Regulation of telomere length and function by a Myb-domain protein in fission yeast

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Telomeres, the specialized nucleoprotein structures that comprise the ends of eukaryotic chromosomes[1,2], are essential for complete replication[3–5], and regulation of their length has been a focus of research on tumorigenesis[6–8]. In the budding yeast *Saccharomyces cerevisiae*, the protein Rap1p binds to telomeric DNA and functions in the regulation of telomere length[9–12]. A human telomere protein, hTRF (human TTAGGG repeat factor) binds the telomere sequence *in vitro*[13] and localizes to telomeres cytologically[14], but its functions are not yet known. Here we use a genetic screen to identify a telomere protein in fission yeast, Taz1p (telomere-associated in *Schizosaccharomyces pombe*), that shares homology to the Myb proto-oncogene DNA-binding domain with hTRF. Disruption or deletion of the *taz1*$^+$ gene causes a massive increase in telomere length. Taz1p is required for the repression of telomere-adjacent gene expression and for normal meiosis or sporulation. It may be a negative regulator of the telomere-replicating enzyme, telomerase[1,3], or may protect against activation of telomerase-independent pathways of telomere elongation[8].

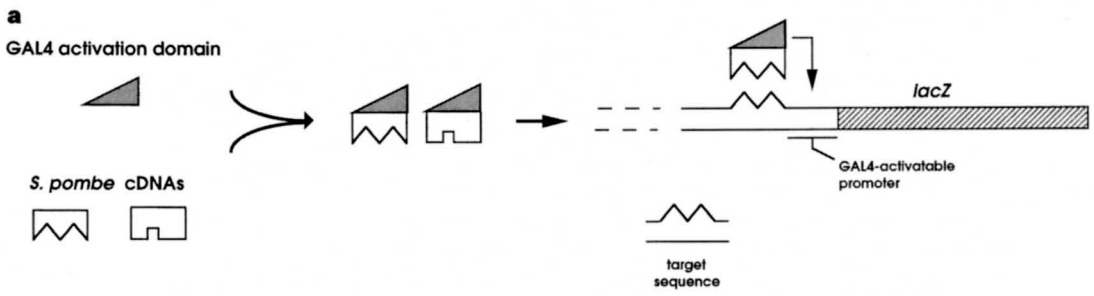
S. pombe, like the budding yeasts, is amenable to genetic analysis, and yet the fission and budding yeasts are sufficiently distant evolutionarily that features present in both genera should be widely conserved in eukaryotes[15]. Furthermore, certain properties of *S. pombe* chromosomes, such as centromere structure and chromosome segregation, are more similar to those of larger eukaryotes[16,17].

To explore the telomere structure of fission yeast, a one-hybrid screen[18] was used to identify proteins that bind specifically to the *S. pombe* telomere DNA sequence (Fig. 1a). This screen had two components: an expression library of *S. pombe* cDNAs, each fused to DNA encoding the *S. cerevisiae* GAL4 activation domain, and a *S. cerevisiae* reporter strain containing a target sequence (a 32-mer of *S. pombe* telomeric sequence[19]; Fig. 1b) located adjacent to the GAL4-activatable promoter of a *lacZ* gene. *S. pombe* proteins that bind to the telomeric target sequence recruit the GAL4 activation-domain fusion to the promoter, thereby activating transcription of the *lacZ* reporter gene and yielding a blue colony upon exposure to X-gal.

Of over a million colonies screened, four blue colonies were identified; these harboured distinct cDNAs, but all four contained the gene *taz1*$^+$. The Taz1 fusion protein did not activate *lacZ* transcription when a scrambled version of the telomeric sequence was used as a target (Fig. 1b), indicating the specificity of Taz1p for telomeric repeats.

To determine more precisely the sequence requirements for Taz1–GAL4 activation, strains with smaller tracts of telomeric sequence placed upstream of the *lacZ* reporter were transformed with the *taz1*$^+$*–GAL4* fusion plasmid (Fig. 1b). Three of the four targets resulted in activation by the Taz1p fusion. The only distinguishing feature of the unactivated, presumably unbound, target was the relative deficit of guanine stretches in the sequence; it contains only two G_2 tracts, whereas the activatable sequences have at least one G_3 tract and a G_4 or two G_2 tracts.

