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DNA microarray analysis of genes involved in p53 mediated apoptosis: activation of Apaf-1

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The transcription regulation activity of p53 controls cellular response to a variety of stress conditions, leading to growth arrest and apoptosis. Despite major progress in the understanding of the global effects of p53 on cellular function the pathways by which p53 activates apoptosis are not well defined. To study genes activated in the p53 induced apoptotic process, we used a mouse myeloid leukemic cell line (LTR6) expressing the temperature-sensitive p53 (val135) that undergoes apoptosis upon shifting the temperature to 32°C. We analysed the gene expression profile at different time points after p53 activation using oligonucleotide microarray capable of detecting ~11 000 mRNA species. Cluster analysis of the p53-regulated genes indicate a pattern of early and late induced sets of genes. We show that 91 and 44 genes were substantially up and down regulated, respectively, by p53. Functional classification of these genes reveals that they are involved in many aspects of cell function, in addition to growth arrest and apoptosis. Comparison of p53 regulated gene expression profile in LTR6 cells to that of a human lung cancer cell line (H1299) that undergoes growth arrest but not apoptosis demonstrates that only 15% of the genes are common to both systems. This observation supports the presence of two distinct transcriptional programs in response to p53 signaling, one leading to growth arrest and the other to apoptosis. The proapoptotic genes induced only in LTR6 cells like Apaf-1, Sumo-1 and gelsolin among others may suggest a possible explanation for apoptosis in LTR6 cells. *Oncogene* (2001) **20,** 3449–3455.

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The tumor suppressor p53 exerts its function mainly through transcriptional activation of target genes, in response to various stress conditions (El-Deiry *et al*., 1992). Recent work showed that mice carrying p53 mutated at the transcription activation domain (codon 25, 26) generated by gene targeting were prone to tumor formation similar to p53 deficient mice (Jimenez *et al*., 2000) demonstrating the importance of the transcription domain in p53 function. The main outcome of p53 activity is cell cycle arrest and apoptosis and various cell lines respond differently to the activation or introduction of p53 (Levine, 1997). It is not clear what controls the cells' choice between growth arrest and apoptosis in response to p53, but a detailed understanding of this decision may provide a better insight into cancer therapy. Recently, the use of DNA microarrays allowed for the global analysis of gene expression that characterizes the state of the cell in response to changing stimuli and to p53 (Polyak *et al*., 1997; Maxwell and Davis, 2000; Yu *et al*., 1999; Zhao *et al*., 2000; Kannan *et al*., 2000; 2001). We used the temperature-sensitive p53 (ts-p53val135), a mutant that acquires wild-type conformation and activity upon changing the temperature from 37°C to 32°C, without the need for *de novo* synthesis (Michalovitz *et al*., 1990). Different cell lines expressing this ts-p53 respond differently at 32°C. For example the human lung cancer cell line H1299 stably expressing this ts-p53 exhibits growth arrest and not apoptosis, whereas the mouse myeloid leukemic cell line LTR6 stably expressing this p53 exhibits marked apoptosis at 32°C (Levy *et al*., 1993; Yonish-Rouach *et al*., 1993). In this study we analysed p53 regulated genes in the LTR6 cells which undergo apoptosis, using oligonucleotide microarrays. We suggest that some apoptotic related genes and particularly Apaf-1 may be responsible for the p53 mediated apoptosis in the LTR6 cell line.

The LTR6 system was analysed previously showing that upon shifting the temperature to 32°C, p53 rapidly causes cell death due to apoptosis, in LTR6 cells (Yonish-Rouach *et al*., 1991). Previous kinetic analysis showed that 50% of the cells were dead by approximately 30 h and DNA fragmentation could be observed as early as 10 h after temperature shift to 32°C (Yonish-Rouach *et al*., 1993). Studies of gene expression using Northern blots, found several genes that were activated (like MDM2) or suppressed (like c-*myc*) after 2 h of incubation at 32°C (Levy *et al*., 1993). We observed change in the morphology of LTR6 cells after 15 h at 32°C, and extensive cell death at 36 h in

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LTR6 cells but not in the parental M1 cell line. Flow cytometric cell cycle analysis revealed apoptosis of LTR6 cells reflected by an increase in the sub-G1 fraction from 6% (at 2 h) to 18% (at 24 h) (Figure 1B) whereas no change was observed in the sub-G1 fraction of M1 cells at 32°C. We therefore decided to follow the profile of gene expression in LTR6 cells using DNA microarrays for various time points between 2 to 12 h after the temperature shift to 32°C. As a control, we used M1 cell line incubated for 2 and 12 h at 32°C.

