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Over-expressed ZF5 Gene Product, a c-*myc*-binding Protein Related to GL1-Kruppel Protein, Has a Growth-suppressive Activity in Mouse Cell Lines

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ZF5 encodes a zinc finger protein, which contains five C2H2-type zinc fingers showing homology with the zinc finger of the Kruppel family, and binds to two sites in the mouse c-*myc* promoter. We report the effect of over-expression of ZF5 on cell growth. $ZnCl_2$ treatment suppressed the growth of a mouse fibroblast cell line (L cells) transfected with the wild-type ZF5 gene driven by the metallothionein promoter. Cells transfected with the wild-type ZF5 gene formed colonies two- to fivefold less efficiently than those transfected with the mutant ZF5 gene in P19, NIH3T3, 3T3-L1 and L cells. Over-expression of ZF5 did not cause c-*myc* down-regulation or arrest of the cell cycle, but increased the DNA content.

Key words: ZF5 — c-*myc*-binding protein — Growth suppressor

The zinc finger motif has been identified in many DNA-binding proteins, including a wide variety of eukaryotic transcriptional factors.[1–4] Several reports have demonstrated that the zinc finger proteins have a broad spectrum of biological functions and the Kruppel gene is known to be related to *Drosophila* segmentation.[5] EGR1[6,7] and EGR2[8,9] proteins have been reported as factors involved in both differentiation of various tissues and the cell cycle. Additionally, the Wilms' tumor gene has been shown to be one of the proto-oncogenes.[10] However, many factors have not been fully characterized as regards their function in cell growth.

Previously, we have cloned a cDNA encoding a C2H2 zinc finger protein, ZF5.[11] This protein contains five Kruppel-type zinc fingers at the C-terminus. ZF5 binds to two GC-rich sites in the mouse c-*myc* promoter (−273 to −267 and −250 to −244); one site is located between PRF[12] and YY1[13] sites, while the second site is immediately 3′ of the YY1 site. It also binds to the herpes simplex virus thymidine kinase promoter (tk), a site shown to bind the zinc finger protein, Sp1.[14] In addition, ZF5 was shown to be a transcriptional repressor in a cotransfection assay using c-*myc* or tk promoters. Recently, Bardwell and Treisman reported that ZF5 has a POZ (poxvirus and zinc finger) domain in the N-terminus and this domain mediates protein-protein interaction.[15]

Here, we report that over-expression of ZF5 resulted in a suppressive effect on the growth of mouse cell lines, and this expression was not accompanied with c-*myc* down-regulation or arrest of the cell cycle.

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MATERIALS AND METHODS

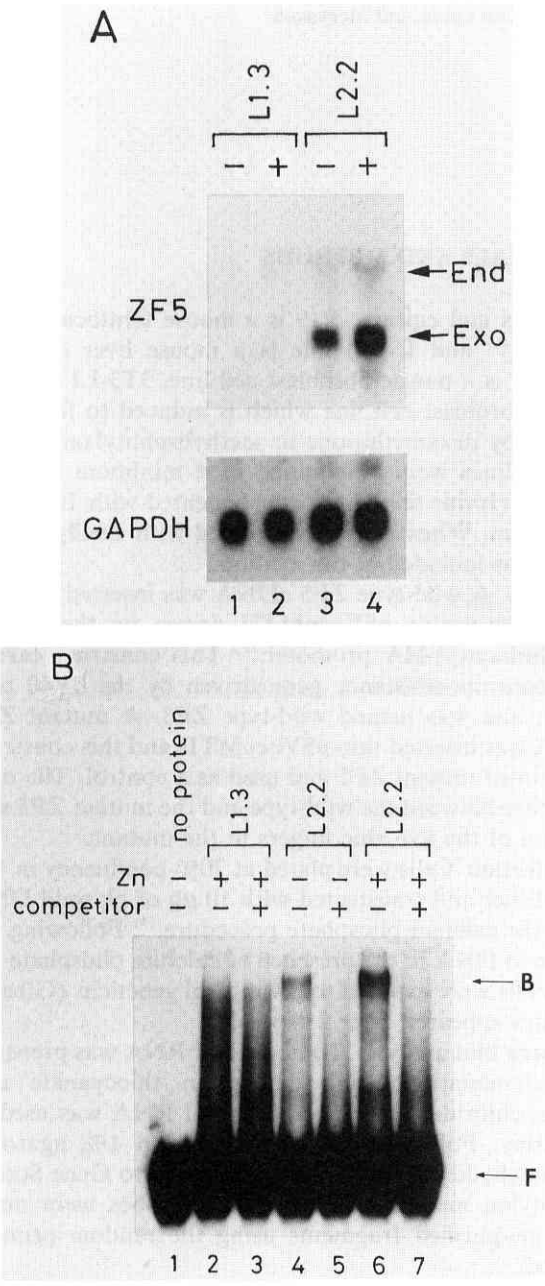
Cell lines and culture P19 is a mouse teratocarcinoma cell line,[16] and L cell line is a mouse liver cell line. NIH3T3 is a mouse fibroblast cell line. 3T3-L1 is also a mouse fibroblast cell line which is induced to form adipocytes by dexamethasone or methylisobutylxanthine.[17] All cell lines were maintained in α minimum essential medium (Irvine Scientific) supplemented with 10% fetal calf serum. When cells were treated with $ZnCl_2$, 50 μM $ZnCl_2$ was included in the medium.

