

Regulatable killing of eukaryotic cells by the prokaryotic proteins Kid and Kis

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Plasmid R1 inhibits growth of bacteria by synthesizing an inhibitor of cell proliferation, Kid, and a neutralizing antidote, Kis, which binds tightly to the toxin. Here we report that this toxin and antidote, which have evolved to function in bacteria, also function efficiently in a wide range of eukaryotes. Kid inhibits cell proliferation in yeast, *Xenopus laevis* and human cells, whilst Kis protects. Moreover, we show that Kid triggers apoptosis in human cells. These effects can be regulated *in vivo* by modulating the relative amounts of antidote and toxin using inducible eukaryotic promoters for independent transcriptional control of their genes. These findings allow highly regulatable, selective killing of eukaryotic cells, and could be applied to eliminate cancer cells or specific cell lineages in development.

Keywords: gene therapy/Kid/Kis/*parD*/regulated cell killing

Introduction

Prokaryotic plasmids have developed genetic systems that increase their stable maintenance in bacterial hosts. One group of them, called killer systems, eliminate bacteria that have lost the plasmid during cell division (Jensen and Gerdes, 1995). *ParD* is a protein killer system of Gram-negative plasmid R1, which is composed of two genes: *kis* (for *killing suppressor*) and *kid* (for *killing determinant*) that encode the antidote (Kis; 10 kDa) and the toxin (Kid; 12 kDa), respectively. Both genes are organized in a bicistronic operon in which the toxin is located downstream of the antidote, and their expression is controlled in several ways: by coupled transcription; by post-transcriptional processing of some of its bicistronic mRNAs to produce *kis+/kid-* and *kis+/kid+* messengers; by overlapping translation of both genes; and by a very tight interaction between Kis and Kid to generate a non-toxic complex that represses its own transcription (Bravo *et al.*, 1987, 1988; Ruiz-Echevarría *et al.*, 1991, 1995). These controls avoid the synthesis of the toxic component if its antidote has not been translated previously and ensure a

balanced production of the antidote relative to the toxin (Figure 1A). Under normal circumstances, both components of this killer system are synthesized at a basal level from their plasmid, allowing the bacterial host to survive. However, the stability of the antidote Kis is lower than that of the toxin Kid (Tsuchimoto *et al.*, 1992). In bacteria that lose the plasmid and thus lack continuous synthesis of the unstable antidote, its more rapid degradation leads to an excess of non-neutralized Kid protein, which is toxic to the host and inhibits its proliferation (Figure 1B).

Pathogenic bacteria have evolved many toxins to attack eukaryotic cells and many of these have had valuable practical applications (Fitzgerald, 1996; Culver, 1997). However, plasmid stability systems differ from these. They have evolved in bacteria to kill bacteria, but under the tight control of effective antidotes. If a similar system could be developed for eukaryotic cells, it would have many applications. For example, gene therapy approaches for selectively killing cancer cells depend on highly selective targeting or expression of toxins to cause maximum damage to cancer cells whilst minimizing damage to normal cells. A higher degree of selectivity could be achieved if the toxin is targeted to tumour cells and non-tumour cells are protected from the action of the toxin by a specific antidote. In this work, we show that Kid inhibits cell proliferation in eukaryotes and kills human cells by apoptosis. Furthermore, we demonstrate that the antidote Kis overcomes the toxic effect of Kid in yeast, *Xenopus laevis* and human cells. We also establish that it is possible to regulate these effects in eukaryotes by means of independent transcriptional regulation of *kis* and *kid*, and we discuss strategies for exploiting these results for selectively killing tumour cells and for selective cell-lineage knock-outs in development.

Results

Expression of Kid inhibits yeast cell growth and co-expression of Kis rescues

The first purpose of this work was to test whether the components of the *parD* system of plasmid R1 could function in eukaryotes. As mentioned above, this killer system is kept silent in *Escherichia coli* by means of a complex genetic and molecular regulatory circuit, based mainly on the bicistronic nature of the *parD* operon. Although some bicistronic operons exist in eukaryotes (McBratney *et al.*, 1993; Cornelis *et al.*, 2000), it would be technically difficult to use them effectively for this purpose. Therefore we decided to study the effect of Kis and Kid in *Saccharomyces cerevisiae* using independent transcriptional control, rather than the native bicistronic nature of the *parD* operon. Budding yeast was transformed with the integrative plasmid p303MKCKd, in which *kis*