Total RNA (10 μg) from 2, 6, 9 and 12 h time points of LTR6 cells and 2 and 12 h time points of M1 cells were processed as previously described (Kannan *et al*., 2001; Kaminski *et al*., 2000a,b) and according to the manufacturer's instruction (Affymetrix, Santa Clara, USA), and were hybridized to Murine Genome U74A® array (Affymetrix). The ratio of gene expression for each sample was calculated by Affymetrix software (Genechip® 3.3 expression analysis) using M1 at 2 h in 32°C as baseline. We selected genes that were changed by more than threefold in at least two time points (404 genes) for our analysis to find out the progressive changes in global gene expression due to p53 activation. Scatter plots of control M1 cells at 2 h and 12 h at 32°C demonstrate a very little difference between the two time points (Figure 2A). Most of the genes are within the twofold range of expression. On the other hand, LTR6 cell line showed progressive modulation in gene expression throughout the time course of the experiment. At 2 h (Figure 2B) there was an obvious increase in scattering of these selected group of genes which was much more pronounced at 9 (Figure 2C) and 12 h (Figure 2D) where many of these genes show up or down regulation of expression by more than the twofold range. To further analyse the profile of gene expression and to distinguish genes activated by p53 from those that are changed in the control cell line we used cluster analysis as previously described (Eisen *et al*., 1998). In this paper only the upregulated genes are discussed.

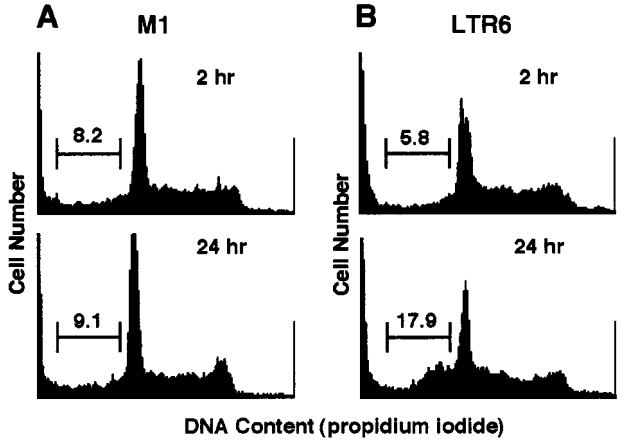

Figure 1 Apoptosis in LTR6 cells incubated for 24 h at 32°C. M1 and LTR6 cells were grown at 37°C and shifted to 32°C and cells samples were collected at various time points and analysed for cell cycle in the fluorescence activated cell sorter (Wang *et al*., 1998). (**A**) Cell cycle analysis of M1 cells at 2 and 24 h in 32°C. (**B**) Cell cycle analysis of LTR6 cells at 2 and 24 h in 32°C. The per cent increase in the Sub-G1 DNA content is shown