DNA sequencing of *taz1*$^+$ revealed its potential to encode a protein of 663 amino acids with a predicted relative molecular



b

Sequence	Colony colour
gatc GGGTTACAAGGTTACGTGGTTACACGGTTACA gatc	blue
gatc GCTATGAATGCAGTAGTCGCGTAGTGTATCGA gatc	white
gatc GGGTTACAAGGTTACG gatc	blue
gatc TGGTTACACGGTTACA gatc	white
gatc GGTTACAGGTTACAGG gatc	blue
gatc GGGTTACAGGGGTTAC gatc	blue

Figure 1 Identification of *taz1*$^+$. **a**, One-hybrid screen for proteins that bind to the telomeric sequence of fission yeast. **b**, Target sequences used in one-hybrid experiments. Underlined are blocks of 5′-GGTTAC-3′, the most frequently occurring repeating unit in *S. pombe* telomeres (most telomere repeats in *S. pombe* conform to the consensus 5′GGTTACA$_{0\text{-}1}$C$_{0\text{-}1}$G$_{0\text{-}6}$-3′; ref. 19). The top sequence is a portion of the telomeric sequence[19] used for the one-hybrid screen. The second line is a randomized version of the first sequence. The colours are for colonies containing the given target sequence when transformed with a *taz1-GAL4* fusion plasmid.

a

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  1  misvQSTETI QKVLENEGDQ QFDKEVVQNS DSNIETGQIS DSLTKAVEER
 51  AETESSSNLS NFTTSESESS KPAYCFNSHS QNMAEGSISI PVISHSMNVE
101  NEVSTAEGQD SRTGESENDQ NAMIVRSIWD IEKASLLVND CQNIANMAEQ
151  KVMMVSAIFS ESSKDIVNPE SFSERLGKET VKDLYEFNEQ LTTKYGLEFR
201  TIFFSYIRKY DAYWCLFEDL EKPLKSIQFF TGLIDLLDNT NKHLTLRSIV
251  LDALLSADEE DSFYGDALVL FEELVIRYFG TDSNPSIDAS EFILSCLPYT
301  SLDALNVVCG QVWKSQKICD FLKSTIGNTS NSPLQLRASF PAFVNAVIHF
351  LLEFKNVRRL ERKDLSVKGM LYDSDSQQIL NRLRERVSGS TAQSADEASG
401  HESDASEDTF SERTLGLNSI DNTEISEVVS LGLVSSALDK ITGLLSADNL
451  SETVSQARDF SHTLSKSLKS RAKSLSQKEA ANRSKLIAKR GDNLRREASL
501  SSEQDDLSED FPPVRESDEQ ESRSGGRSSA MRVSIERSAA RSGTRRSQGN
551  PYEGYRTRRK WTDEEENELY EMISQHGCCW SKIIHIQKLE NGPLKTFGPT
601  QIKDKARLIK ARFMKQNRLQ ELYSKSLNWK NVTVGQAYCE LHKIPYIEAT
651  PPLLREELVN YQS
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b

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              Helix 1        Helix 2               Helix 3
Taz1p   RTRRKWTDEEENELYEMISQHG-CCWSKIIHIQKLENG---PLKTFGPTQIKDKARLIKAR
Tbf1p   KAKRTWSKEEEEALVEGLKEVG-PSWSKILDLYGPGGKITENLKNRTQVQLKDKARNWKLQ
hTRF    RKRQAWLWEEDKNLRSGVRKYGEGNWSKILLHY--------KFNNRTSVMLKDRWRTMKKL
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Figure 2 Sequence and Myb homology of Taz1p. **a**, Sequence of Taz1p. Amino acids deduced from sequences of cDNAs identified in the one-hybrid screen (upper case). The first four amino acids (lower case) were not present in any of the Taz1-Gal4 fusion proteins and were subsequently identified by matching the deduced sequence with portions of the *S. pombe* genome sequenced by the Sanger Centre sequencing project (found on the *S. pombe* blast server; the gene is encompassed by cosmid c16A10D; ref. 30). Amino acids 112–663 were present in all of the Taz1-Gal4 fusions identified in the one-hybrid screen. The region with homology to Myb DNA binding domains is boxed. The arrow indicates the region of the Taz1p coding sequence that is missing in the *taz1*$^-$ disruption strains. **b**, Alignment of Taz1 protein sequence to the Myb-related (telobox) domains of the human TRF protein[14] and *S. cerevisiae* Tbf1p[20]. Amino acids in Taz1p that show identity to at least one of the other two sequences are indicated by bold type. The α-helices 1, 2 and 3 are proposed on the basis of a recent structure-sequence alignment of Myb domain, homeodomain and telomere-binding proteins by D. Rhodes (manuscript in preparation); helix ends are uncertain. The dashed line between helices 2 and 3 denotes a region of length variability[20].

