

# Position-effect variegation in *Drosophila*: the modifier Su(var)3-7 is a modular DNA-binding protein

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Received July 18, 2001; revised October 2, 2001; accepted October 8, 2001

**An increase in the dose of the *Su(var)3-7* locus of *Drosophila* augments heterochromatin-promoted variegated silencing. The deduced protein sequence of *Su(var)3-7* reveals seven widely spaced zinc fingers. We found that *Su(var)3-7* has affinity for DNA *in vitro* and that the minimal protein sequence requirement for DNA binding is any module containing two zinc fingers and the interval between them. As *Su(var)3-7* is a heterochromatin-associated protein, we tested its affinity for various satellite DNA sequences *in vitro*. The AATAT and 353-bp elements have the highest affinity. If affinity for satellite DNAs contributes to the presence of *Su(var)3-7* in heterochromatin, a general affinity for DNA, or sequences yet to be determined, suggests a function in the genomic silencing of position-effect variegation: expansion of heterochromatin, whether continuous by spreading or discontinuous by pairing with sequence elements scattered through euchromatin, could use the affinity of *Su(var)3-7* for DNA.**

## INTRODUCTION

In 1930, Müller described a number of X-ray-induced mutants characterized by a mosaic phenotype. These mutants are chromosomal rearrangements relocating the variegating genes next to pericentric heterochromatin (Schultz, 1936). Two types of models were put forward to explain the phenomenon of position-effect variegation (PEV). Schultz was the first to propose that inactivation by heterochromatin could spread from the rearrangement breakpoint, and Zuckerkandl (1974) proposed that the spreading depends on the dose of building blocks of heterochromatin. The other school of thought also has ancient roots. Pontecorvo suggested in 1944 that heterochromatin could form from folding of DNA at any region comprising repetitive

sequences (Pontecorvo, 1944). Recent observations of discontinuous expansion of silencing and of long-distance effects raise the possibility that sequence elements dispersed in the genome could anchor the formation of heterochromatin by association with pericentric heterochromatin (Dorer and Henikoff, 1994; Talbert and Henikoff, 2000). Dominant modifiers of PEV provide an avenue to identifying the proteins involved in PEV (Reuter and Spierer, 1992). Indeed, three loci with a haplo-suppressor triplo-enhancer phenotype were found to encode heterochromatin-associated proteins. These include *Su(var)2-5* (Eisenberg *et al.*, 1990), *Su(var)3-7* (Cléard *et al.*, 1997) and *Su(var)3-9* (Tschiersch *et al.*, 1994; Aagaard *et al.*, 1999).

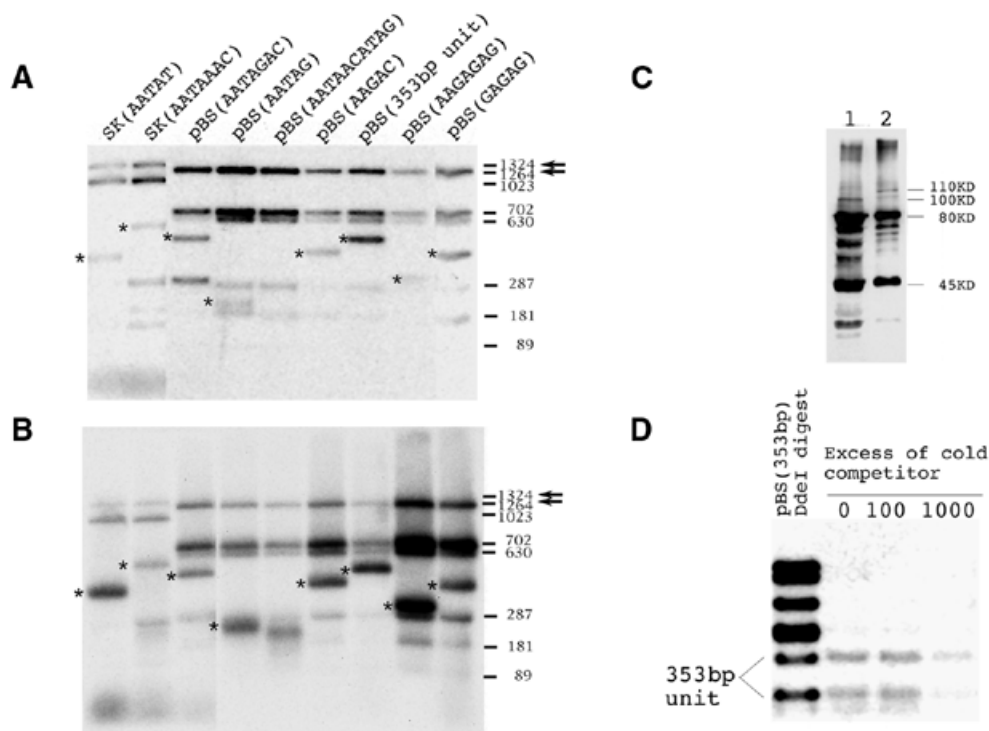
Attempts at explaining PEV require that at least one component binds to DNA. Silencing should reflect the amounts of this component, and therefore the haplo-suppressor and triplo-enhancer modifier loci are first-choice candidates. Moreover, as PEV affects different genes in a variety of rearrangements, this component should bind DNA with affinity to a wide range of sequences. *Su(var)3-7* is a good candidate, as its amino-acid sequence encodes seven zinc fingers, which are motives known to bind DNA (Harrison, 1991). In this study, we found that *Su(var)3-7* associates with DNA *in vitro* and that each pair of zinc fingers forms a DNA-binding domain.

