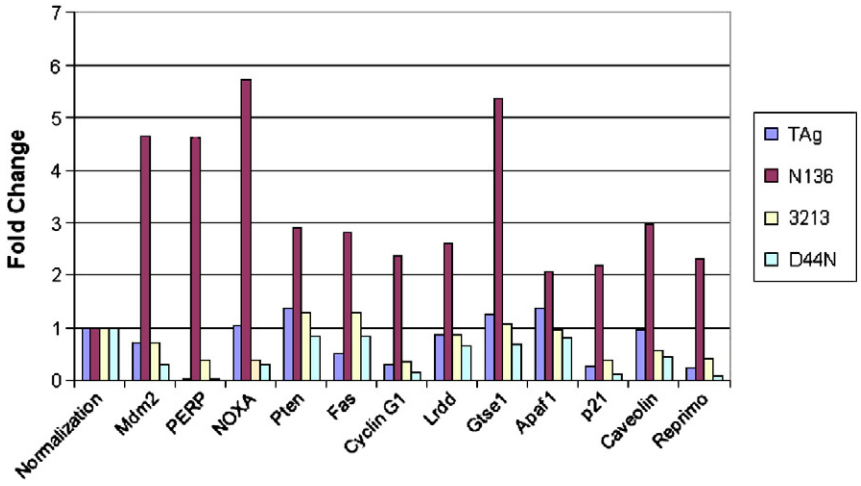

Fig. 3. TAg^{N136} upregulates p53 target genes. Fold change of the specified genes were determined by microarray analysis for the respective category: TAg^{wt} (TAg), TAg^{N136} (N136), TAg^{3213} (3213) and TAg^{D44N} (D44N). "Normalization" represents fold change of 1 (not regulated) in each category.

expressing TAg^{wt} or mutants as well as MEFs abortively infected with SV40 virions (Fig. 7A). We found phosphorylation of Stat1 at Tyr701 in MEFs infected with SV40 (lane 2) as well as MEFs expressing TAg^{wt} (lane 3) or TAg^{D44N} (lane 6). However, Stat1 were not phosphorylated in MEFs stably expressing TAg^{N136} (lane 4) or TAg^{3213} (lane 5). In addition, the steady-state levels of Stat1 protein was increased in MEFs infected with SV40 or stably expressing TAg^{wt}. Stat1 was also phosphorylated at Tyr701 in BJ cells expressing TAg^{wt} (Fig. 7B). Thus, T antigen-induced STAT1 phosphorylation followed the same genetics as ISG induction.

Regulation of immune response genes by TAg requires interaction with p53

TAg directly binds to the DNA-binding surface of p53 thus blocking its ability to bind promoters and to regulate gene expression (Bargonetti et al., 1993; Jiang et al., 1993). As a consequence, complex formation between TAg and p53 interferes with the expression of p53-regulated genes involved in several biological processes such as cell cycle (p21, cyclin G1), DNA repair (GADD45), apoptosis (Bax) and signal transduction (IGF-BP3). We found that MEFs expressing TAg^{N136}, a truncation mutant unable to bind p53, did not show induction of immune response genes (Fig. 5A). To determine if p53 binding to TAg is required for ISG regulation, we used TAg mutant Patch-1 (Ahuja et al., 2009). Mutations comprising patch-1 specifically alter the p53–TAg interface and thus fail to bind and inactivate p53 (Ahuja et al., 2009). Fig. 8 shows that Patch-1 is defective for the upregulation of ISGs. We conclude that T antigen binding and perhaps stabilization of p53 is required for ISG induction.

Discussion

SV40 is thought to induce transformation in part by acting on key transcriptional regulators and thereby altering cellular gene expression. Thus, SV40 TAg antagonizes the ability of Rb-proteins to repress E2F-dependent gene expression leading to the expression of genes required for cell cycle entry and progression while simultaneously blocking p53-dependent transcription and consequently inhibiting apoptosis. Consistent with this view, we previously reported that TAg upregulates E2F-dependent genes in both primary MEFs and transgenic murine enterocytes and that p53-dependent transcription was not induced in either of these systems (7). However, these studies were limited by the use of an Agilent mouse cDNA array which only included 8462 genes of the estimated 21,000 mouse genes.