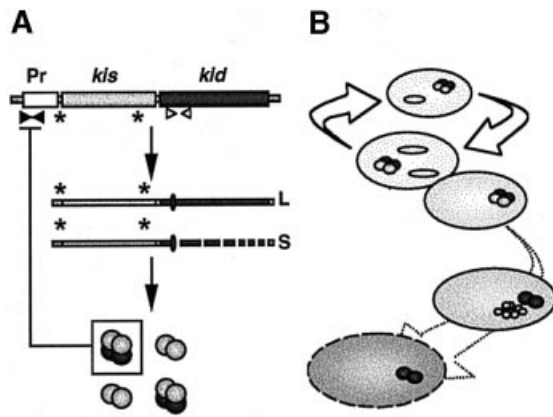


Fig. 1. Mechanism of action of protein prokaryotic killer systems and genetic organization of the *parD* killer system of plasmid R1. (A) The *parD* killer system of R1 is organized in a bicistronic operon that ensures that transcription of the antidote *kis* occurs before that of the toxin *kid*. After transcription, some bicistronic mRNAs are degraded by exonucleolytic processing from their 3' end up to an inverted repeat that folds in a stem-loop structure (white arrowheads). This ensures an excess of monocistronic mRNAs that encode only for the antidote. Translation of *kid* starts from a Shine-Dalgarno sequence (indicated by asterisks) that overlaps with the 3' end of the *kis* gene. Thus, *kid* translation can start only if the antidote has been translated previously. All these strategies ensure that, under normal circumstances, the antidote is always in excess over the toxin and they allow the formation of a non-toxic protein complex that interacts with an inverted repeat located in the *parD* promoter (black arrows) to repress its own transcription. (B) Toxicity of protein prokaryotic killer systems is neutralized in plasmid-containing cells by continuous synthesis of an unstable antidote (light grey spheres). Loss of the plasmid during bacterial division prevents further production of the antidote and its preferential degradation allows the toxin to exert its lethal effect on bacterial segregants (dark grey spheres).

expression is repressed in the presence of methionine and *kid* expression is activated in the presence of Cu^{2+} (Figure 2A). Yeast growth was severely inhibited in this transformant in the presence of methionine and Cu^{2+} , but not in their absence or in the presence of Cu^{2+} only (Figure 2B). These results indicate that expression of Kid inhibits cell proliferation in *S.cerevisiae* and that co-expression of its antidote, Kis, protects against inhibition. Importantly, they also indicate that antidote alone has no apparent side effects on yeast cell viability.

Microinjected Kid inhibits cell proliferation in frog embryos and kills human cells; Kis protects

Next, we injected purified proteins to see whether Kid inhibits cell proliferation in *X.laevis* embryos and whether Kis protects from that effect. Two-cell embryos of *X.laevis* were microinjected near the animal pole of one of the blastomeres with Kid protein, or an active fusion of the Kis protein (maltose-binding protein fused to Kis, MBPKis), or both proteins or buffer alone. The effects of these injections on subsequent cell divisions were followed with time (Figure 3A). Kid-injected blastomeres failed to develop normally unlike the non-injected half of the embryo. On the other hand, blastomeres injected with MBPKis, MBPKis and Kid, or buffer alone progressed normally in all cases until at least mid-blastula (Figure 3B). Nuclear staining of sections of the different embryos showed that cells injected with Kid underwent a limited number of divisions (Figure 3C).

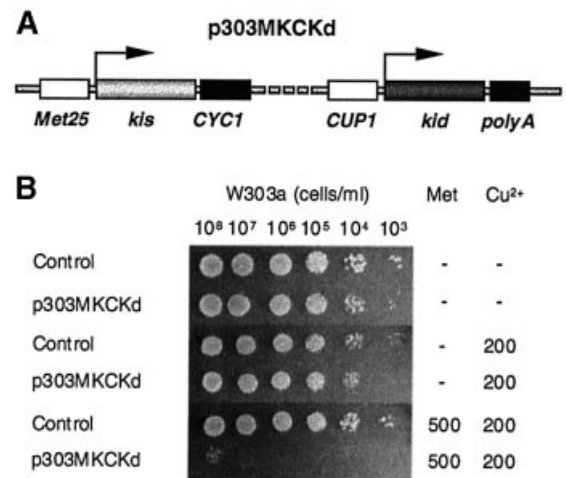


Fig. 2. Independent transcriptional control of *kis* and *kid* allows activation of the *parD* system in *S.cerevisiae* inhibiting cell proliferation conditionally. (A) Scheme depicting plasmid p303MKCKd. (B) Analysis of growth rates of budding yeast transformed with p303MKCKd or with pRS303 (control) in solid media supplemented with the indicated amounts (μM) of methionine and/or Cu^{2+} . Samples were diluted in sterile water at the cell densities indicated and $10 \mu\text{l}$ of each dilution were used for each dot. Abbreviations: Met25, methionine repressible promoter; CUP1, copper inducible promoter; CYC1, CYC1 terminator.