In processing the data for cluster analysis, we scaled up to 100 all the expression values that were less than 100 (and therefore may be due to noise). We then calculated ratios for all the pair-wise comparisons between different experiments. The resulting data were subjected to complete linkage cluster analysis (Eisen *et al*., 1998). Figure 3 depicts the results of the cluster analysis that contain three sections. The first section used M1 at 32°C for 2 h as control, the second section used M1 at 12 h as control and the third one used LTR6 at 2 h as control. In this last section only three experiments (LTR6 at 6, 9 and 12) were selected ignoring the changes in LTR6 in the first 2 h of incubation at 32°C and therefore, the intensity of changes is somewhat weaker. The cluster analysis revealed, six main clusters (A – F shown in Figure 3) and it is of interest that regardless of the control baseline chosen, the up and down regulated genes remain similar (Figure 3 cluster B, C, D and E). However, cluster F shows a group of genes that were up regulated only if M1 at 2 h was used as baseline. The list of these genes showed that they contain predominantly ribosomal protein genes indicating that the up regulation of these genes is p53 independent and is due to the temperature shift to 32°C and can be considered as a general response to stress. However, in contrast to increased expression level at 12 h in the control M1 cells, these ribosomal genes showed a marked down regulation in the LTR6 cells between 2 and 12 h time points presumably due to the effect of p53. Similarly, one can conclude that the cluster A contains genes that were down regulated due to temperature change, regardless of p53 function.

Clusters C, D and E contain 173 genes that are up regulated in all three sections due to p53 activation. It is clear that cluster C and E contain genes with delayed expression compared to those in cluster D. For example, at 2 h, most of the genes at cluster C and E are unchanged or even down regulated (black or green color) whereas cluster D contain many up regulated genes in red color for the same time point comparison. Obviously in section 3 of Figure 3, the elevated expression of cluster D gene is lower than that of cluster C and E since the base line of LTR6 at 2 h might already have increased expression for these genes. Hence cluster D contain predominantly early p53 activated genes and cluster C and E contain middle and late p53 activated genes. This pattern was also demonstrated in human colon cancer cell line with inducible p53 (Zhao *et al*., 2000). For supplementary information on the detailed figures and tables about these clusters, visit our web site at URL *http://www.weizmann.ac.il/home/ligivol/apoptosis.html*.

To compile a list of genes consistently modulated by p53, we increased the stringency of the criteria and only those genes with greater than threefold induction or repression in at least three time points of the