Plasmids A wild-type ZF5 cDNA was inserted into the expression vector pSVneoMTII driven by the human metallothionein-IIA promoter.[18] This construct carries the neomycin-resistance gene driven by the SV40 promoter, and was named wild-type ZF5. A mutant ZF5 cDNA was inserted into pSVneoMTII and this construct was named mutant ZF5 and used as a control. The only difference between the wild-type and the mutant ZF5 was deletion of the five zinc fingers in the mutant.

Transfection Cells were plated at 70% confluency in 100 mm^2 dishes and transfected with 10 μg of plasmid DNA using the calcium phosphate procedure.[19] Following exposure to DNA in the presence of calcium phosphate for 24 h, cells were exposed to 0.4 mg/ml geneticin (Gibco). Colonies appeared after 2–4 weeks.

Northern blot analysis Total cellular RNA was prepared by sedimentation using guanidium thiocyanate and cesium chloride[20] and 20 μg of total RNA was used in the assay. Following electrophoresis on 1% agarose/formaldehyde gel, RNA was transferred to Gene Screen Plus nylon membrane (Du Pont). Probes were made from gel-purified fragments using the random priming method.

Preparation of nuclear extracts Nuclear extracts were prepared from L cells. Nuclei were prepared by a modified version of the procedure of Storb *et al.*[21] After treatment with 50 μM $ZnCl_2$ for 24 h, cultured cells were harvested and washed with ice-cold phosphate-buffered saline (PBS). Washed cell pellets were suspended in a detergent lysis buffer (12.5 mM Tris, pH 7.5, 12.5 mM KCl, 3.75 mM $MgCl_2$, 0.5% NP-40, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.2 mM EDTA, 30% sucrose) and cells were lysed by tilting. Nuclei were harvested and washed with RSB (10 mM NaCl, 10 mM Tris, pH 7.5, 10 mM $NgCl_2$). Nuclei were suspended in an appropriate volume of extraction buffer A (10 mM HEPES, pH 8.0, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 200 mM NaCl, 5% glycerol) and an equal volume of extraction buffer B (extraction buffer A with 600 mM NaCl) was added. Nuclei were extracted on ice for 30 min. The nuclear extract was clarified by ultra-