mass of 74,600 (M_r 74.6K) (Fig. 2a). The carboxy-terminal region of the protein contains a 57 amino-acid region of homology with the Myb-related DNA-binding domain from the human telomere protein hTRF (Fig. 2b). This presumed helix–turn–helix motif is also known as the 'telobox' (ref. 20) because it is present in other proteins that bind telomere-like sequences *in vitro*, including the *S. cerevisiae* protein Tbf1p (refs 20–22). These proteins, including Taz1p, contain a single Myb-related domain near their C termini, which distinguishes them from several transcription factors containing multiple Myb domains[20]. The crystal structure of the *S. cerevisiae* Rap1 protein reveals two regions of structural homology to the Myb DNA-binding domain, despite it having only limited sequence homology[23]. Outside the Myb-related domain, Taz1p is acidic and has only slightly more homology with hTRF and Tbf1p (21–22% identity) than with randomly chosen proteins.

To explore the functions of Taz1p *in vivo*, a disruption of *taz1*$^+$ was made in which two-thirds of the gene was deleted (Fig. 2a) and replaced with a selectable marker, the *ura4* gene. Dissection of tetrads derived from the resulting heterozygous *taz1*$^+$/*taz1*$^-$ diploids showed that the two *ura4*$^+$ *taz1*$^-$ spores were viable and that *taz1*$^-$ haploids grow vegetatively at the same rate as cells containing wild-type *taz1*$^+$. A complete deletion of *taz1*$^+$ was also constructed and found not to affect viability.

Telomere length increased dramatically when *taz1*$^+$ was disrupted (Fig. 3) or deleted (data not shown). Two of the three *S. pombe* chromosomes contain *ApaI* restriction sites just centromere-proximal to their telomeric repeats[19]. Digestion of wild-type DNA with *ApaI* gave a broad distribution of telomeric fragments centred around 300 base pairs. In DNA from cells carrying the *taz1* disruption, the telomere band was shifted to a smear of ~2.5–4.5 kilobases. This striking increase in the abundance of telomeric DNA is further underscored by the greater intensity of telomeric hybridization relative to the signal for wild-type telomeric DNA. A similar increase in the intensity of hybridization to the rDNA-adjacent telomeres on chromosome III, which do not contain telomere-proximal *ApaI* sites, was also observed (Fig. 3); an increase in length of fragments as large as the rDNA telomeric fragments may not be detectable with the gel system used. Despite the continual presence

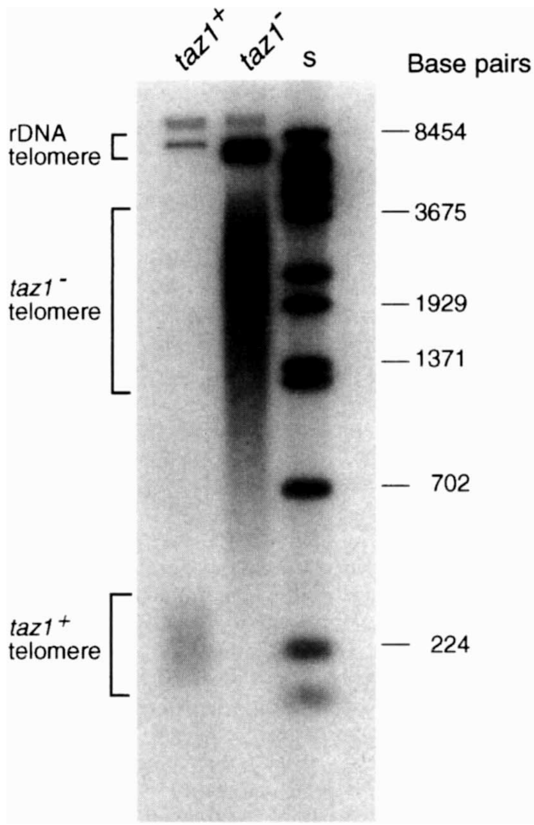

Figure 3 Elongated telomeres in *taz1*$^-$ strains. Southern blot of genomic DNA digested with *ApaI*; *taz1*$^+$ denotes strain CF11 containing the intact *taz1*$^+$ gene; *taz1*$^-$ denotes a strain of the same genotype as CF11 with the exception of the *taz1*$^-$ disruption. Ethidium bromide staining confirmed that approximately equal quantities of DNA were loaded in these two lanes. S, *BstEII* digest of bacteriophage lambda DNA. Identical results were obtained when *taz1*$^+$ was disrupted or deleted in other strains.