We have now extended these studies by the use of mouse whole genome arrays and by including three key mutants (TAg^{N136}, TAg^{3213} and TAg^{D44N}) in addition to TAg^{wt} in the analysis. TAg^{3213} and TAg^{D44N} are defective for Rb protein inactivation but retain the ability to bind p53, while TAg^{N136} is defective for p53 interaction but retains the ability to inhibit the Rb proteins. In addition, to eliminate genes whose regulation may be altered as a consequence of cell line

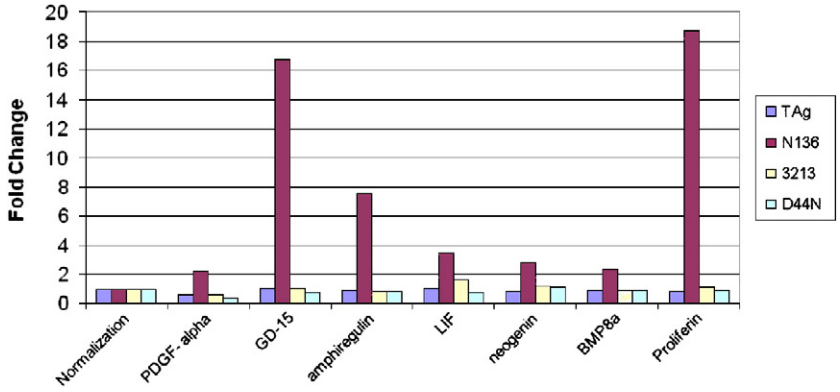

Fig. 4. Upregulation of growth factors by TAg^{N136}. Fold change of the specified genes were determined by microarray analysis for the respective category: TAg^{wt} (TAg), TAg^{N136} (N136), TAg^{3213} (3213) and TAg^{D44N} (D44N). "Normalization" represents fold change of 1 (not regulated) in each category.

Table 1
Immune response genes regulated by SV40, TAg and mutants.