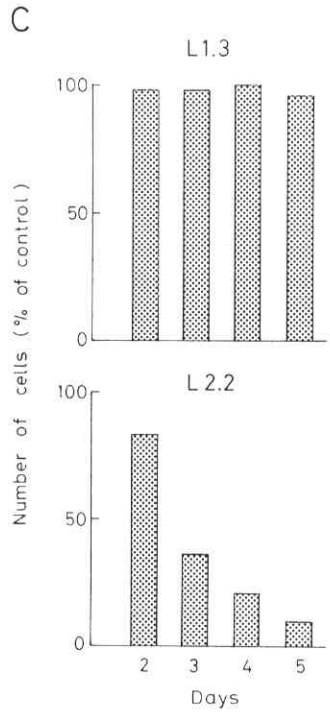


Fig. 1. A) Northern blot analysis using total RNA (30 μg) of L1.3 and L2.2 cells, which had been treated with 50 μM $ZnCl_2$ for 24 h. ZF5 cDNA (zinc finger coding region, 450 bp) and rat glyceraldehyde phosphate dehydrogenase (GAPDH) were used as probes. "End" and "Exo" represent the endogenous and exogenous ZF5 expression, respectively. Presence (+) or absence (−) of $ZnCl_2$ (50 μM) is shown. B) Electrophoretic mobility shift assay using the ZF5 binding site on the c-*myc* promoter as a probe and the nuclear extract (1 μg) from L1.3 or L2.2 cells, treated with 50 μM $ZnCl_2$ for 24 h. For the competition assay, 100-fold molar excess of unlabeled ZF5 oligonucleotides (see "Materials and Methods") was added. "Zn" indicates treatment with 50 μM $ZnCl_2$. Probe complexed with exogenous ZF5 (B) and free probe (F). "Competitor" indicates the ZF5 oligonucleotides and the presence (+) or absence (−) of the competitor is shown. C) Growth rate of L1.3 and L2.2 cells. Cells were counted at daily intervals for 5 days. The data are expressed as a percentage of the number of cells obtained without 50 μM $ZnCl_2$. The values represent the average of duplicate samples with standard deviations up to 10%.

centrifugation (35,000 rpm) for 30 min, dialyzed against chromatography buffer A (20 m*M* HEPES, pH 8.0, 0.1 m*M* EDTA, 100 m*M* NaCl, 20% glycerol, 0.5 m*M* DTT, 0.2 m*M* PMSF), quick-frozen in liquid N_2 and stored at −70°C. Protein concentration was measured by the method of Bradford[22] with bovine serum albumin as a standard.

Electrophoretic mobility shift assay (EMSA) 5′-End-labeled mouse c-*myc* fragment (−230 to −270) was used as a probe. Binding reaction were performed in 15 μl of binding buffer (10 m*M* Tris, pH 7.5, 50 m*M* NaCl, 5% glycerol, 1 m*M* DTT, 100 μM $ZnSO_4$) containing 2–4 μg of poly(dA-dT)·poly(dA-dT) (Pharmacia) and 0.1 to 0.5 ng of labeled probe DNA. Binding mixtures were incubated at 22°C for 20 min prior to loading onto 4.5% acrylamide gel in 0.25×TBE (23 m*M* Tris, 23 m*M* borate, 0.5 m*M* EDTA, 5% glycerol). Gels were electrophoresed at 100 V at 22°C.

Flow cytometric analysis Cells were trypsinized and the cell number was counted with a Coulter counter and adjusted to 1×10^6 cells/ml. The cells were fixed with 70% ethanol at −20°C for more than 2 h and washed with PBS, following treatment with DNase-free RNase A (Sigma) for 30 min. After staining with 50 μg/ml propidium iodide (Sigma), the cells were analyzed in a Cytotron-Absolute Flow Cytometry System (Ortho Diagnostic Systems, Johnson-Johnson Co.). Twelve thousand cells were counted for analysis. The percentage of the cells in each cell cycle stage (G1, S and G2/M) was calculated with a Multicycle computer program (Phoenix Flow Systems).

RESULTS

We wished to examine the effects of ZF5 on cell growth. For this purpose, we prepared several clones stably transfected with the wild-type ZF5, and one of them was designated L2.2. One clone similarly transfected with the vector alone was named L1.3 and used as a control. ZF5 expression was studied by Northern blot analysis using the zinc finger motifs of ZF5 cDNA as a probe. As shown in Fig. 1A, a small amount of exogenous ZF5 transcript was already detected in L2.2 cells without $ZnCl_2$ treatment (lane 3), but not in L1.3 cells (lane 1), whereas the band of endogenous ZF5 transcript was very weak. When these clones were treated with 50 μM $ZnCl_2$ for 24 h, the exogenous ZF5 transcript significantly increased in L2.2 cells (lane 4). This result indicates that the exogenous ZF5 gene was stimulated by $ZnCl_2$ treatment.