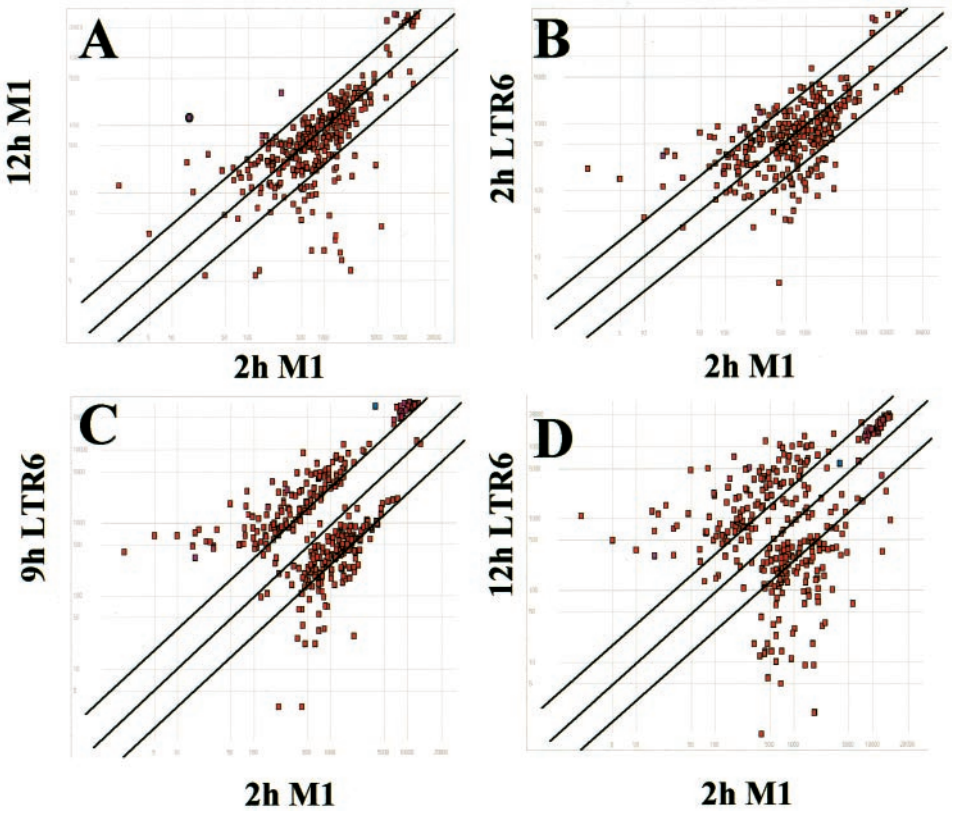

Figure 2 Scatter plots of 404 genes that were regulated by p53 in LTR6 cells. Genes whose expression changed by more than threefold in at least two of the time points were selected for this analysis. (**a**) Scatter plot of these genes in control M1 cells after 2 and 12 h at 32°C. (**b, c, d**) Scatter plots of these genes in LTR6 cells after 2 h (**b**), 9 h (**c**) and 12 h (**d**) at 32°C compared to M1 cells at 32°C for 2 h. The two lines parallel to the diagonal in each graph, represents the twofold ratios of expression level

experiment were selected. This resulted in 91 genes up regulated (Table 1) and 44 genes down regulated (Table 2) (for Table 2, see supplementary information in the above URL) in LTR6 cells compared to M1 cells at 32°C for 2 h. The 91 up regulated genes described in Table 1 are represented in the clusters C, D and E (Figure 3). As expected, many of these up and down regulated genes are involved in apoptosis and cell cycle regulation. Also pronounced is the number of genes involved in other cellular functions such as DNA repair/replication, metabolism and signal transduction. More than 20 genes listed in Tables 1 and 2 are ESTs without identified function yet (for Table 2 see the above URL).

The most clear result from the analysis of ~11 000 probes on the microarray used in this study, is the large number and heterogeneity of the p53 regulated genes. This was observed in several other systems using other cell types like colon (Yu *et al*., 1999), or bladder (Maxwell and Davis, 2000) derived tumor cells, where 1–2% of the genes analysed by the microarray probes were found to be modulated by p53. The functional classification of these genes (Tables 1 and 2 (see supplementary information for Table 2)) indicates that many of them do not show obvious connection to the main outcome of p53 activity, which is growth arrest, apoptosis and repair of DNA damage. In order to understand some of this heterogeneity we compared the list of p53 regulated genes in the LTR6 with another system we studied recently, the human lung cancer cell line H1299 expressing the same ts-p53 (Kannan *et al*., 2001). Only 15% of the up regulated genes (represented in bold letters in Table 1) are common to the two systems. This indicates that many of the p53 regulated genes are cell type specific, and may therefore depend on cell specific co-activators (Shikama *et al*., 1999). For example, the transactivation of MSH (mismatch repair) gene requires both p53 and c-jun or p53 and UV irradiation (Scherer *et al*., 2000). Another factor that determine the heterogeneity of the p53 activated genes may be the differences in the activation state of p53. It has been shown that the phosphorylation state of p53 affects its activity as a transcription factor (Oda *et al*., 2000). Since there are many phosphorylation and other post-translation modification sites, it is possible that p53 is present in the cell as a collection of phosphorylated isomers with some different transactivation properties (Vousden, 2000).

Another conclusion from the functional classification of the regulated genes suggests that p53 activates concerted opposing signals to affect a particular