Specific function	Biological process	Description	Entrez gene ID	Gene symbol	SV40	TAg	N136	3213	D44N
Transcription	Interferon type I biosynthetic process	Interferon regulatory factor 7	54123	Irf7	29.1	4.2	1.0	0.8	5.7
		Interferon dependent positive acting transcription factor 3 gamma	16391	Irf9	3.7	2.8	0.9	0.7	2.4
	JAK/Stat signaling	Signal transducer and activator of transcription 1	20846	Stat1	5.8	3.1	1.5	0.5	2.0
		Signal transducer and activator of transcription 2	20847	Stat2	7.5	1.9	1.0	0.5	1.5
	Cytokine and chemokine signaling	N-myc (and STAT) interactor	64685	Nmi	3.5	4.3	1.4	1.2	2.3
	PML body	Promyelocytic leukemia	18854	Pml	1.8	2.5	3.1	1.4	1.9
		Nuclear antigen Sp100	20684	Sp100	4.5	7.2	1.1	1.1	2.2
RNA binding	Interferon-induced	2′–5′ oligoadenylate synthetase 1C	114643	Oas1c	2.5	2.4	2.2	1.5	2.5
		2′–5′ oligoadenylate synthetase-like 1	231655	Oasl1	16.0	2.7	1.2	1.1	3.5
		2′–5′ oligoadenylate synthetase 1A	246730	Oas1a	13.8	18.9	4.0	1.3	15.1
		2′–5′ oligoadenylate synthetase 2	246728	Oas2	9.5	9.1	2.1	1.2	4.2
		2′–5′ oligoadenylate synthetase-like 2	23962	Oasl2	82.4	16.3	0.6	0.4	7.1
Binding to other proteins	Interferon-induced	Interferon-induced protein with tetratricopeptide repeats 1	15957	Ifit1/ISG56	55.7	7.7	2.0	0.5	4.6
		Interferon-induced protein with tetratricopeptide repeats 2	15958	Ifit2/ISG54	4.3	2.4	1.7	0.9	2.1
		Interferon-induced protein with tetratricopeptide repeats 3	15959	Ifit3/ISG49	13.5	4.3	1.1	0.4	3.0
		Interferon activated gene 202B	26388	Ifi202b	7.7	3.3	1.2	0.8	2.3
		Interferon activated gene 203	15950	Ifi203	10.8	2.7	1.2	1.0	2.0
		Interferon activated gene 204	15951	Ifi204	8.3	3.2	0.9	1.1	1.8
NC*	Interferon-induced	Interferon-induced protein 35	70110	Ifi35	3.6	3.1	1.8	1.0	2.1
		Interferon-induced protein 44	99899	Ifi44	49.8	17.0	1.1	0.5	6.5
		Interferon, alpha-inducible protein 27	76933	Ifi27	21.6	21.5	3.4	1.6	6.5
		Interferon gamma inducible protein 47	15953	Ifi47	9.0	4.2	0.9	0.9	1.9
Helicase	Interferon-induced	Interferon-induced with helicase C domain 1	71586	Ifih1	22.6	8.9	3.0	0.6	4.7
	Interferon type I biosynthetic process	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	230073	Ddx58/RIG-I	5.5	4.2	1.2	0.9	1.6
Catalytic activity	Virus response	Radical S-adenosyl methionine domain containing 2	58185	Rsad2	37.9	12.4	1.5	1.2	6.7
GTPase	Interferon-induced	Myxovirus (influenza virus) resistance 1	17857	Mx1	11.4	4.1	1.1	0.9	1.8
		Myxovirus (influenza virus) resistance 2	17858	Mx2	8.0	3.2	0.9	1.0	2.5
		GTPase, very large interferon inducible 1	74558	Gvin1	3.4	2.5	1.7	0.5	1.1
		Interferon inducible GTPase 1	60440	Iigp1	23.2	6.8	0.3	0.3	2.8
		Interferon inducible GTPase 2	54396	Iigp2	10.1	8.5	1.0	0.6	4.4
		Interferon gamma induced GTPase	16145	Igtp	15.0	8.1	1.7	0.8	4.0
Protein kinase	Interferon-induced	Eukaryotic translation initiation factor 2-alpha kinase 2	19106	Eif2ak2/PKR	3.1	2.3	1.1	0.9	2.0
Ubiquitin like/protein binding	Interferon-induced	Interferon, alpha-inducible protein	100038882	Isg15	33.2	9.8	1.7	0.4	4.1
Exonuclease	Interferon-induced	Interferon-stimulated protein	57444	Isg20	3.8	1.9	1.1	1.6	1.8
Adenosine deaminase	Interferon-induced	Adenosine deaminase, RNA-specific	56417	Adar	5.2	5.0	1.8	0.9	2.5
Receptor	Response to microbial infections	Toll-like receptor 3	142980	Tlr3	3.4	3.9	1.8	1.1	2.6
	Cell adhesion	Lectin, galactose binding, soluble 9	16859	Lgals9	3.5	3.9	0.9	1.1	2.4
	Cell proliferation	CD274 antigen	60533	Cd274	2.4	2.6	1.3	1.3	1.2
	Interferon-induced	RIKEN cDNA 5830458K16 gene	67775	Rtp4	28.3	7.4	1.6	0.4	4.3
Endopeptidase	Antigen presentation	Proteosome (prosome and macropain) subunit, beta type 8 (large multifunctional peptidase 7)	16913	Psmb8	10.6	5.5	1.5	0.4	2.6
		Proteosome (prosome and macropain) subunit, beta type 9 (large multifunctional peptidase 2)	16912	Psmb9	4.1	6.3	3.6	0.6	2.4
MHC class II presentation	Antigen presentation	Ia-associated invariant chain	16149	Cd74	1.1	2.1	0.8	1.9	1.4
MHC class I presentation	Antigen presentation	Histocompatibility 2, K1, K region	14972	H2-K1	6.1	4.5	2.0	1.6	3.4
		Histocompatibility 2, D region locus 1	14964	H2-D1	3.5	4.2	3.0	1.5	2.5
		Histocompatibility 2, K1, K region	100044874	LOC100044874	6.9	5.2	2.0	1.8	3.9
		Histocompatibility 2, T region locus 23	15040	H2-T23	5.1	3.6	3.0	0.9	2.0
		Histocompatibility 2, D region locus 1	14980	H2-L	2.4	2.2	1.6	0.9	1.5
GTP binding	Interferon-induced	Guanylate nucleotide binding protein 2	14469	Gbp2	11.7	2.8	1.8	0.5	1.6
		Macrophage activation 2 like	100702	Mpa2l	18.3	8.5	0.8	0.6	3.1
Transferase activity	Protein localization	Poly (ADP-ribose) polymerase family, member 9	80285	Parp9	5.3	4.0	1.2	0.5	2.3
	Virus response	Zinc finger CCCH type, antiviral 1	78781	Zc3hav1	3.8	2.8	1.0	1.2	2.1
Cyclic-nucleotide phosphodiesterase activity	IFN-induced	SAM domain and HD domain, 1	56045	Samhd1	2.5	2.3	1.4	1.8	2.0
dTTP biosynthesis	LPS response	Thymidylate kinase family LPS-inducible member	22169	Tyki	39.6	8.5	0.7	0.8	4.6
		Placenta-specific 8	231507	Plac8	11.9	2.0	0.1	0.5	0.3
Phospholipid binding	Defense response	Lymphocyte antigen 6 complex, locus E	17069	Ly6e	3.5	2.3	1.1	1.7	1.4
Ubiquitin ligase	Interferon-induced	Deltex 3-like (Drosophila)	209200	Dtx3l	6.5	3.7	1.3	0.7	2.6
Peptidase	Interferon-induced	Ubiquitin specific peptidase 18	24110	Usp18	49.0	20.4	1.8	0.7	9.7
Protein/nucleic acid binding	Interferon-induced	Tripartite motif protein 34	94094	Trim34	3.4	2.2	1.2	0.8	0.9

List of selected immune response genes showing differential regulation (in fold change) by SV40, TAg and mutants compared to wild-type MEFs.
NC* = Not classified.