## RESULTS

### Su(var)3-7 binds DNA

To test the affinity of *Su(var)3-7* for DNA, we constructed a glutathione *S*-transferase (GST) *Su(var)3-7* fusion protein. The construct encodes 1047 of the 1169 amino acids of the complete protein. One hundred and twenty-one amino acids

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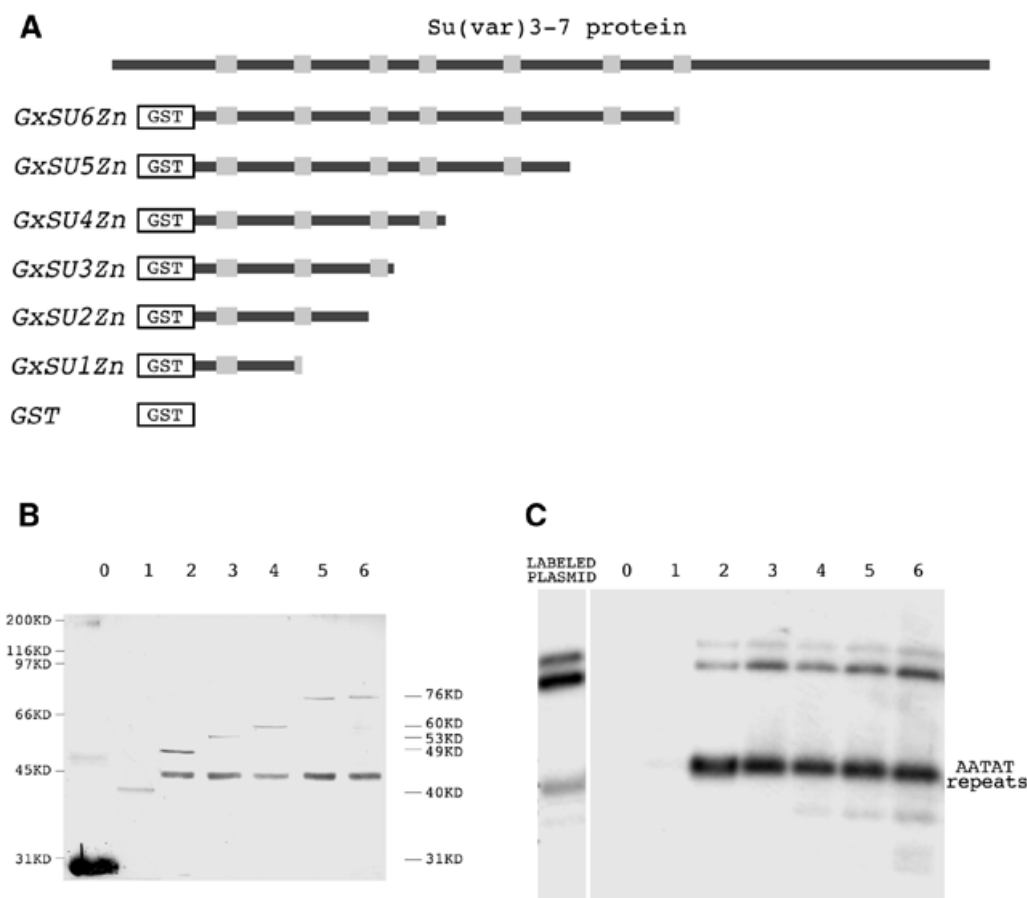
**Fig. 1.** Binding of different DNA probes by GST-Su(var)3-7. (A) Each lane of a 2.5% agarose gel corresponds to a digest of a <sup>32</sup>P-labelled recombinant plasmid. The satellite DNA insert is marked by a star and identified above each lane. The arrows indicate the plasmid fragments used to estimate the relative affinity. (B) Fragments selected by the GST-Su(var)3-7 fusion protein in the absence of poly(dI-dC). Same reaction and probe as in (A). (C) Western blot of the GST-Su(var)3-7 constructs containing seven (lane 2) or six (lane 1) zinc fingers. Both lanes show a minor band of the correct size (110 and 100 kDa, respectively). The major products are truncated proteins, one of 80 kDa (corresponding to a protein with five zinc fingers) and one of 45 kDa (ending just after the second finger). (D) Competition for binding of labelled plasmid pBS(353 bp) by GST-Su(var)3-7. The binding reaction mixtures contain poly(dI-dC) at a final concentration of 0.1 μg/μl and ~10 ng of labelled plasmid, and 0, 1.0 or 10.0 μg of the same, but unlabelled, digested plasmid was added. In this experiment, the pBS(353 bp) plasmid was cut with *Eco*RI, *Hind*III and *Dde*I, the latter splitting the insert into two fragments.