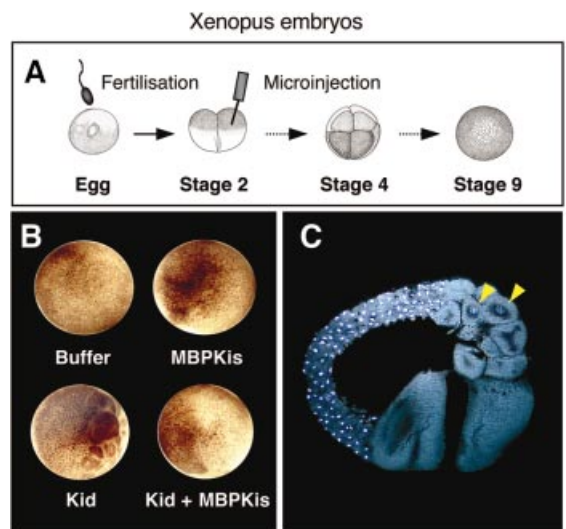


Fig. 3. Kid inhibits cell proliferation in *X.laevis* embryos; Kis neutralizes this effect. (A) Scheme depicting microinjection experiments of *Xenopus* embryos. One blastomere of a two-cell embryo was injected with either Kid, MBPKis, Kid plus MBPKis or buffer. Development was allowed to proceed until the control uninjected embryos reached stage 9 (late blastula). (B) Representative embryos from the experiment shown in (A) when buffer, MBPKis, Kid, or Kid plus Kis proteins were microinjected. Almost identical results were obtained with all the embryos injected with Kid (43 in total), MBPKis (15 in total) and Kid plus MBPKis (19 in total). (C) A section of one embryo microinjected into one blastomere with Kid protein. Embryos were fixed, paraffin-embedded, sectioned and stained for DNA. The uninjected half embryo developed normally whereas the Kid-injected half showed very few cells, most of which were anucleate. The yellow arrowheads indicate the only two nuclei present in the Kid-injected half embryo.

These results encouraged us to test the effects of Kis and Kid microinjection in human cells. Thus, we performed

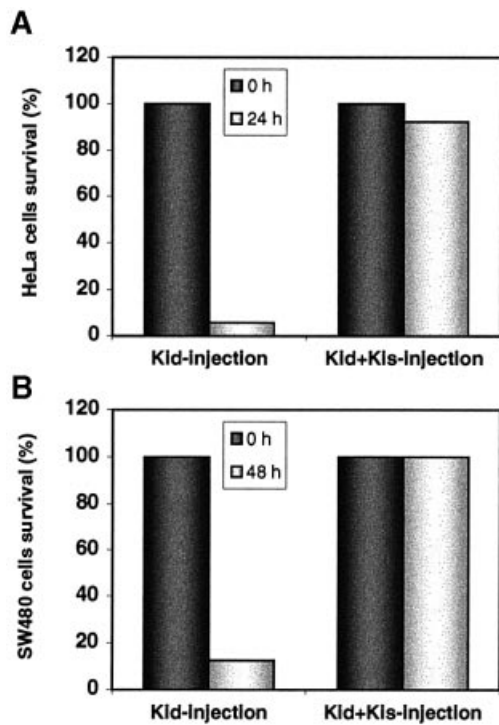


Fig. 4. In the absence of its antidote Kis, toxin Kid kills human cells. (A) Plot showing that only 9 out of 154 HeLa cells (5.8%) survived 24 h after Kid microinjection, whereas 155 out of 168 HeLa cells (92.5%) did so after Kid plus MBPKis microinjection. (B) Plot showing that only 9 out of 72 SW480 cells (12.5%) survived 48 h after Kid microinjection, whilst 65 out of 65 (100%) of these cells did so when they were microinjected with Kid plus MBPKis.

similar experiments using HeLa and SW480 cells. Microinjection of Kid into these cells dramatically decreased their survival and eventually led to the death of all Kid-injected cells. This effect was completely abolished when Kis was pre-incubated with Kid before injection (Figure 4) and absent when only buffer was injected instead (data not shown).

Kid-mediated toxicity and Kis-mediated survival can be regulated in human cells

The results so far demonstrate that the consequences of Kid toxicity are similar in both eukaryotes and prokaryotes. Kid inhibits cell proliferation whilst Kis neutralizes the toxicity of Kid. They also demonstrate that, in yeast, it is possible to substitute the complex prokaryotic regulatory circuits that regulate *parD* in *E.coli* by separate control of transcription of the antidote and the toxin. We asked whether independent transcriptional control of *kis* and *kid* would regulate cell killing or cell survival in human cells. For that purpose two plasmids, pNATHA1i and pNATHA2i, were constructed. Their design was based on previous observations of relative transcription from a constitutive cytomegalovirus (CMV) early promoter compared with a tetracycline repressible promoter in the presence or the absence of tetracycline (or its analogue doxycycline) in HeLa Tet Off cells (Yin *et al.*, 1996) (Figure 5A).