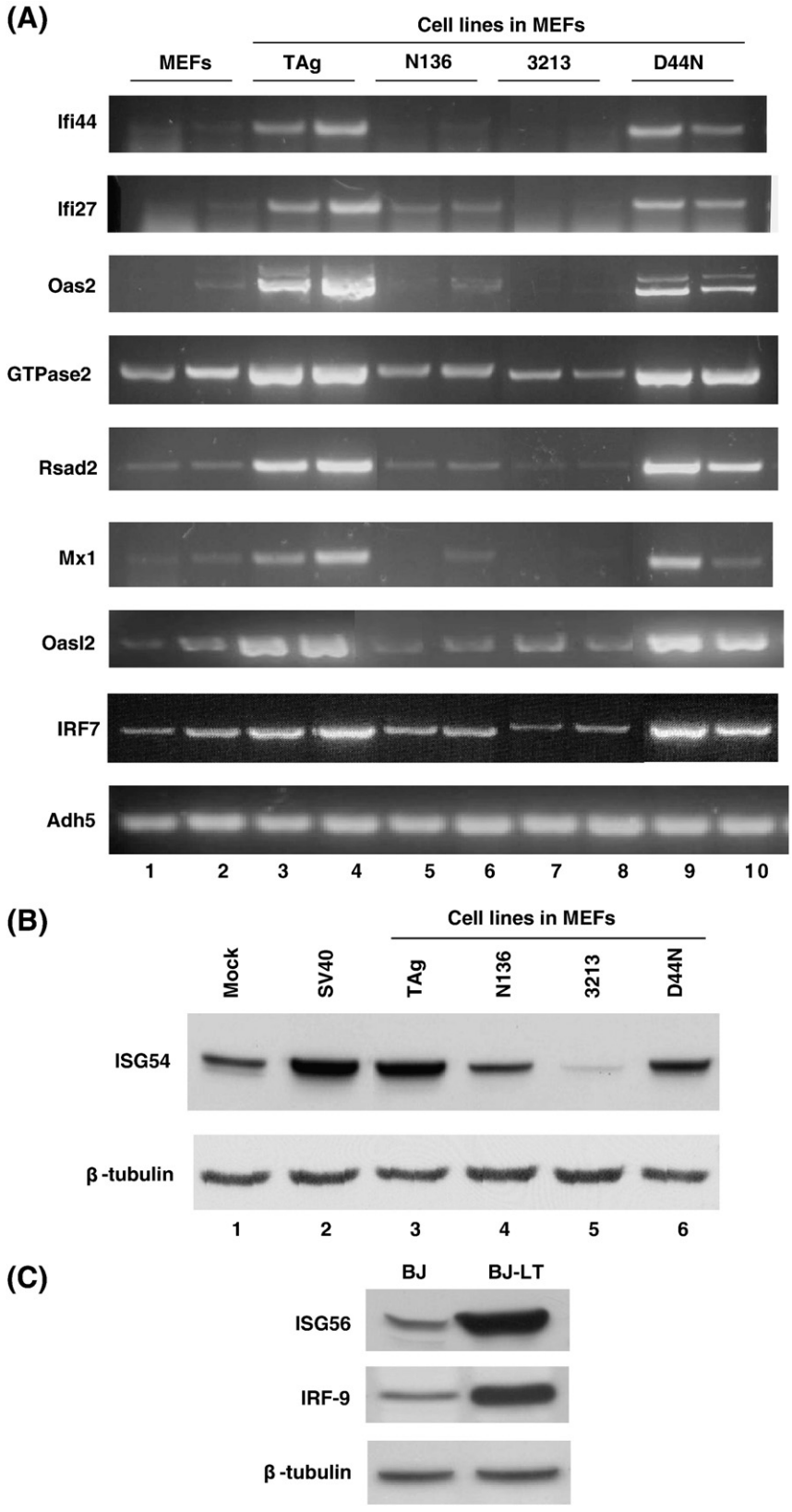

Fig. 5. Upregulation of interferon-stimulated genes (ISGs) by TAg. (A) Transcript levels of ISGs were analyzed in normal MEFs and MEFs expressing TAg^{wt} (TAg), TAg^{N136} (N136), TAg^{3213} (3213) and TAg^{D44N} (D44N). Two independent clones of each cell line were used for this experiment. cDNAs were reverse-transcribed from equal amounts of total RNA and subjected to PCR using specific primers. Transcript level of alcohol dehydrogenase 5 (Adh5) was used as a loading control. (B) MEFs were infected with SV40 (lane 2) or stably expressing TAg or its mutants (lanes 3–6) were used. Whole-cell extracts from these cells were subjected to immunoblot for ISG54. Mock (lane 1) represents normal MEFs. β-tubulin was used as a loading control. (C) Whole-cell extracts from BJ cells (human fibroblast) expressing empty vector or TAg were subjected to immunoblots for ISG56 and IRF-9. β-tubulin was used as a loading control.

establishment, we have analyzed gene expression in MEFs abortively infected with SV40.