from the N-terminus are missing, but they are known to be dispensable for the modifier of variegation function (Reuter *et al.*, 1990; Cléard *et al.*, 1995). The fragment was cloned in an expression vector and introduced in *Escherichia coli*. The expressed fusion protein is shorter than expected, probably because of premature arrest of translation. From its size on western blot (Figure 1), we estimate that the major protein component is interrupted before the sixth zinc finger. Nonetheless, the protein expressed contains five of the seven zinc fingers, and we decided to use it for DNA-binding assays *in vitro*. In a preliminary test, we determined by southwestern blot that the GST-Su(var)3-7 protein retains *Drosophila* genomic DNA (data not shown). As Su(var)3-7 is a heterochromatin-associated protein (Cléard *et al.*, 1997), we tested potential targets, namely satellite DNA sequences. Nine plasmids containing different *Drosophila* satellite sequence DNA (Lohe and Brutlag, 1986) were digested to separate the insert from plasmid DNA and were labelled. The mixture was incubated with the GST-Su(var)3-7 protein bound to a solid matrix through its GST moiety. Figure 1 shows an autoradiogram of the fragments generated by digestion (A) compared with the same fragments selected by the Su(var)3-7 protein (B). In the absence of a non-specific competitor such as poly(dI-dC), binding is detected with essentially all the DNA fragments, but

there is a strong enrichment for some of the satellite DNA. Enrichment was quantified by scanning the autoradiograms to verify the qualitative differences visible in Figure 1 (data not shown). Except for the repeats of the sequence motif GAGAG, the satellite DNA insertions are preferentially selected by the fusion protein. Affinity of Su(var)3-7 is 8-fold stronger for AATAT repeats, and the 353-bp unit, than for AATAAAC repeats. Figure 1 also shows a competition experiment designed to further assess the binding affinity for satellite DNA compared with plasmid DNA. Increasing amounts of cold competitor (the same, but unlabelled digest) were added. The binding to plasmid is readily competed out, but association of Su(var)3-7 to the satellite DNA fragments is still visible with a 1000-fold excess of identical cold fragments.

### A module of two zinc fingers is necessary and sufficient for DNA binding

The deduced sequence of Su(var)3-7 reveals a number of repeated motifs (Reuter *et al.*, 1990; Cléard *et al.*, 1995). Among them, the seven zinc fingers separated by large intervals stand out. We have shown above that a segment of the protein comprising five fingers does bind DNA. In order to delineate the



**Fig. 2.** Binding of the AATAT satellite by different GST–Su(var)3-7 derivatives. **(A)** The Su(var)3-7 protein is represented as a black bar within which the zinc fingers appear as grey boxes. **(B)** Western blot of the different recombinant proteins schematized in **(A)**, bound to glutathione–Sepharose 4-B. Lanes 0–6 correspond to proteins with zero to six zinc fingers revealed with anti-GST antibody. **(C)** Autoradiogram of the agarose gel after electrophoresis of the 300-bp AATAT repeats fragment retained by the same proteins. The amounts of protein fragments in the binding assay were those deposited on the western blot in **(B)**, and the experiment was performed in the presence of 0.1 µg/µl of the non-specific competitor poly(dI–dC).