Since we wished to test whether or not the over-expressed ZF5 gene product acts as a functional protein, we performed EMSA using the ZF5 binding site of mouse c-*myc* gene as a probe. As shown in Fig. 1B, a DNA-protein complex was observed using nuclear extract from L2.2 cells (lanes 4 and 6), but not with extract from L1.3 cells (lanes 2 and 3). Interestingly, this complex was already apparent in the extract from untreated L2.2 cells, in accordance with the results of Northern blot analysis. ZF5 oligonucleotides containing ZF5-binding site competed for this complex (lanes 5 and 7). Furthermore, the band of this complex was significantly stronger with extract from $ZnCl_2$-treated cells (lane 6) than from untreated cells (lane 4). Therefore, we speculate that exogenously expressed ZF5 can bind to the ZF5-binding site and acts as a functional protein. However, to conclude definitively that a protein is a DNA-binding protein, either it is necessary to use pure protein or the complex must be eliminated or super-shifted by pretreatment with a specific antibody. Finally, in order to examine the growth properties of L1.3 and L2.2 cells, we measured the growth rate of both clones under $ZnCl_2$ treatment. Fig. 1C shows the average of duplicate experiments; the growth of L2.2 cells was suppressed in the presence of $ZnCl_2$ compared with that of untreated cells. On the other hand, L1.3 cells were not affected by this treatment.

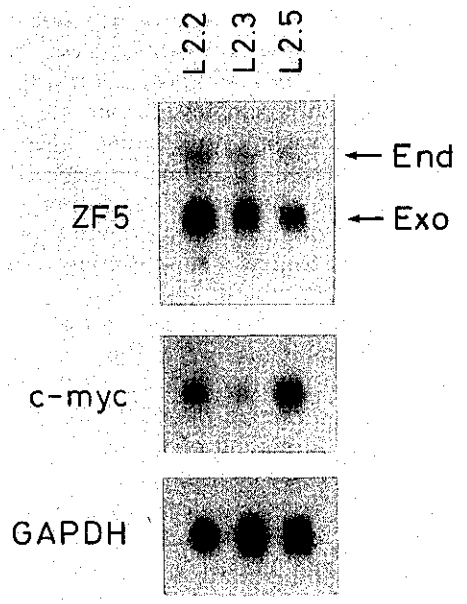


Fig. 2. Northern blot analysis using total RNA from L cell clones. Cells were treated with 50 μM $ZnCl_2$ for 24 h. We used mouse c-*myc* exon 3 (370 bp) as a c-*myc* probe. Other experimental details were the same as described in the legend to Fig. 1A.

To confirm the above results, we tested the growth properties using other L cell clones, L2.3 and L2.5, which were transfected with the wild-type ZF5 gene. As shown in Fig. 2, both clones expressed the exogenous ZF5 tran-

scripts as well as L2.2 cells after $ZnCl_2$ treatment for 24 h. We also measured the growth suppression of L2.2, L2.3 and L2.5 cells. Table I demonstrates that the growth of all three clones was suppressed, but the degree of growth suppression was not directly correlated with the amount of ZF5 mRNA. The c-*myc* expression also did not parallel the ZF5 expression. These results indicate that over-expressed ZF5 acted as a growth suppressor in L cells, although the degree of suppression varied among clones.

If ZF5 is truly suppressive for cell growth, one would expect that a high expression level of this gene would lead to suppression of cell growth in other mouse cell lines. To examine this issue, we tested the effect of the wild-type ZF5 on the colony formation of various cell lines. The mutant ZF5 was used as a control (data not shown). The appropriate constructs were transfected into teratocarcinoma cell line P19, L cell line and two mouse fibroblast cell lines, NIH3T3 and 3T3-L1. Subsequently geneticin-resistant colonies were counted. Cells transfected with the wild-type ZF5 formed two- to fivefold fewer colonies than those transfected with the mutant ZF5, although the efficiency varied among different cell lines (Table II). In

Table I. Relationship between Growth Suppression and the Expression of ZF5 or c-*myc* in L Cell Clones

L cell clone	ZF5 mRNA	c-*myc* mRNA	Percent of control
L2.2	2.9	2.6	10
L2.3	1.1	1	45
L2.5	1	4.3	29

"ZF5 mRNA" and "c-*myc* mRNA" are presented as the ratio of ZF5 or c-*myc* to GAPDH expression as determined with a Bio-Imaging Analyzer (Fuji Film). "Percent of control" is presented as the ratio of cell number in the presence to that in the absence of $ZnCl_2$ (50 μM). Cells were counted after 5 days of culture. The values represent the average of duplicate experiments with standard deviations up to 29%. Other experimental details are the same as described in the legends to Fig. 1A, C and Fig. 2.