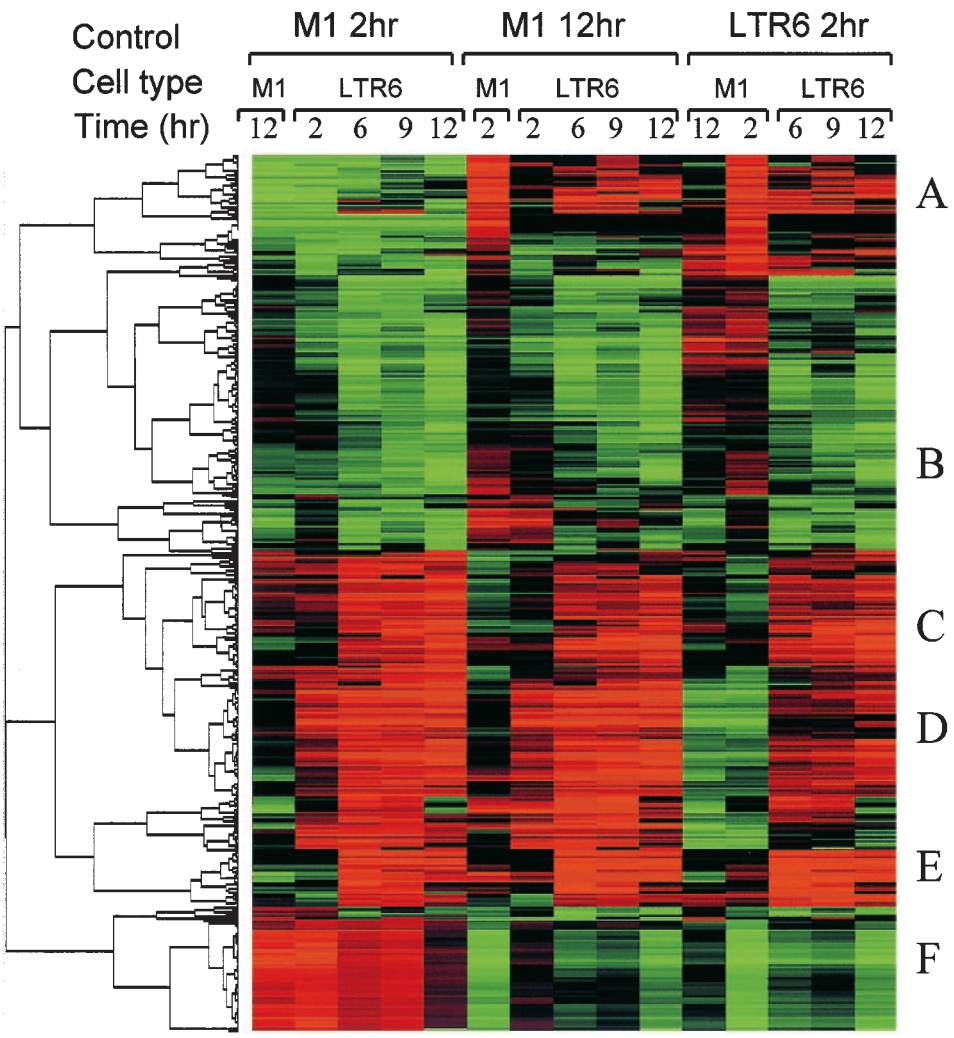

Figure 3 Cluster analysis of gene expression profile following temperature shift to 32°C and activation of p53. A total of 404 genes selected for this analysis were clustered by all pair-wise comparisons, on the basis of their expression kinetics. The three sections represent comparison to M1 cells after 2 h at 32°C (left), M1 cells after 12 h at 32°C (middle) and LTR6 cells after 2 h at 32°C (right). The 404 genes were clustered into six groups (A–F) according to their pattern of expression. The degree of redness and greenness represent induction and repression respectively. Genes whose expression changed by more than threefold in at least two of the time points were selected for this analysis. (See Kaminski *et al*., 2000a,b for details)

pathway. For example, the up regulation of cell cycle inhibitors (e.g. p21waf, Table 1) is accompanied by the down regulation of growth stimulators (e.g. cyclin D1, *myc*, Table 2, see supplementary information on URL).

A central question emerging from our analysis is why the same activated p53 leads to growth arrest in one cell type and to apoptosis in another? The apoptosis related genes in LTR6 show several genes that drive cells to apoptosis, e.g. Fas (Owen-Scaub *et al*., 1995), and gelsolin (Kothakota *et al*., 1997), but these genes are also activated in H1299. However the function of gelsolin may be different in murine and human cells. Gelsolin, is a substrate of caspase 3 and it is cleaved during Fas-mediated apoptosis (Kothakota *et al*., 1997). The caspase cleaved gelsolin fragment can depolymerize actin filaments and cause apoptosis in mouse cells but can be an inhibitor of apoptosis in human cells by closing mitochondrial anion channel VDAC (Kusano *et al*., 2000). It is therefore possible that although gelsolin is activated by p53 in both systems, it has an opposite effect in each of them and may explain, in part, the difference in the phenotype of these cells upon activation of p53. Also important are the genes that are induced only in murine LTR6 cells and not in human H1299 cells (these genes are represented by * in Tables 1 and 2). Interestingly, TNFR18, Sumo-1 and Apaf-1 are up regulated only in LTR6 and not in H1299. These genes were shown to be very effective in driving cells to apoptosis and may explain in part, the different consequence of p53 activity in H1299 and LTR6 cells. In H1299 cells only TNFR6 (Fas) is up regulated whereas in LTR6 both TNFR6 and TNFR18 are up regulated which may amplify the apoptotic signals. The up regulation of Sumo-1 is particularly relevant since Sumo-1 modification of p53 increases its transcriptional activity by at