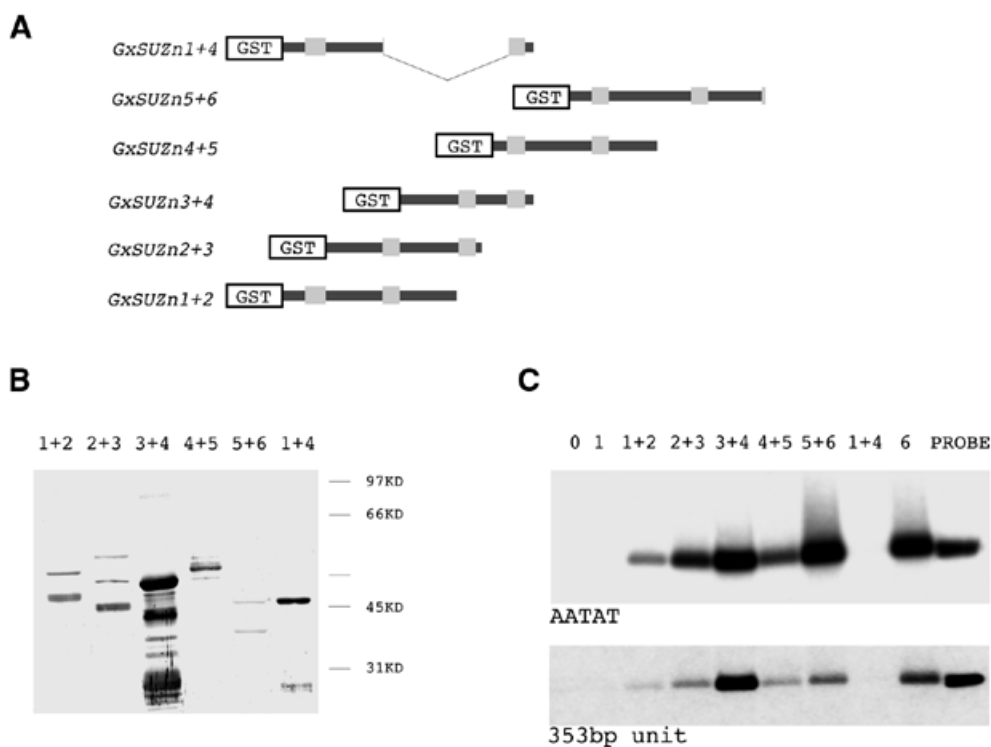
protein sequence required for DNA binding, we constructed a panel of proteins with a stepwise reduction in length and consequently in the number of zinc fingers (Figure 2A). The DNA sequence tested was the AATAT repeat. Figure 2C illustrates the results. Stepwise reduction from five down to two fingers does not affect significantly the binding. In contrast, the fragment containing only one complete finger, and the spacer between this one and the next, does not bind the satellite DNA. The same was observed with a segment comprising the last finger, namely the seventh, and the entire C-terminus part of the protein (data not shown).

From these results, one could argue that the requirement is not for a pair of fingers, but that the second or the seventh finger plays a specific role. To refine the analysis, we designed five additional constructs representing a collection of different pairs of zinc fingers, including an artificial one, made of the first and the fourth finger (Figure 3A). The segments were tested for interaction with the AATAT and the 353-bp satellite DNAs. The results are shown in Figure 3. Any pair of zinc fingers, together with the spacer sequence, binds efficiently both the AATAT and

the 353-bp satellites. Differences in intensity of the bands seem to be parallel to the amounts of protein in the assay (data not shown). There is one notable exception to the rule of the pair of fingers. The construct GxSUZn1+4 comprises two zinc fingers, but, in contrast to all others of its kind, it is unable to retain DNA. In this construct, however, the cystein doublet at the base of the finger has become a cystein triplet because of the cloning strategy. The sequence -C-X-X-C- is mutated to -C-X-X-C-X-X-C-, and this probably renders the finger non-functional. This result reinforces the observation that a DNA-binding module must comprise two functional fingers.