HeLa Tet Off cell line was stably transfected with plasmids *kis+* (pNATHA1i+) and *kis+/kid+* (pNATHA2i+)

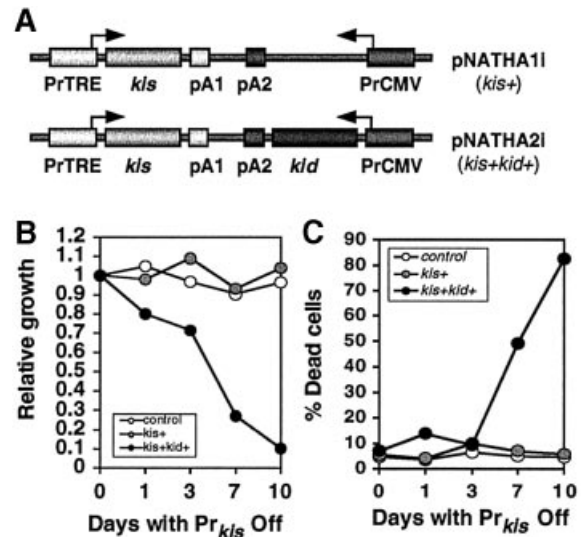


Fig. 5. Independent transcriptional control of *kis* and *kid* allows regulated inhibition of cell proliferation and cell death in HeLa cells. (A) Scheme depicting plasmids pNATHA1i and pNATHA2i, in which *kid* (if present) is constitutively expressed from a CMV promoter whilst *kis* expression is repressed in Tet Off cell lines by doxycycline addition to the culture media. (B) Time course comparing the relative growth of HeLa Tet Off (control), *kis+* (pNATHA1i+) and *kis+/kid+* (pNATHA2i+) stably transfected cells in the presence (Pr *kis* Off) and the absence (Pr *kis* On) of doxycycline. Each line represents a comparison of the cell proliferation rate in the cultures without doxycycline (Pr *kis* On) with the rate for cells grown in the presence of doxycycline (Pr *kis* Off). Control HeLa cells and pNATHA1i (*kis+*) cells grow identically with or without *kis* transcription. In contrast, relative growth decreases over time when *kis* transcription is repressed by doxycycline in pNATHA2i (*kis+/kid+*) cells. (C) Cell death rates for the experiment shown in (B). At least 250 cells were assessed at each time point.

and clones were selected in the absence of doxycycline so that the promoter for the antidote *kis* is On (Pr *kis* On). Figure 5B shows that conditions that switch Kis synthesis Off (Pr *kis* Off) inhibited proliferation only in cells that contain *kid* (pNATHA2i+). Furthermore, as expected from the results shown in Figure 4, this inhibition of proliferation was associated with widespread cell death when Kid is expressed without Kis beyond three days (Figure 5C and see below) and with total cell death after 15 days (data not shown). These results demonstrate that, as in the case of *S.cerevisiae*, inhibition of cell proliferation can be modulated in human cells by independent transcriptional control of *kis* and *kid*. Importantly, and as seen previously in the case of yeast and *X.laevis*, expression of *kis* alone has no apparent phenotypic effects in HeLa cells.

Kid triggers premature cell death by apoptosis in HeLa cells

Figures 4 and 5C show that excess Kid promotes widespread death in HeLa cells. This observation raised the interesting question of whether the lethal effect of Kid in these cells was due to activation of apoptosis. This pathway, which exists in human cells (Evan and Littlewood, 1998), is absent from *S.cerevisiae* (Shaham *et al.*, 1998) and the first stages of *Xenopus* embryonic development (Hensley and Gautier, 1997). To address this question, samples studied in Figure 5 were stained with propidium iodide and fluorescein-linked Annexin-V, an early marker for apoptosis, and analysed by confocal