Table 1 Classification of p53 up-regulated genes in LTR6 cells

Mouse accession no.	*Description*	*2 h*	*6 h*	*9 h*	*12 h*	*Human accession no.*
	Apoptosis					
AB021961	**p53**	**66.4**	**102.4**	**74.6**	**57.8**	**AI909620**
M83649	**Fas antigen/TNFR6**	**4.7**	**13.8**	**18.1**	**54.5**	**X89101**
U82532	TNFR18	1.6	10.3	12.9	11.3	AI923712
J04953	**Gelsolin**	**4.4**	**6.1**	**6**	**2.9**	**X04412**
*Z16410	btg1	2	4.6	5.7	9	X61123
AW060710	EST = PIG8 (Etoposide induced)	2.2	3.6	4.9	6.2	R11732
*X74504	T10 mRNA/human sentrin/SUMO-1	1.7	3.7	3.3	4.3	U83117
AF064071	Apaf-1	1.3	3.1	3.1	7.2	AL135220
	Angiogenesis					
AI840158	EST = Angiopoietin like 2	2.6	24.8	21.3	37	AF125175
	Cell cycle					
*AJ010108	cytosolic adenylate kinase	3.6	15.7	24.5	60	J04809
AW048937	**EST = p21waf**	**2.8**	**7.2**	**13.6**	**23**	**U09579**
AI853375	**EST = MDM2**	**4.6**	**9.3**	**8.5**	**14.3**	**M92424**
M36579	S100 calcium-binding protein A4	2.2	5.3	5.8	9	M80563
*U95826	cyclin G2	−1.2	3.3	4.6	5.3	U47414
AI835968	**EST = Ras inhibitor Rin1**	**−1.5**	**3.4**	**4**	**4.8**	**L36463**
M64292	**B-cell translocation gene 2, anti-proliferative**	**2.6**	**3.6**	**3.1**	**4.1**	**U72649**
	DNA repair/replication					
U40796	ERCC5	3.6	9.7	13.2	58.7	AW502004
X05862	**H2B and H2A histone genes (291A)**	**−1.4**	**4.4**	**3.6**	**3.9**	**Z83336**
	Receptors/ECM					
*Z11886	Notch gene homolog 1, (*Drosophila*)	2.1	8	15	26.4	AI566271
U07634	Eph receptor A2	5.5	5.3	10	5.8	M59371
	Growth factor inhibitor					
*M74180	hepatocyte growth factor-like protein gene	1.3	3.1	6.7	16	U37055
	Metabolism					
*AI843222	EST = Alpha-L Fucosidase precursor	1.6	3.5	9.6	19.3	M29877
U87240	**Mannosidase 2, alpha B1**	**2.6**	**7.5**	**8**	**7.6**	**U37248**
*AF007267	phosphomannomutase Sec53p homolog	1.7	6.7	7.4	11.3	U86070
*L10244	Spermidine	2.4	3.8	6.2	7.8	U40369
AJ006341	peroxisomal integrel membrane protein PMP34	1.5	4.9	5.3	8.6	AI871429
AJ010338	hypothetical protein/human Cubilin	1.5	4.2	4.6	21.2	AF034611
M31690	**Arginosuccinate synthetase 1**	**1.8**	**3.9**	**4.4**	**4.4**	**AA069289**
M69260	Lipocortin 1/human Annexin A1	2.1	4.3	3.1	4.4	ASW379702
*AI838704	EST = Xanthine dehydrogenase	1.4	6	5.9	9	U39487
AW124360	EST = similar to phosphate/PEPyruvate translocator	4	4.7	3.8	6.1	
	Neuronal growth					
*AI840733	EST = Prostaglandin synthase	5.8	12	13.2	20.6	M98539
AW124113	Biotin Carboxylase/human neuronal acidic protein	1.1	3.4	6.3	4.9	AI422580
*X04653	Ly-6 alloantigen/human dihydropyrimidinase	4.8	4.7	5.2	2.9	D78014
AF085192	neural visinin-like protein 3 (NVP-3)	1.5	4.7	3.7	5	AI391924
	Signal transduction					
AW227620	EST = Aryl-hydrocarbon receptor interacting protein	3.2	7	10.7	23.1	U78521
D50264	Phosphatidylinositol glycan, class F	3.1	9.2	10.1	15.7	AW015279
AI850194	EST = UNC-51 like kinase 1	2.9	9.1	7.6	11.2	AL046256
*D78382	DNA for tob family/transducer of ERBB2	2.7	6	6.2	9.3	D38305
AF024637	TYRO protein tyrosine kinase binding protein	3.6	5	4.9	3.8	AI299346
	Transcription					
AF012923	p53-inducible zinc finger protein (Wig-1) mRNA	4.9	15.7	25.5	24.4	AI457344
AF006492	friend of GATA-1 (FOG)	1.7	4.4	4.6	4.1	
	Other					
U21673	T-complex-associated testis expressed 3	−1.4	3.4	4.9	7.4	AA781436
*M32023	Selenium binding protein 1	3.5	3.4	4.7	3.3	U29091
AW123286	**EST = BPM1/human Plectin 1**	**2.6**	**3.1**	**4.2**	**4.4**	**Z54367**
*AI838601	EST = Vamp2/human synaptobrevin 2	2	4.3	3.9	4.1	M36205