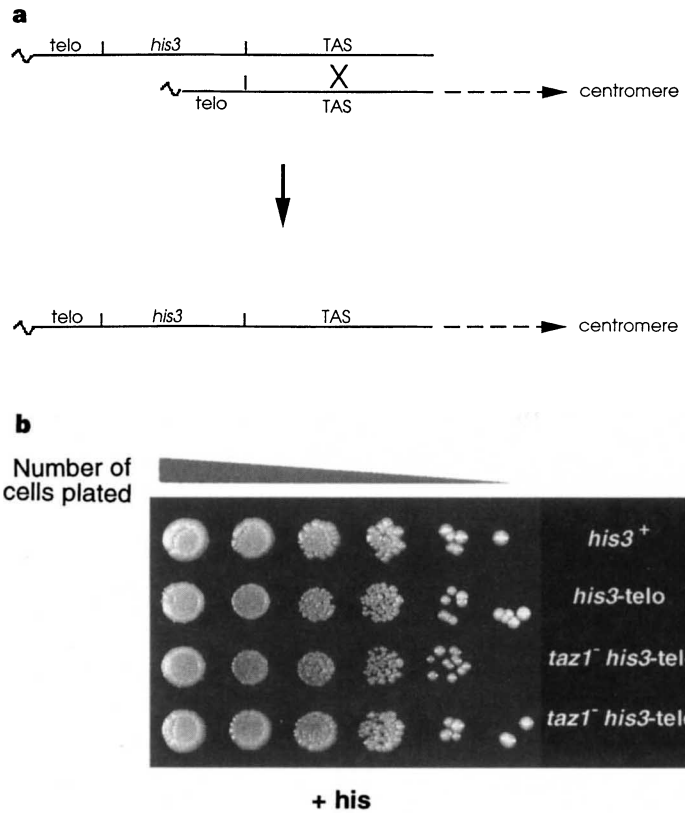

Figure 4 Alleviation of the telomere position effect in *taz1*$^-$ cells. **a**, Construction of the *his3*-telo reporter strain. A fragment containing the *his3*$^+$ gene cloned between TAS and telomere repeat sequences was transformed into a *his3*$^-$ *lot1*$^-$ strain; *lot1*$^-$ allows expression of telomere-adjacent genes (E.R.N. and R.C.A., unpublished data). Chromosome breakage in the TAS region generated the *his3*-telo strain, which was verified by Southern blot analysis and pulsed-field gel electrophoresis (data not shown). **b**, Derepression of expression of the telomeric *his3* gene upon *taz1* disruption. Tenfold serial dilutions of overnight cultures of the strains indicated were spotted onto plates either containing (+ his) or lacking (– his) histidine.

of longer telomeres, *taz1*$^-$ cells thrive for many generations, even after extensive subculturing.

The large and immediate increase in the number of telomeric DNA repeats when *taz1*$^+$ was disrupted or deleted suggests possible functions for the protein. (1) Taz1p might act as a negative regulator of telomerase. For example, Taz1p might bind directly or indirectly to the telomerase enzyme to inhibit its processivity, or it might limit the accessibility of telomerase to telomeric DNA. In support of this latter possibility, we have characterized a discrete non-nucleosomal chromatin structure at the telomeres of fission yeast, and observed the disruption of this structure in *taz1*$^-$ cells (J.P.C. and T.R.C., manuscript in preparation). (2) All *taz1*$^-$ cells might immediately activate some telomerase-independent, recombination-based mechanism of telomere repeat synthesis; such pathways have previously been observed as rare events in populations of yeast undergoing senescence[24].