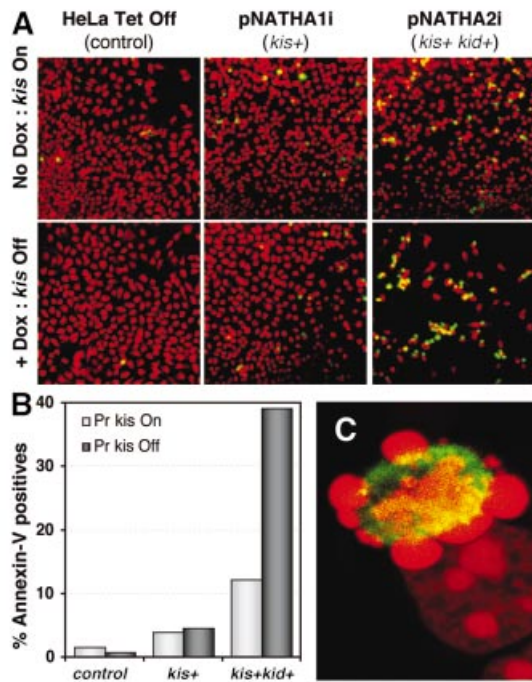


Fig. 6. Expression of *kid* without *kis* causes widespread and premature cell death by apoptosis in HeLa cells. (A) Low power fields of control HeLa Tet Off, *kis*+ (pNATHA1i+) and *kis*+/*kid*+ (pNATHA2i+) cell cultures grown in the absence (Pr *kis* On) and the presence (Pr *kis* Off) of doxycycline for 10 days and stained with propidium iodide (red) and FITC-linked Annexin-V (green). (B) Percentage of Annexin-V positive cells in the different cultures analysed in (A). (C) Magnified image of one *kis*+/*kid*+ (pNATHA2i+) cell grown in the presence of doxycycline (Pr *kis* Off) for 10 days and stained as in (A).

microscopy. After 10 days of growth, HeLa Tet Off cell line and *kis*+ (pNATHA1i+) stable transfectant cells showed a similar small percentage of apoptotic cells in both the absence or presence of doxycycline. In contrast, the percentage of Annexin-V positive *kis*+/*kid*+ (pNATHA2i+) cells increased almost four-fold upon addition of doxycycline to the growth medium for 10 days (Figure 6A and B). A similar analysis upon longer exposures to doxycycline was not possible as, consistently, all pNATHA2i (*kis*+/*kid*+) cells were dead beyond day 10. A magnified view of one of these cells further confirmed apoptosis by morphological criteria (Figure 6C). These results clearly show that widespread cell death observed upon prolonged exposure to Kid in HeLa cells is due to activation of the apoptotic program.

Discussion

The *parD* system of plasmid R1 has evolved to provide a finely balanced mechanism for regulated bacterial cell killing. This system has evolved to ensure the selfish maintenance of the R1 plasmid (Bravo *et al.*, 1987, 1988; Ruiz-Echevarría *et al.*, 1991, 1995; Tsuchimoto *et al.*, 1992). When the plasmid is present, the antidote exceeds the toxin and the cell survives. If the plasmid is lost during cell division, the antidote decays and the residual toxin kills the plasmid-free daughter cell; thus, the possession of the plasmid can be likened to holding a hand grenade with the pin pulled; drop it and it kills you. Here we report the

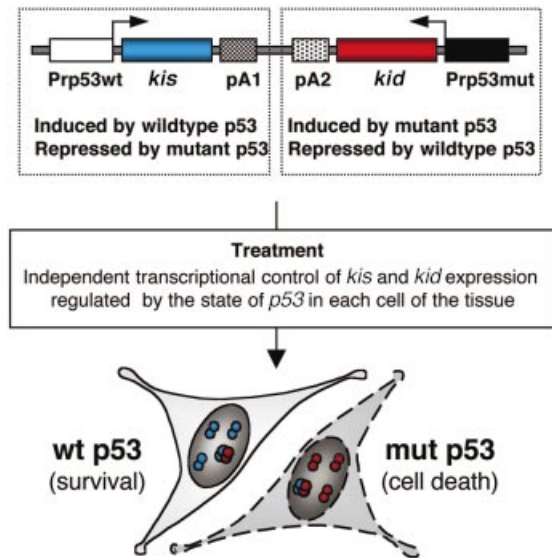


Fig. 7. Regulated expression of Kid and Kis for selective killing of cancer cells. Scheme depicting a possible approach for anti-cancer gene therapy based on the transcriptional activation of the *kis* gene by wild-type *p53* and reinforced by activation of the *kid* toxin gene by *p53* bearing a common mutation. Blue spheres, Kis; red spheres, Kid.

surprising finding that the protein components of this prokaryotic system can be used to manipulate cell proliferation or cell survival in a wide range of eukaryotic organisms. Kid inhibits their proliferation and equally important, Kis antagonizes this inhibition. To achieve these effects, it was necessary to place *kis* and *kid* under control of eukaryotic promoters. We show in this paper that independent control of both genes using eukaryotic promoters still allows regulatable arrest of cell proliferation and, in some cases, cell death.