Continued

Table 1 (*Continued*)

Mouse accession no.	*Description*	*2 h*	*6 h*	*9 h*	*12 h*	*Human accession no.*
AF111172	lysosomal pepstatin insensitive protease (Cln2)	−1.4	3.4	3.8	6.8	AF039704
AB000636	Procollagen, type XIX, alpha 1	1.5	5.6	3.6	4.8	D38466
M16465	Calpactin I light chain	1.7	3.2	3.4	3.1	AA857434
AF004941	S100A3 calcium binding protein	1.3	3.8	3.3	4.6	Z18948
D00622	Low density lipoprotein receptor related protein	2.2	3.3	3.2	5.5	M63959
*AI853036	EST = Calnexin	1.5	4	3.1	4.4	L60284
AI850335	EST = similar to AF117229	2.2	7.1	14.7	53	
AI849939	EST = Flj20500 hypothetical protein	6.5	9.7	14.5	10.3	AA533627
AI846934	EST = KIAA0188	3.6	8.7	10.1	10.4	AW502927
AA419684	EST = KIAA0188	1.2	9.1	9.9	6	AW502927
AI119347	EST = DD112 gene	1.3	5.3	8.6	7.2	
AJ222580	B99 protein/G2 and S phase expressed	6.1	8.8	7.6	3.5	AA355797
*U22262	Apolipoprotein B editing complex 1	2.8	6.1	7.4	10.1	L26234
AW049897	EST = Fln29	3.4	6.8	6.4	8.3	AW385093
AI465965	**EST = IgG Fc binding protein**	**1.3**	**4**	**6.3**	**26.7**	**D84239**
Y12713	MuERV-L gag, pol and dUTPase genes	−1.2	4.7	6.3	10.1	AW069343
AA624599	EST = Retrovirus related ENV polyprotein	−2.1	6.1	5.8	4.4	
AA763368	EST = KIAA0247	4.1	5.5	5.4	7.5	AI149255
AI852970	EST = HSPC034	−1.1	4.5	5.4	7.5	AA975396
AV250661	EST = imap (immunity associated protein)	1.9	4.8	5	6.1	
U36488	embryonic stem cell phosphatase (Esp)	−2.1	5	5	21.4	
AB025406	sid23p/rat Destrin (actin depolymerising factor)	1.5	3.2	3.7	4.7	S65738
AB011370	Ankhzn mRNA	2	3.8	3.4	6	
M90388	**protein tyrosine phosphatase (70zpep)**	**1.9**	**3.3**	**3.3**	**4.3**	**AW504119**
AV342910	EST = weekly similar to Semaphorin C	1.1	14.3	12.4	3.7	AL390081
AA266467	EST = weekly similar to Semaphorin C	1	8.4	10.1	5.9	AL390081
AA260145	EST = weekly similar to env protein	1.1	3.6	4.8	7.6	
AA796690	EST	1.4	3.8	3.4	5.6	AK027160
AI854863	EST	1.7	6.7	11.3	32.1	
AI594426	EST	1.6	5.1	8.6	6.1	
AI553536	EST	2.6	6.4	7.1	13.3	
AW122061	EST	2	3.6	6.7	12.4	
AW045710	EST	3.8	6.6	6.7	4.8	
C79615	EST	1.3	5.6	6.2	12.1	
AI839880	EST	1	4.6	6.1	8.8	
AW125272	EST	−1.9	6.8	5.7	4.4	
AI181346	EST	1	3.5	5.6	29	
AI847631	EST	2.1	4.8	5.3	6.7	
AI642245	EST	−1	4.4	5.1	7.4	
AI841606	EST	−1.2	3.3	4.2	16.5	
AW123796	EST	1.2	3.2	4	4.4	
AI593759	EST	1.9	3.9	3.9	4.7	
AA407599	EST	−1.5	4.7	3.7	4.6	
AW211760	EST	1.7	3.3	3.7	6.3	
AW124231	EST	1.6	3.3	3.2	5.5	