TAg induces E2F-dependent transcription and blocks expression of p53-target genes

Studies of transgenic enterocytes expressing wild-type or mutant TAg demonstrated that nearly all TAg-dependent gene regulation in these cells can be explained by the inactivation of the Rb proteins and resultant upregulation of E2F-dependent gene transcription. Neither wild-type nor the mutant TAg's affected the expression of p53-regulated genes, consistent with the lack of p53 expression in this cell type (Markovics et al., 2005).

In this report we examined the consequences of wild-type and mutant TAg's in MEFs. Consistent with our studies in enterocytes we found that TAg induction of E2F-dependent transcription depends on both a functional J domain and Rb-binding LXCXE motif, and that the first 136 amino acids of TAg, that contain both of these elements are capable of upregulating these genes. In contrast to enterocytes, MEFs upregulated p53-dependent genes in response to TAg^{N136}. However, p53-dependent genes were not induced, or in some cases were repressed, by TAg^{wt} and by TAg^{3213} and TAg^{D44N}, each of which retains the ability to bind p53.

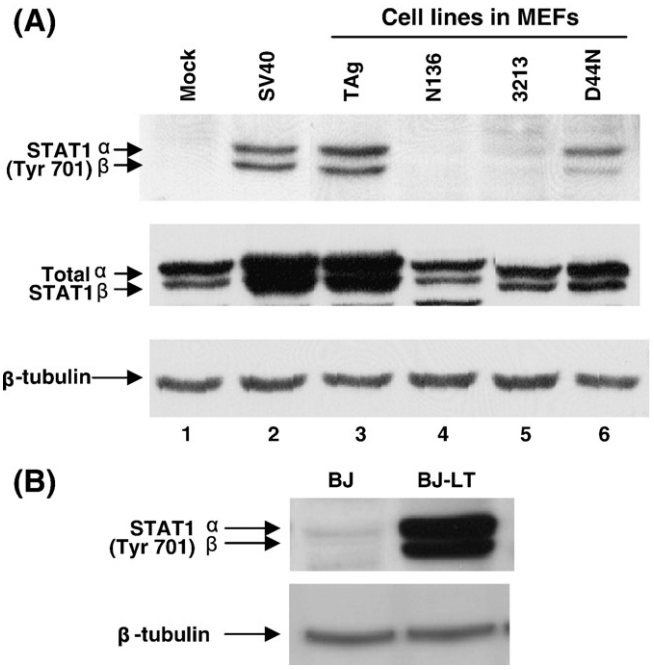

Fig. 7. Activation of STAT1 by TAg. (A) MEFs infected with SV40 (lane 2) or stably expressing TAg or its mutants were used (lanes 3–6). Whole-cell extracts from these cells were subjected to immunoblots for phospho-specific (Tyr701) STAT1 and total STAT1. Mock (lane 1) represents normal MEFs. β-tubulin was used as a loading control. (B) Whole-cell extracts from BJ cells (human fibroblast) expressing empty vector (BJ) or TAg (BJ-LT) were subjected to immunoblots for phospho-specific (Tyr701) STAT1. β-tubulin was used as a loading control.

(A)

Interferons	Fold change by TAg expressing MEFs	Fold change by SV40 infected MEFs
IFN α1	0.9	0.9
IFN α2	0.8	1.0
IFN α4	1.3	1.0
IFN α5	1.0	1.1
IFN α6	1.1	0.8
IFN α9	1.0	0.8
IFN α11	1.0	1.0
IFN αB	0.9	0.8
IFN β1	1.0	1.2
IFN γ	1.0	1.0
IFN Zeta	1.7	2.0

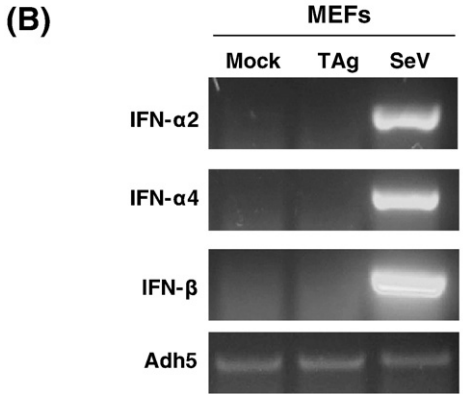

Fig. 6. Interferon mRNAs are not increased in MEFs expressing TAg. (A) The table represents the fold change of the interferon genes, determined by microarray analysis in MEFs expressing TAg or infected with SV40 virions. (B) Transcript levels of Interferon α2, α4 and β1 were analyzed in normal MEFs and MEFs expressing TAg^{wt} (TAg). MEFs infected with Sendai virus were used as a positive control. cDNAs were reverse-transcribed from equal amounts of total RNA and subjected to PCR using specific primers. Transcript level of alcohol dehydrogenase 5 (Adh5) was used as a loading control.