### Defining a consensus DNA sequence for binding

We addressed this question using two approaches. First, we performed footprints experiments using the 353-bp unit, which also contains three AATAT repeats. The DNA fragments were protected by an increase in protein concentration, but we could not detect a specific footprint (data not shown). Secondly, we tried to select fragments of *Drosophila* genomic DNA with a



**Fig. 3.** Binding of the AATAT and 353-bp satellites by different zinc finger pairs derived from Su(var)3-7. **(A)** Different parts of the fusion proteins are schematized as in Figure 2. **(B)** Western blot of the proteins corresponding to (A), after purification on glutathione–Sepharose 4-B, revealed with an anti-GST antibody. Lanes are named according to the pair of fingers present in the fusion proteins. **(C)** Autoradiography of the agarose gel after electrophoresis of the 300-bp AATAT repeats fragment and the 500-bp insert containing the 353-bp unit bound by the GST-tagged protein constructs. The amounts of protein fragments in the binding assay were those deposited on the western blot in (B), and the experiment was carried out in the presence of 0.1 µg/µl of the non-specific competitor poly(dI–dC).

Su(var)3-7 fragment comprising two zinc fingers according to Cuvier *et al.* (1998). Following five cycles of selection and PCR amplification, 60 fragments were sequenced (data not shown). Although repeated elements were predominant, we were unable to define a consensus sequence.

## DISCUSSION

PEV was first characterized as an effect *in cis* of blocks of pericentric heterochromatin onto euchromatic genes (Weiler and Wakimoto, 1995). In addition to its cytological definition, the main characteristic of heterochromatin is its concentration of satellite DNA and of middle repetitive elements (Lohe and Brutlag, 1986). With the exception of genes naturally embedded in heterochromatin, the phenotypes in PEV result from the silencing of genes normally in euchromatin. The different models invoke linear expansion of heterochromatin, discontinuous expansion allowing for ‘skipping genes’ and sequestration at distance by pairing of sequences within euchromatin with others of their kind in heterochromatin (Weiler and Wakimoto, 1995). These models could be reconciled by assuming that there are sequences scattered throughout the genome that have a potential affinity for association with heterochromatin. When in the proximity of a large block of heterochromatin, possibly because of random movements or higher concentrations of associated ‘nucleating’ factors, these sequences could bind

heterochromatin constituents and eventually fuse with the bulk of pericentric heterochromatin. A higher concentration of components would favour these interactions and the resulting expansion of the repressed state. The candidate to play this role is obviously the haplo-suppressor triplo-enhancer of PEV.

The results reported here lead us to propose that Su(var)3-7 is one of the links between these scattered sequences and the pericentric heterochromatin. However, there are other candidates. HP1 seems to have a non-specific affinity for DNA and nucleosomes (Zhao *et al.*, 2000) and interacts with histones in mammals (Nielsen *et al.*, 2001). In *Drosophila*, HP1 associates with a DNA-binding protein ORC (Shareef *et al.*, 2001) and induces ectopic chromosomal loops (Seum *et al.*, 2001). Su(var)3-9 might also play a central role, as its mutations are epistatic to those in HP1 and Su(var)3-7 (G. Reuter, personal communication). Interpretation of this result is delicate, however, as the authors used *Su(var)2-5* and *Su(var)3-7* haplo-insufficient mutants. Finally, DDP1, a single-stranded nucleic-acid-binding protein, associates with satellite DNA and co-localizes with HP1 (Cortés *et al.*, 1999), and Prod, a satellite DNA-binding protein, was also proposed to function in heterochromatin compaction (Török *et al.*, 2000).

The preference of Su(var)3-7 *in vitro* for some satellite DNA sequences, followed by a lesser affinity for others, and an apparent general affinity for DNA, suggests a mechanism



whereby the increase in dose might cause the expansion of heterochromatin-induced silencing. At the 'physiological' dose, Su(var)3-7 associates only with heterochromatin sequences, but at higher concentrations it binds to other sequences in the genome and to other partners to mediate heterochromatin formation. This nucleation is facilitated by proximity of large blocks of pericentric heterochromatin, thus explaining the spreading effect. This mechanism allows for the discontinuous expansion observed in a number of instances (for cytological evidence, see Belyaeva and Zhimulev, 1991; for genetic evidence, see Talbert and Henikoff, 2000, and references therein). Variegation induced by insertion of a tandem array of repeated elements in euchromatin (Dorer and Henikoff, 1994) is also explained if Su(var)3-7 has a particularly good affinity for them. Future research should now be addressed at identifying targets of Su(var)3-7 in euchromatin. For example, we have shown that Su(var)3-7 associates at the locus of a large insertion of the AAGAG satellite DNA, a sequence we have not tested *in vitro*, and affects variegation of a neighbouring gene (Delattre *et al.*, 2000).