Table II. Colony Formation after Transfection Using the Wild-type or the Mutant ZF5 Construct

Cell line	No. of geneticin-resistant colonies formed	
	Mutant ZF5	Wild-type ZF5
P19	184	59
NIH3T3	153	28
L cell	51	23
3T3-L1	206	67

For each experiment, two 56 cm^2 dishes were used and the total colonies were counted after 3 to 4 weeks of selection with geneticin (0.4 mg/ml). The figures represent the average of two dishes, with standard deviations up to 20%.

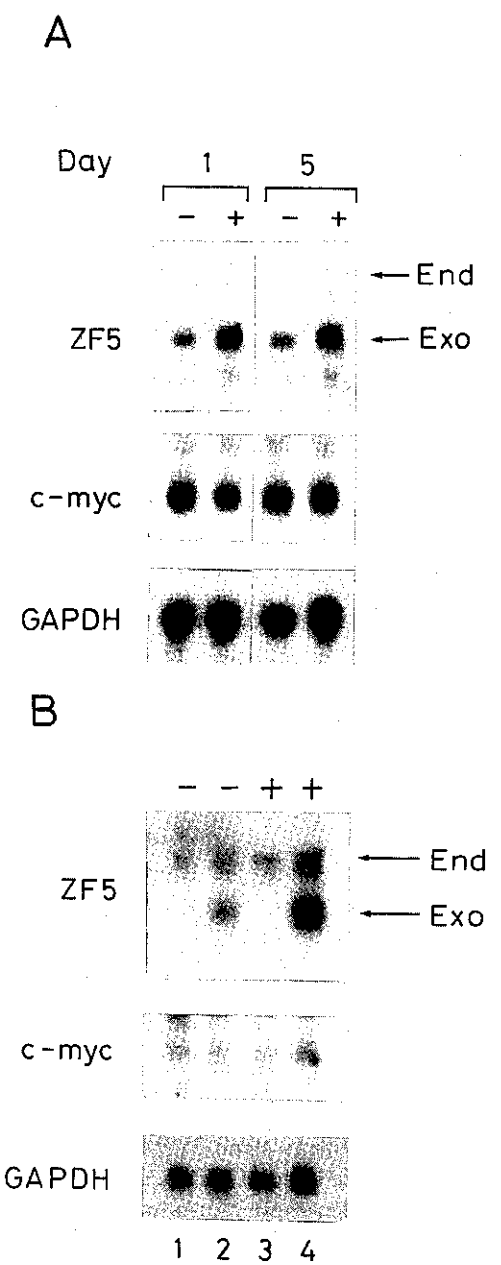


Fig. 3. Northern blot analysis using total RNA from L2.2 cells cultured in the presence (+) or absence (−) of $ZnCl_2$ (50 μM). Other experimental details were the same as described in the legends to Fig. 1A and Fig. 2. A) L2.2 cells. B) NIH3T3 cells. N1.3 cells (lanes 1 and 3) and N2.5 cells (lanes 2 and 4).

all cell lines tested, the number of colonies after transfection with the vector alone was similar to that produced by cells transfected with the mutant ZF5 (data not shown). These results indicate that the over-expressed ZF5 also suppressed the clonal growth of these mouse cell lines.

Since ZF5 binds on the c-*myc* promoter and represses its transcription, as shown in our previous report,[11] we expected that ZF5 over-expression would be accompanied with c-*myc* down-regulation. So, we examined whether or not an excess amount of ZF5 expression leads to c-*myc* down-regulation using L2.2 cells with or without $ZnCl_2$ treatment. As shown in Fig. 3A, exogenous ZF5-transcripts significantly increased after $ZnCl_2$ treatment on days 1 and 5, but c-*myc* transcripts did not change. We could not detect any change of c-*myc* expression after $ZnCl_2$ treatment on days 2, 3 and 4 (data not shown). Using NIH3T3 cells, we prepared one clone, N2.5, stably transfected with the wild-type ZF5 (Fig. 3B). Another clone transfected with the vector alone was named N1.3. When these clones were treated with $ZnCl_2$, we could detect a significant increase in ZF5 expression but not in c-*myc* expression (lanes 2 and 4). Therefore we conclude that ZF5 over-expression did not lead to c-*myc* down-regulation.