Induction of immune response genes by TAg requires the LXCXE motif and p53 binding

Previously we noticed that TAg regulated a set of immune response genes in MEFs and that these same genes were not regulated by TAg in transgenic enterocytes (7). The use of the whole genome

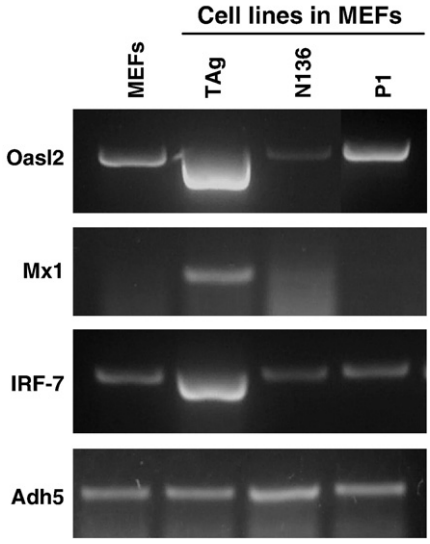

Fig. 8. Regulation of immune response genes by TAg requires interaction with p53. Transcript levels of Interferon-stimulated genes including Oasl2, Mx1 and IRF-7 were analyzed in normal MEFs and MEFs expressing TAg^{wt} (TAg), TAg^{N136} (N136) or p53 binding defective mutant, Patch-1 (P1). cDNAs were reverse-transcribed from equal amounts of total RNA and subjected to PCR using specific primers. Transcript level of alcohol dehydrogenase 5 (Adh5) was used as a loading control.

mouse array in this study clearly shows the upregulation of a large number of immune response genes by SV40 T antigen (Table 1). These genes represent two major classes: (1) Interferon-stimulated genes (ISGs), such as the OAS family, MX1, ISG56, ISG54, ISG15, Cig5, GTPase, P200 gene family and PKR; and, (2) genes involved in interferon induction and signaling such as IRF-7, IRF-9, RIG-1, STAT1 and STAT2. This collection of genes was upregulated in both MEFs abortively infected with SV40 as well as in MEFs stably expressing TAg indicating that their altered regulation is not an artifact arising from cell line establishment.

The genetic data suggests that the immune response genes are regulated by a common mechanism. All of these genes are regulated by TAg^{wt} and by TAg^{D44N} but not by TAg^{N136} or TAg^{3213}. This suggests a requirement for both the LXCXE motif and a TAg function or functions carboxy-terminal to amino acid 136. The inability of TAg^{3213} to regulate the immune response genes suggests that TAg binding to Rb family members plays a role in this effect. However, the inactivation of Rb proteins by TAg is thought to be J domain dependent so the observation that TAg^{D44N} is capable of regulating these genes argues against this possibility. Perhaps the LXCXE motif can also act on Rb proteins in a J domain-independent manner. Alternatively, the LXCXE motif may target cellular proteins other than Rb and these unknown targets may play a role in regulating the immune response genes.

TAg^{N136} is fully able to inactivate Rb family members, yet it is unable to induce ISGs. This indicates that the regulation of immune response genes requires one or more activities residing in the carboxy-terminal region of TAg. One candidate activity is the ability of TAg to bind p53 and we found that a p53 binding defective mutant of TAg (Patch-1) is unable to upregulate ISGs (Fig. 8). This indicates that the TAg–p53 interaction is necessary for ISG induction. At present we cannot distinguish between a "loss of function" model, in which p53 normally functions to repress ISG induction and TAg blocks this p53 action, or a "gain of function" model in which the TAg–p53 complex is actively involved in ISG expression. Bocchetta et al. have shown that TAg–p53 complexes are required to activate the insulin-like growth factor-I promoter (Bocchetta et al., 2008). Consistent with a "gain of function" hypothesis, enterocytes, which lack p53 expression, do not show induction of immune response genes (Cantalupo et al., 2009; Rathi et al., 2009). In either case, binding of TAg to p53 alone is not sufficient for ISG induction as TAg^{3213} can bind to p53 but is unable to induce the immune response genes. Collectively, our mutant analysis suggests cooperation between LXCXE motif of TAg and binding with p53 in the regulation of immune response genes.