A second interesting finding in this report is the modular structure of Su(var)3-7. From its deduced sequence, Su(var)3-7 encodes seven zinc fingers rather regularly spaced over 700 amino acids, starting from the N-terminus of the 1169 amino acid long protein (Cléard *et al.*, 1995). These fingers are very similar and are each preceded by a tryptophan motif. By testing a variety of segments of the protein for binding to DNA, we have determined that the minimal requirement is a pair of fingers separated by a spacer sequence. Although we have not tested all the possible combinations, we propose that each finger and its immediate neighbourhood is exchangeable with any other and that a combination of two with any of the spacers provides DNA-binding properties. Obviously, there are a number of other constraints to accommodate. For example, Su(var)3-7 is part of a larger complex containing HP1 (Cléard *et al.*, 1997; Delattre *et al.*, 2000). The affinity of Su(var)3-7 *in vivo* may differ from that observed *in vitro* by its participation in a large complex of proteins. Another speculation to assess is an alternative role for Su(var)3-7: the multimodular structure of Su(var)3-7 could be instrumental in the DNA compaction characteristic of silent heterochromatin.

## METHODS

**The Su(var)3-7 fusion protein.** Various fragments of the Su(var)3-7 cDNA (Cléard *et al.*, 1995) were cloned in-frame downstream of the gene encoding the enzyme GST in the vectors pGEX-3X, pGEX-4T-1 and pGEX-4T-2 from Pharmacia. The resulting clones were introduced by transformation in the *E. coli* BL21 strain. Expression was induced by 0.1 mM IPTG (final concentration). After 4 h of growth, cells were collected by centrifugation and suspended in 5% of the initial volume of ice-cold PBS prior to disruption by mild sonication. Lysis was completed by incubation for 30 min on ice after the addition of Triton X-100 to a final concentration of 1%. Debris were removed by centrifugation at 10 000 r.p.m. for 10 min at 4°C. For binding on glutathione-Sepharose 4-B (Pharmacia) and purification, the lysate was gently mixed for 30 min at room temperature with beads (50 µl per ml of lysate) equilibrated in PBS. Unbound protein was removed by several washes with PBS.

All proteins used in binding assays were analysed by SDS-PAGE and western blot with anti-GST and/or anti-Su(var)3-7 antibodies.

**DNA probes and DNA binding assays.** Plasmids containing satellite DNA sequences (Lohe and Brutlag, 1986) were digested to separate the insert. The digestion mixture was treated with shrimp alkaline phosphatase followed by heat inactivation. When an isolated insert instead of the mixture of fragments was used as a probe, the reaction was submitted to electrophoresis on agarose gel and the fragment of interest purified by GeneClean (Bio 101). For the labelling reaction, 50 ng of fragment or whole digest were incubated for 30 min at 37°C in 50 µl of 1 × PNK buffer, 50 mM DTT, 10 µCi [ $\gamma^{32}$ P]dATP and 10 U of polynucleotide kinase. The probe was purified on a spin column. A typical reaction was performed in 25 µl final volume using 2.5 µl of matrix bound protein and 22.5 µl of a mixture containing 10 000 c.p.m. of radiolabelled fragment (~0.5 ng) and 2.5 µg poly(dI-dC) in NETN (20 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) (Kaelin *et al.*, 1991). The reaction was incubated for 30 min at room temperature. After removing the unbound material, beads were washed three times with 1 ml of NETN containing NaCl at a final concentration of 0.2 M. After the last wash and addition of 40 µl of loading buffer (10 mM Tris-Cl pH 7.5, 0.025% Bromophenol Blue, 3% glycerol, 0.1% SDS), beads were heated for 10 min at 80°C, pelleted by centrifugation and the supernatant loaded onto a 2.5% agarose gel. After electrophoresis, the gel was dried and exposed for autoradiography.

## ACKNOWLEDGEMENTS

We thank Rakesh Mishra for his expert advice on binding assays. This work was supported by grants from the Swiss National Science Foundation and by the State of Geneva, Switzerland.

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DOI: 10.1093/embo-reports/kve243