To investigate the possibility that ZF5 plays some role in the cell cycle, we performed Coulter-counter analysis using $ZnCl_2$-treated or untreated L cells. Fig. 4 shows a representative result. The DNA content of the L1.3 cells was decreased by $ZnCl_2$ treatment, whereas that of L2.2 cells was increased. This result indicates that the DNA content of L cells was augmented by the over-expressed ZF5 gene product. Surprisingly, L2.2 cells were not inhibited throughout the cell cycle.

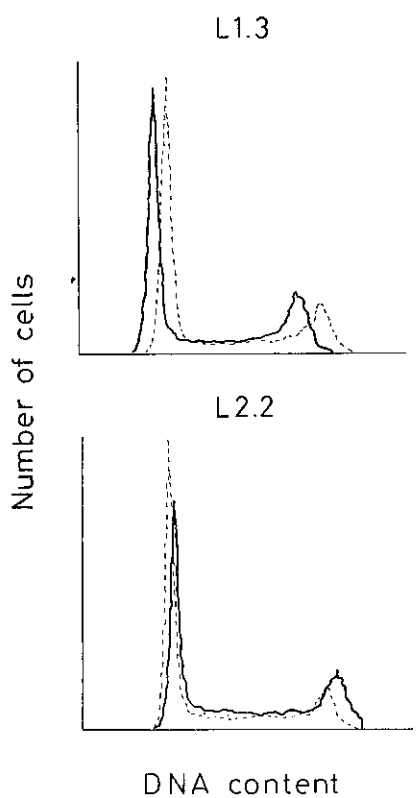


Fig. 4. Cell cycle analysis of L1.3 and L2.2 cells. Histograms of the flow cytometric data obtained after 5 days of culture are presented. Control cells (broken line). $ZnCl_2$-treated cells (solid line).

DISCUSSION

In this report, we demonstrate that a newly identified Kruppel family protein, ZF5, acts as a suppressive factor for proliferation of mouse cell lines, and also that cells transfected with the wild-type ZF5 gene form fewer colonies than those transfected with a mutant gene. In addition, the ZF5 over-expression is not accompanied with c-*myc* down-regulation or cell cycle arrest at any specific point.

As shown in our previous report, expression of ZF5 is ubiquitous, though very weak, in all cell lines tested and ZF5 gene product binds to GC-rich sites, which appear ubiquitously on promoters.[11] These results indicate that ZF5 may regulate many genes and play a fundamental role in transcription. Therefore, expression of ZF5 at high levels may be deleterious to cells by overwhelming the normal regulatory processes. In fact, over-expressed ZF5 induced the growth suppression of L cell clones (see Table I) and also suppressed the colony formation of several mouse cell lines, although the degree of suppression varied among cell lines (see Table II). This suggests that different amounts of ZF5 could be required for growth suppression in each cell line.

It was shown that introduction of wild-type p53 gene suppressed the growth of various types of cells carrying endogenous mutant p53 gene, or lacking p53 gene.[23-27] This indicates that the presence of the mutant or the absence of the wild-type protein is essential for the growth of cells with a defective or mutant p53 gene. The suppression of cell growth by the wild-type RB is consistent with this scenario,[28-30] though the effects of Kruppel family proteins on cell growth have not yet been explored. Our results demonstrate for the first time that one of the Kruppel family proteins has a growth-suppressive effect on mammalian cell lines.

We showed that ZF5 represses the mouse c-*myc* promoter by using chloramphenicol acetyl transferase (CAT) assay.[11] However, even when ZF5 gene was over-expressed, the level of c-*myc* expression did not

change (see Fig. 3A, B). This result indicates that ZF5 acts as a repressor on the c-*myc* promoter only *in vitro*, but not *in vivo*. Many other proteins also bind in the region of the ZF5-binding site[12, 13] and we think that the overall regulatory mechanisms must be complex. Much more work is needed before the *in vivo* role of ZF5 can be understood. Furthermore, we have preliminary results which suggest that ZF5 activates other promoters (unpublished data), whereas over-expression of Max is known to suppress the activity of Myc/Max dimers.[31] We speculate that over-expressed ZF5 acts as an activator of the Max promoter, while over-expressed Max suppresses the Myc function, and this effect induces the cell growth-suppressive activity.