Regulated expression of Kid and Kis for selective killing of cancer cells and for targeted cell ablation

Gene therapy approaches for selectively killing cancer cells depend on highly selective targeting or expression of toxins to cause maximum damage to cancer cells, whilst minimizing damage to normal cells. Prokaryotic toxins have been used for this purpose previously (Fitzgerald, 1996), but Kid has the advantage that it can be antagonized by a specific protein antidote in eukaryotic organisms. Unlike simple toxins (Culver, 1997), which require highly specific gene delivery or expression in cancer cells (Zullo *et al.*, 1998), the approach suggested by this work offers the opportunity to both regulate the expression and antagonize the effect of Kid by activating the expression of Kis in non-tumour cells. Transcriptional regulators known to be inactivated in many human cancers, such as *p53* (Hainaut and Hollstein, 2000), could be used to induce *kis* expression. If so, combining this approach with efficient delivery vehicles might allow a regulated and selective strategy for gene therapy of cancer (Figure 7). Our results show that Kid-induced cell death does not depend on the presence of functional *p53*, as SW480 cells, which express a mutant P53 protein, die 48 h after injection of Kid (Figure 4B). The recent determination of the structure of Kid (Hargreaves *et al.*, 2002) opens the

possibility of designing small drugs that mimic its effects on human cells.

We have shown that Kid inhibits cell proliferation not only in somatic cells, but also in embryonic cells. Thus, an additional use for this approach to regulated inhibition of cell proliferation is the opportunity to perform highly regulated knockouts of cell lineages during development. Targeted ablation has been successfully used in developmental studies (Booth *et al.*, 2000; Lee *et al.*, 2000). Once again, tissue-specific promoters and conditional promoters could be used to tune the relative expression of *kis* and *kid* to allow selected cell knockouts at specific stages of development. This approach could have value in studies of development as well as in studies of differentiation, organogenesis or degenerative disorders.

Materials and methods

Plasmids construction

p303MKCKd was constructed in two steps. First, *kis* was amplified by PCR and cloned into the *Sma*I site of p424Met25 (Mumberg *et al.*, 1994). The *Pvu*II fragment of this plasmid containing *kis* was exchanged by the equivalent fragment of pRS303 (Sikorski and Hieter, 1989) to generate p303MK. Second, *kid* was subcloned from pCIKid (see below) into pSAL1 (Mascorro-Gallardo *et al.*, 1996) by exchanging their small *Sca*I-*Xho*I fragments. A blunt-ended *Bam*HI fragment from this plasmid was subcloned in blunt-ended *Aat*II-digested p303MK to generate p303MKCKd. For pMALKis (MBPKis overproducer), *kis* flanked by an *Eco*RI site at 3' was obtained by PCR using the oligonucleotides 5'-ATGCATACCAACCCGACTG-3' and 5'-TCGGAATTTCAGATTTCCTCCTG-3'. This PCR product was cloned into *Xmn*I and *Eco*RI sites of pMAL-c2 (BioLabs). For pGCHisKisKid (Kid overproducer), *kiskid* DNA flanked by *Nde*I and *Bam*HI was obtained by PCR using the oligonucleotides 5'-GGAATTCCATATGCATACCAACCCGACT-3' and 5'-CGGGATCTCAAGTCAGAATAGTGGACAGG-3'. This PCR product was cloned into *Nde*I and *Bam*HI sites of pET15b (Invitrogen) before subcloning its *Nco*I-*Bam*HI small fragment into the same sites of pRG80-recA-Nhis (Giraldo *et al.*, 1998). For pNATHA plasmids, *kis* was flanked by *Eco*RI and *Xba*I by PCR using the oligonucleotides 5'-CGGAATTCATGCATACCAACCCGACTG-3' and 5'-CTCTAGATCAGATTTCCTCCTGACC-3'. This PCR product was cloned into pTRE (Clontech) digested with *Eco*RI and *Xba*I. For pNATHA2i plasmid, *kid* flanked by *Xho*I and *Eco*RI sites was amplified by PCR using the oligonucleotides 5'-CCGCTCGAGATGGAAGAGGGG-AAATCT-3' and 5'-CGGAATTCCTCAAGTCAGAATAGTGGACAGG-3'. This PCR product was cloned in pCI-neo (Promega) digested with *Xho*I and *Eco*RI. Neomycin resistance was deleted from pCI-neoKid (and from parental pCI-neo) by *Bst*XI and *Sma*I digestion and religation. Resultant plasmids lacking the neomycin resistance gene were digested with *Bgl*II and *Bam*HI. The CMV promoter-containing fragments resulting from these digestions were blunt ended and subcloned into a blunt-ended *Hind*III pTREKis to obtain pNATHA1i and pNATHA2i plasmids. Tail-to-tail orientations between CMV- and tetracycline-dependent transcriptional units were chosen for pNATHA1i and pNATHA2i.