TAg activates STAT1 in the absence of interferon production

Interestingly, TAg is capable of inducing the downstream interferon pathway without affecting the levels of IFN-α or IFN-β. We found that TAg induces STAT1 Tyr701 phosphorylation suggesting that TAg can activate the interferon signaling pathway independent of interferon production. One possibility is p53-dependent activation of STAT1 by c-Abl1 tyrosine kinase instead of the classical JAK–STAT pathway as reported by Youlyouz-Marfak et al. (2008). Furthermore, TAg-mutants capable of inducing ISG expression also induce STAT1 phosphorylation while mutants defective for ISG induction do not induce STAT1 phosphorylation. This suggests that the primary mechanism by which TAg induces ISGs is through STAT1 signaling.

In conclusion, we have shown that SV40-transformed MEFs have activated the interferon pathway in the absence of interferon production. The biological consequences of this activation remain unclear. However, one practical consequence of this observation is in the common use of TAg in immortalization of MEFs obtained from knockout mice. Our observation clearly demonstrates that this practice should be critically evaluated while testing functional consequences of gene knockouts, especially in the studies involving genes that modulate innate immune signaling pathways.

Several herpesviruses, such as Epstein–Barr virus, herpes simplex virus, Kaposi's sarcoma-associated herpesvirus and HCMV have been shown to activate IFN-responsive genes, such as MxA and OAS (Browne et al., 2001; Mossman et al., 2001; Poole et al., 2002; Ruvolo et al., 2003; Zhu et al., 1998). However, in these cases the induction occurs in the context of a productive infection and the effects of the ISGs are later mitigated by other viral functions. One interesting question that arises is how TAg expressing MEFs are able to survive in the presence of high levels of ISGs which are known to create growth inhibitory environment. Future studies are needed to identify the cellular target(s) on which TAg acts to elicit ISG expression and to explore the connection between ISGs and transformation by TAg.

Materials and methods

Isolation of primary fibroblasts, cell culture conditions, and establishment of cell lines

Mouse embryo fibroblasts (MEFs) were harvested from 13.5-day-old FVB embryos as described previously (Markovics et al., 2005) and grown in DMEM (Mediatech, cat# 10-013-CV) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, cat# SH30071.03), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, cat# 15140-122). MEFs were grown at 37 °C in 5% CO_2. Stable MEF cell lines were made with pRSVB-neo plasmid that expresses a Geneticin (G418) resistance gene and a cDNA or genomic version of the wild-type or mutant T antigens (N136, 3213 (E107K and E108K) and 5110 (D44N)) under the control of the Rous sarcoma virus (RSV) promoter (Srinivasan et al., 1997). Primary cultures of MEFs were transfected with above plasmids using Lipofectamine reagent (Invitrogen), according to the manufacturer's instructions. Upon selection in culture with 0.4 mg/ml of G418 (Invitrogen, cat# 11811-031), transformed colonies (foci) were selected and individually grown and several independent cell lines were established.

SV40 infection

For abortive SV40 infections, confluent MEFs were infected with SV40 at a multiplicity of infection 10 pfu/cell (M.O.I = 10) as described (Tremblay et al., 2001). Cells were incubated for an additional 3 to 4 days before the cells were harvested with trypsin. The cell pellet was washed three times with cold PBS/EDTA and frozen at −80 °C for protein and RNA extraction.

RNA extraction

Wild-type MEFs and MEFs expressing TAg^{wt} and TAg-mutants were grown to confluence and fed with fresh medium. After 2 days, the cells were harvested and the cell pellets were washed with cold PBS/EDTA three times. Two independent wild-type MEFs, three independent MEFs expressing TAg^{wt} and mutants were used to prepare RNA. Three independent mock and SV40 infections were conducted. Total RNA from transfected or infected MEFs was isolated using the RNeasy kit according to the manufacturer's protocol (Qiagen Inc.). Genomic DNA contamination was eliminated by digesting the RNA with RNase-free DNase. The yield (at absorbance A260) and purity (A260/A280 ratio) of each RNA sample were determined by using a spectrophotometer (Eppendorf).

Microarrays

Total RNA was sent to the Genomics and Proteomics Core Laboratories (University of Pittsburgh) for hybridization to the Mouse 430 2.0 whole genome array (Affymetrix) which contains

Table 2
Primers used in this study.