As shown in Fig. 4, there was no single point in the cell cycle at which the cell growth was blocked. We assume that ZF5 suppressed the growth throughout the cell cycle. The nature of ZF5 DNA-binding to GC-rich regions indicates that ZF5 may work on many genes in the cell cycle, repressing some and activating others. Change in cell volume has been shown to be an early event in the commitment of Friend erythroleukemia cells[32] and the spontaneous differentiation of an EC cell line,[33] so the increase in cell volume of L cells may be related to differentiation induction by over-expressed ZF5.

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REFERENCES

1) Miller, J., McLachlan, A. D. and Klug, A. Repetitive zinc-binding domains in the protein transcription factor IIIA from *Zenopus* oocytes. *EMBO J.*, **4**, 1609–1614 (1985).
2) Schuh, R., Aicher, W., Gaul, U., Cote, S., Preiss, A., Maier, D., Seifert, E., Nauber, U., Schroder, C., Kemler, R. and Jackle, H. A conserved family of nuclear proteins containing structural elements of the finger protein encoded by Kruppel, a *Drosophila* segmentation gene. *Cell*, **47**, 1025–1032 (1986).
3) Chowdhury, K., Deutsch, U. and Gruss, P. A multigene family encoding several "finger" structures is present and differentially active in mammalian genomes. *Cell*, **48**, 771–778 (1987).
4) Ruppert, J. M., Kinzler, K. W., Wong, A. J., Bigner, S. H., Kao, F. T., Law, M. L., Seuanez, H. N., O'Brien, S. J. and Vogelstein, B. The GLI-Kruppel of human genes. *Mol. Cell. Biol.*, **8**, 3104–3113 (1988).
5) Rosenberg, U. B., Schroder, C., Preiss, A., Kienlin, A., Cote, S., Riede, I. and Jackle, H. Structural homology of the product of the *Drosophila* Kruppel gene with *Xenopus* transcription factor IIIA. *Nature*, **319**, 336–339 (1986).
6) Christy, B. A., Lau, L. F. and Nathans, D. A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with "zinc finger" sequences. *Proc. Natl. Acad. Sci. USA*, **85**, 7857–7861 (1988).
7) Sukhatme, V. P., Cao, X. M., Chang, L. C., Tsai-Morris, C. H., Stamenkovich, D., Ferreira, P. C., Cohen, D. R., Edwards, S. A., Shows, T. B., Curran, T., LeBeau, M. M. and Adamson, E. D. A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. *Cell*, **53**, 37–43 (1988).
8) Chavrier, P., Zerial, M., Lemaire, P., Almendral, J., Bravo, R. and Charnay, P. A gene encoding a protein with zinc fingers is activated during G0/G1 transition in cultured cells. *EMBO J.*, **7**, 29–35 (1988).
9) Joseph, L. J., LeBeau, M. M., Jamieson, G. A., Jr., Acharya, S., Shows, T. B., Rowley, J. D. and Sukhatme, V. P. Molecular cloning, sequencing, and mapping of EGR2, a human early growth response gene encoding a protein with "zinc-binding finger" structure. *Proc. Natl. Acad. Sci. USA*, **85**, 7164–7168 (1988).
10) Gessler, M., Poustka, A., Cavenee, W., Neve, R. L., Orkin, S. H. and Bruns, G. A. Homozygous deletion in Wilms tumors of a zinc-finger gene identified by chromosome jumping. *Nature*, **343**, 774–778 (1990).
11) Numoto, M., Niwa, O., Kaplan, J., Wong, K., Merrell, K., Kamiya, K., Yanagihara, K. and Calame, K. Transcriptional repressor ZF5 identifies a new conserved domain in zinc finger proteins. *Nucleic Acids Res.*, **21**, 3767–3775 (1993).
12) Kakkis, E. and Calame, K. A plasmacytoma-specific factor binds the c-myc promotor region. *Proc. Natl. Acad. Sci. USA*, **84**, 7031–7035 (1987).
13) Shi, Y., Seto, E., Chang, L. S. and Shenk, T. Transcriptional repression by YY1, a human GLI-Kruppel-related protein, and relief of repression by adenovirus E1A protein. *Cell*, **67**, 377–388 (1991).
14) Kadonaga, J. T., Carner, K. R., Masiarz, F. R. and Tjian, R. Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. *Cell*, **51**, 1079–1090 (1987).
15) Bardwell, V. J. and Treisman, R. The POZ domain: a conserved protein-protein interaction motif. *Genes Dev.*, **8**, 1664–1677 (1994).
16) Jones-Villeneuve, E. M., Rudnicki, M. A., Harris, J. F. and McBurney, M. W. Retinoic acid-induced neural differentiation of embryonal carcinoma cells. *Mol. Cell. Biol.*, **3**, 2271–2279 (1983).
17) Russell, T. R. and Ho, R. Conversion of 3T3 fibroblasts