Yeast growth determination

Several fresh colonies of W303a strain (*MATa*, *ade2-1*, *trp1-1*, *can1-100*, *leu2-3*, *112*, *his3-11*, *ura3*, *psi+*) transformed with pRS303 (control) or p303MKCKd were diluted in sterile water to 10⁸ cells/ml. Ten microlitres of this and four serial 1/10 dilutions were placed on SD plates without histidine, supplemented or not with 500 μ M methionine and/or 200 μ M CuSO₄, as indicated in Figure 2, and grown at 23°C for 48 h.

Protein purification

MBPKis was expressed in DH5 α and purified by affinity chromatography through an amylose resin (BioLabs) in 20 mM HEPES pH 7.5, 100 mM KCl, 1 mM DTT and 10% ethyleneglycol, following manufacturer's instructions. Kid was expressed in TG1 upon induction of the culture with Nalidixic acid (25 μ g/ml; Merck). The soluble fraction was precipitated with 60% ammonium sulphate and resuspended in 20 mM Tris-HCl pH 7.5, 500 mM KCl and dialysed against the same buffer to eliminate the

ammonium sulphate. The dialysed fraction was loaded in Fast-Flow chelating Sepharose column (Pharmacia) activated with Ni²⁺. A gradient of 0–6 M guanidinium chloride (GnCl) in 20 mM Tris-HCl pH 7.5 was applied to the column until elution of denatured Kid. Kid was diluted in 20 mM HEPES pH 7.5, 6 M GnCl, 150 mM KCl, 20 mM β -mercaptoethanol, 0.2 mM EDTA and 1.2% CHAPS and refolded by dialysis against 150 mM KCl, 20 mM HEPES pH 8.0, 10 mM β -mercaptoethanol, 0.1 mM DTT and 10% ethyleneglycol. Soluble protein was concentrated in 3K Centricron tubes.

Protein microinjections

For embryo microinjections, 160 and 720 ng of Kid and MBPKis, respectively, dialysed in 20 mM Tris-HCl pH 8.0 and 50 mM KCl were mixed with each other or with dialysis buffer in 4 μ l and incubated on ice for 10 min. Fifty nanolitres of each mix were microinjected into one of the cells of dejellied two-cell embryos of *X.laevis*. Embryos were incubated in 4% ficoll 400 in MBS buffer at 18°C until mid-blastula was reached in the non-injected controls. Embryos were photographed, fixed and embedded in paraffin wax for sectioning as described previously (Butler *et al.*, 2001). Sections were mounted and stained with Hoechst 33258 or with propidium iodide and analysed by microscopy. For HeLa and SW480 microinjections, proliferating cells were asynchronously grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and cultured in small chambers. Medium was replaced with warmed CO₂-independent medium (Gibco/BRL) for microinjection. MBPKis and Kid were diluted in water to 2.7 and 2.3 pmol/ μ l, respectively, and supplemented with 1 mg/ml Texas Red Dextran (70 000 MW; Molecular Probes) and injected into cells. For the Kis and Kid sample, proteins were pre-mixed at those concentrations prior to injection. After injection, cells were returned to normal DMEM for continuing growth. Images were collected on a Bio-Rad 1024 confocal microscope for determination of survival rates.

Stable transfections

HeLa Tet Off cell line (Clontech) was co-transfected with pNATHA plasmids and pTKHyg (Clontech) by the lipofectamine method (Gibco). Stable clones were selected following manufacturers' recommendations.

Calculation of cell growth and cell death rates for HeLa cells

For cell proliferation and cell death experiments, control HeLa Tet Off, *kis+* (pNATHA1i+) and *kis+/kid+* (pNATHA2i+) cells were each cultured in six plates at equally low density and in selective medium. Doxycycline (0.1 μ g/ml) was added to five of the plates of each sample 10, 7, 5, 3 or 1 days before the end of the experiment. Dead and mitotic cells were added back to the plates when medium was changed. All cells were resuspended in PBS, and stained with Trypan Blue to count total and dead cells. At least 250 cells for each sample were counted in a haemocytometer.

Annexin-V staining

Cells were plated at identical cell densities in the absence or the presence of 0.1 μ g/ml doxycycline and grown for 10 days on coverslips. Cells were stained with fluorescein-linked Annexin-V (green; Clontech) as recommended by the manufacturer, and then fixed and DNA stained with propidium iodide (red) before analysis.