Gene	Forward (F) or reverse (R)	Primer sequence (5′ to 3′)	Product size (bp)	Annealing temp. (°C)	Extension time (s)
Adh	F	TGCACCACCAACTGCTTAG	152	58	30
	R	GATGCAGGGATGATGTTC			
Ifi44	F	GAGAGAACAGGGAATGAAGAAGGC	134	52.4	30
	R	CCAACAGAATTGCGATTGGTCC			
Ifi27	F	CCATAGCAGCCAAGATGATGTCTG	121	55	30
	R	GCATTTGTTGATGTGGAGAGTCC			
Oas2	F	AAAACCAACCGCTCCCAGTTCGTC	488	57.1	30
	R	GCAATGTCAAAGTCATCTGTGCC			
GTPase2	F	CTTCCACCTGCTTGTTCTTTGG	266	55.4	30
	R	TCACAGTTTCCTCAGTGCTGGG			
Rsad2	F	CAATCACACCCAGCAGCAGTTAG	209	54.4	30
	R	AGCGATGCCTCAGAACACAGTG			
Mx1	F	CAGCACCTGAAAGCCTACTACCAG	135	53.6	30
	R	GGTGTCCTGTAAAAGCTGAAGCATC			
Oasl2	F	TTACAGAACAGCCAGAGCTATACGG	548	56.1	40
	R	CAAGGGAGATAGATTTACGTCCACG			
IRF7	F	ACACCATCTACCTGGGTTTTG	243	54	60
	R	TTGGGATTCTGAGTCAAGGC			
IFN-α2	F	CATCTGCTGCTTGGAATACAACC	197	56.7	30
	R	GGGGCTGTGTTTCTTCTCTCTCAG			
IFN-α4	F	TCAATGACCTCAAAGCCTGTGTG	211	56.6	30
	R	CACTCCTCCTCACTCAGTCTT			
IFN-β	F	AAGAGTTACACTGCCTTTGCCATC	138	53.2	30
	R	AAACACTGTCTGCTGGTGGAGTTC			

45,101 probesets representing 21,635 unique genes. CEL files for each array were converted into RMA expression values using BRB-Array Tools (Rich Simon, National Cancer Institute, http://linus.nci.nih.gov/BRB-ArrayTools.html). An average fold change ratio (experimental/control) and a one-sample T-test was calculated for each probe set. The total number of genes which were selected showed three fold up or downregulation in an individual experimental class (TAg^{wt} / TAg^{N136}/ TAg^{3213}/ TAg^{D44N}). We applied additional criteria of present and absent calls provided by Affymetrix data files. To be included for consideration, an upregulated gene needs to be present in all the replicates of an experimental class and a downregulated gene needs to be present in all the replicates of the control class (wild-type MEFs). Microarray data submitted to GEO, under the accession number: GSE20620.

Reverse transcription PCR (RT-PCR) analysis

cDNA synthesis from 1 μg of total RNA was performed using Superscript II Reverse Transcriptase (Invitrogen). PCR was performed with equal amounts of cDNA using GoTAq polymerase (Promega) for 25 cycles with specific primers for the different transcripts. Amplification with primers for the alcohol dehydrogenase 5 (Adh5) transcript was used as a normalizing control. PCR reaction products were resolved through a 2% agarose gel in 1× TAE and stained with GelStar (Cambrex Bio Science). The cDNA was amplified with PCR using primers specific for each gene as shown in Table 2. To ensure that these reactions were within the linear range of the assay, we optimized the number of cycles required to obtain non-saturated signals. Exponential amplifications of PCR products were obtained as follows: 2 min at 94 °C; a series of 25 cycles at 94 °C for 30 s, variable annealing temperatures for 30 s, and 72 °C for 30 s, and a final extension step of 5 min at 72 °C. The annealing temperatures and product sizes for each gene are described in Table 2. The products were resolved on 2% agarose gels and stained with GelStar (BioWhittaker Molecular Applications).

Immunoblot analysis

Lysates were prepared from 2-day post-confluent cells in lysis buffer [50 mM HEPES pH 7.9, 400 mM KCl, 0.5 mM EDTA, 0.1% NP40, 10% glycerol, 1 mM DTT, 0.5 mM Na_3VO_4, 0.5 mM NaF, 1 μg/ml pepstatin and a protease inhibitor tablet (Roche)] for 30 min on ice, then centrifuging the lysate for 10 min at 4 °C. Protein concentration was determined by Bradford assay (Bio-Rad). Appropriate dilutions of the following primary antibodies were used: anti-STAT1 (Cell Signaling); anti-Phospho-STAT1 (Tyr701) (Cell Signaling), anti-ISG54 (Thermo Fischer); anti-ISG56 (a gift from Dr. Saumendra N. Sarkar), anti-IRF-9 (C-20; Santa Cruz), anti-β-tubulin-HRP (Santa Cruz). Goat anti-mouse A2554 and goat anti-rabbit A0545 (Sigma) were used as secondary antibodies.

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