into adipose cells: triggering of differentiation by prostaglandin F2alpha and 1-methyl-3-isobutylxanthine. *Proc. Natl. Acad. Sci. USA,* **73,** 4516–4520 (1976).
18) Kaneko-Ishino, T., Kume, T. U., Sasaki, H., Obinata, M. and Oishi, M. Effect of c-myc gene expression on early inducible reactions required for erythroid differentiation *in vitro*. *Mol. Cell. Biol.,* **8,** 5545–5548 (1988).
19) Graham, F. L. and van der Eb, A. J. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology,* **52,** 456–467 (1973).
20) Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry,* **18,** 5294–5299 (1979).
21) Storb, U., Wilson, R., Selsing, E. and Walfied, A. Rearranged and germline immunoglobulin kappa genes: different states of DNase I sensitivity of constant kappa genes in immunocompetent and noimmune cells. *Biochemistry,* **20,** 990–996 (1981).
22) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **72,** 248–254 (1976).
23) Baker, S. J., Markowitz, S., Fearon, E. R., Willson, J. K. and Vogelstein, B. Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science,* **249,** 912–915 (1990).
24) Mercer, W. E., Shields, M. T., Amin, M., Sauve, G. J., Appella, E., Romano, J. W. and Ullrich, S. J. Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53. *Proc. Natl. Acad. Sci. USA,* **87,** 6166–6170 (1990).
25) Casey, G., Lo-Hsueh, M., Lopez, M. E., Vogelstein, B. and Stanbridge, E. J. Growth suppression of human breast cancer cells by the introduction of a wild-type p53 gene. *Oncogene,* **6,** 1791–1797 (1991).
26) Isaacs, W. B., Carters, B. S. and Ewing, C. M. Wild-type p53 suppresses growth of human prostate cancer cells containing mutant p53 alleles. *Cancer Res.,* **51,** 4716–4720 (1991).
27) Ramqvist, T., Magnusson, K. P., Wang, Y., Szekely, L., Klein, G. and Wiman, K. G. Wild-type p53 induce apoptosis in a Burkitt lymphoma (BL) line that carries mutant p53. *Oncogene,* **8,** 1495–1500 (1993).
28) Huang, H. J., Yee, J. K., Chen, P. L., Bookstein, R., Friedmann, T., Lee, E. Y. and Lee, W. H. Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells. *Science,* **242,** 1563–1566 (1988).
29) Goodrich, D. W., Wang, N. P., Qian, Y. W., Lee, E. Y. and Lee, W. H. The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. *Cell,* **67,** 293–302 (1991).
30) Hinds, P. W., Mittnacht, S., Dulic, V., Arnold, A., Reed, S. I. and Weinberg, R. A. Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell,* **70,** 993–1006 (1992).
31) Amati, B. and Land, H. Myc-Max-Mad: a transcription factor network controlling cell cycle progression, differentiation and death. *Curr. Opin. Genet. Dev.,* **4,** 102–108 (1994).
32) Loritz, F., Bernstein, A. and Miller, R. G. Early and late volume changes during erythroid differentiation of cultured Friend leukemic cells. *J. Cell. Physiol.,* **90,** 423–438 (1977).
33) Pfeiffer, S. E., Jakob, H., Mikoshiba, K., Dubois, P., Guenet, J. L., Nicolas, J. F., Gaillard, J., Chevance, G. and Jakob, F. Differentiation of a teratocarcinoma line: preferential development of cholinergic neurons. *J. Cell Biol.,* **88,** 57–66 (1981).