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References

- Booth,G.E., Kinrade,E.F.V. and Hidalgo,A. (2000) Glia maintain follower neuron survival during *Drosophila* CNS development. *Development*, **127**, 237–244.
- Bravo,A., de Torrónategui,G. and Díaz,R. (1987) Identification of

- components of a new stability system of plasmid R1, *parD*, that is close to the origin of replication of this plasmid. *Mol. Gen. Genet.*, **210**, 101–110.
- Bravo,A., Ortega,S., de Torrontegui,G. and Díaz,R. (1988) Killing of *Escherichia coli* cells modulated by components of the stability system *parD* of plasmid R1. *Mol. Gen. Genet.*, **215**, 146–151.
- Butler,K., Zorn,A. and Gurdon,J.B. (2001) Non-radioactive *in situ* hybridisation to *Xenopus* tissue sections. *Methods*, **23**, 303–312.
- Cornelis,S., Bruynooghe,Y., Van Huffel,S., Tinton,S. and Beyaert,R. (2000) Identification and characterisation of a novel cell cycle-regulated internal ribosome entry site. *Mol. Cell*, **5**, 597–605.
- Culver,K.W. (1997) Suicide genes for the treatment of cancer. In Strauss,M. and Barranger,J.A. (eds), *Concepts in Gene Therapy*. Walter de Gruyter, New York, NY, pp. 469–486.
- Evan,G. and Littlewood,T. (1998) A matter of life and death. *Science*, **281**, 1317–1322.
- Fitzgerald,D. (1996) Why toxins! *Semin. Cancer Biol.*, **7**, 87–95.
- Giraldo,R., Andreu,J.M. and Díaz-Orejas,R. (1998) Protein domains and conformational changes in the activation of RepA, a DNA replication initiator. *EMBO J.*, **17**, 4511–4526.
- Hainaut,P. and Hollstein,M. (2000) *p53* and human cancer: the first ten thousand mutations. *Adv. Cancer Res.*, **77**, 81–137.
- Hargreaves,D., Santos-Sierra,S., Giraldo,R., Sabariego-Jareño,S., de la Cueva-Méndez,G., Boelens,R., Díaz-Orejas,R. and Rafferty,J.B. (2002) Structural and functional analysis of the Kid toxin protein from *E.coli* plasmid R1. *Structure*, **10**, 1425–1433.
- Hensley,C. and Gautier,J. (1997) A developmental timer that regulates apoptosis at the onset of gastrulation. *Mech. Dev.*, **69**, 183–195.
- Jensen,R.B. and Gerdes,K. (1995). Programmed cell death in bacteria: proteic plasmid stabilization systems. *Mol. Microbiol.*, **17**, 205–210.
- Lee,K.J., Dietrich,P. and Jessell,T.M. (2000) Genetic ablation reveals that the roof plate is essential for dorsal interneuron specification. *Nature*, **403**, 734–740.
- Mascorro-Gallardo,J.O., Covarrubias,A.A. and Gaxiola,R. (1996) Construction of a CUP1 promoter-based vector to modulate gene expression in *Saccharomyces cerevisiae*. *Gene*, **172**, 169–170.
- McBratney,S., Chen,C.Y. and Sarnow,P. (1993) Internal initiation of translation. *Curr. Opin. Cell Biol.*, **5**, 961–965.
- Mumberg,D., Muller,R. and Funk,M. (1994) Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression. *Nucleic Acids Res.*, **22**, 5767–5768.
- Ruiz-Echevarría,M.J., Berzal-Herranz,A., Gerdes,K. and Díaz-Orejas,R. (1991) The *kis* and *kid* genes of the *parD* maintenance system of plasmid R1 form an operon that is autoregulated at the level of transcription by the co-ordinated action of the Kis and Kid proteins. *Mol. Microbiol.*, **5**, 2685–2693.
- Ruiz-Echevarría,M.J., de la Cueva,G. and Díaz-Orejas,R. (1995) Translational coupling and limited degradation of a polycistronic messenger modulate differential gene expression in the *parD* stability system of plasmid R1. *Mol. Gen. Genet.*, **248**, 599–609.
- Shaham,S., Shuman,M.A. and Herskowitz,I. (1998) Death-defying yeast identify novel apoptosis genes. *Cell*, **92**, 425–427.
- Sikorski,R.S. and Hieter,P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*, **122**, 19–27.
- Tsuchimoto,S., Nishimura,Y. and Ohtsubo,E. (1992) The stable maintenance system *pem* of plasmid R100: degradation of PemI protein may allow PemK protein to inhibit cell growth. *J. Bacteriol.*, **174**, 4205–4211.
- Yin,D.X., Zhu,L. and Schimke,R.T. (1996) Tetracycline-controlled expression system achieves high-level and quantitative control of gene expression. *Anal. Biochem.*, **235**, 195–201.
- Zullo,S.A., Caplen,N.J. and Blaese,R.M. (1998) Gene therapy for cancer. In Xanthopoulos,K.G. (ed.), *Gene Therapy*. NATO ASI series, H105. Springer-Verlag, Berlin, Germany, pp. 133–145.

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