The list contains genes from LTR6 cells that showed over threefold induction in at least three time points compared to M1 cells at 2 h at 32°C. The human accession numbers were extracted from the Unigene and Genecards databases. The ratios of gene expression at different time points (2, 6, 9 and 12 h at 32°C) are shown. Accession numbers in bold indicate the genes that are similarly induced in both murine LTR6 and human H1299 cells (Kannan *et al*., 2001). *Indicates genes that are induced only in murine LTR6 and not in human H1299 cells, although they are present in both human and murine DNA chips

least twofold. Lysine 386 in human p53 is the site for Sumo-1 modification. When this site was mutated (K386R) no effect of Sumo-1 on increased p53 transactivation was observed (Rodriguez *et al*., 1999; Gostissa *et al*., 1999), and the mutant p53 has a lower apoptotic activity (Muller *et al*., 2000). Hence the activation of Sumo-1 and its binding to wild-type p53, may amplify the apoptotic signal.

Another important gene upregulated in LTR6 is Apaf-1, the mammalian homologue of *C. elegans CED-4*. The transcriptional activation of Apaf-1 in LTR6 at 32°C was also confirmed by Northern blot analysis (data not shown). Apaf-1 is a central element in the mitochondrial pathway of apoptosis. Apaf-1 participates in the CASP9-dependent activation of CASP3, through oligomerization of the CASP9/Apaf-1 (apoptosome) complex with cytochrome-*c*. Apaf-1 deficient mice exhibit reduced apoptosis, hyperproliferation of neuronal cells and embryonal lethality at 16.5 embryonic days (Cecconi *et al*., 1998; Yoshida *et al*., 1998). Disruption of Apaf-1 in cells dramatically reduced p53-dependent apoptosis and facilitates oncogenic transformation (Soengas *et al*., 1999). Our results suggest that Apaf-1 is a downstream effector of p53 in the apoptotic pathway. The proapoptotic genes induced by p53 in LTR6 (Table 1) may collectively

provide the necessary signals to induce apoptosis. It is, however, noteworthy that the activation of Apaf-1 by p53 may be the most important factor in the pathway of p53- driven apoptosis in this system.

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