In *S. cerevisiae* and *S. pombe*, genes that are transcriptionally active when located internally become subject to reversible transcriptional repression when placed adjacent to a telomere[25,26]. To test the involvement of Taz1p in telomeric repression, we used *S. pombe* strain FY1778, in which the endogenous *his3*$^+$ gene was deleted and cloned adjacent to a chromosomal telomere (Fig. 4a). The *his3*$^+$ gene in the resulting *his3*-telo strain is repressed to the extent that it gives an ~200,000-fold reduction in the number of colonies that grow on plates lacking histidine (Fig. 4b). In marked contrast, *his3*-telo cells in which *taz1*$^+$ has been disrupted grow equally well on plates containing or lacking histidine. Thus disruption of *taz1*$^+$ in *his3*-telo cells leads to nearly complete derepression of the telomeric *his3* gene.

The reduction in telomeric repression seen in *taz1*$^-$ cells could result from either the absence of *taz1*$^+$ or the increased length of *taz1*$^-$ telomeres, which might be expected to widen the distance between a telomere-adjacent gene and the protein complex that would repress its transcription. In support of the former, disruption of telomeric chromatin structure in *taz1* mutants (J.P.C. and T.R.C., manuscript in preparation) suggests that such repressive telomeric complexes may be absent or defective. In addition, when *taz1*$^+$ and *taz1*$^-$ haploids mate, telomeres remain longer than normal (~800 bp) for many (>130) generations, although the telomere position effect is quickly restored. Thus we think it likely that Taz1p is involved in establishing or maintaining a telosome structure that is required for telomere position effect.

The effect of *taz1* disruption on transcription appears to be analogous to that observed for a mutant of the *S. cerevisiae* Rap1 protein (*rap1-17*) in that *rap1*-17 cells exhibit both telomere lengthening and derepression of telomere-adjacent transcription[27].

Although vegetative growth of *taz1*$^-$ cells is normal, sexual reproduction is aberrant. Mating mixtures of homothallic *taz1*$^+$ cells (Fig. 5, left) contained normal haploid and diploid cells, as well as numerous asci in which meiosis and sporulation had occurred (filled arrows in Fig. 5, left); these asci usually contained four clearly visible, round spores. In contrast, mating mixtures in which *taz1* had been disrupted (*taz1::ura4* × *taz1::ura4*) or deleted were markedly deficient in healthy-looking asci. Most *taz1*$^-$ asci contained fewer than four distinct spores (open arrows in Fig. 5, right), which were often shaped aberrantly. Occasionally, *taz1*$^-$ asci contained four spores of normal appearance (filled arrow in Fig. 5, right), and some cells in the *taz1*$^+$ mixture may have corresponded to aberrant asci. Of the spores that did form in *taz1*$^-$ homozygous crosses, spore viability was only ~5% of that measured for *taz1*$^+$ crosses. Thus Taz1p is required for either efficient meiosis or sporulation. Telomeres may be important for meiotic processes, as telomeres mediate the attachment of chromosomes to spindle pole bodies and lead chromosome movement during the premiotic phase in *S. pombe*[28]. It is tempting to speculate that Taz1p may be involved in the interactions between chromosomes and spindle proteins, and that disruption of these interactions leads to defective meiosis.

The sequence specificity displayed by Taz1p in one-hybrid experiments and the presence of a known telomere DNA-binding motif,

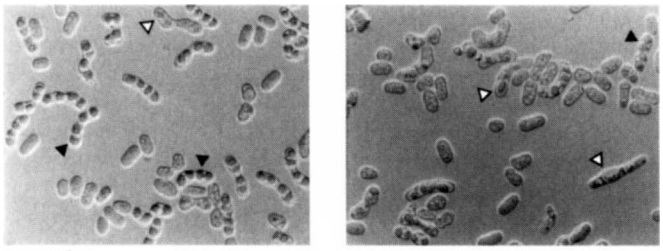

Figure 5 Meiosis/sporulation defect in *taz1*$^-$ cells. The *taz1*$^+$ gene was disrupted in strain FY1778, which is of the h^{90} homothallic (switchable) mating type. Nomarski images of homozygous *taz1*$^+$ and *taz1*$^-$ crosses after 3 days of incubation at 30 °C on malt-extract plates. Filled arrowheads indicate asci containing four distinct spores; open arrowheads indicate aberrant asci.

the Myb domain, suggest that Taz1p is recruited to telomeres by direct DNA binding. The many roles of Taz1p may be mediated either by modulating the conformation of telomeric DNA and its accessibility to other proteins, or by recruiting additional proteins to telomeres. For example, the striking increase in telomere length seen in *taz1*$^-$ cells could be explained if Taz1p normally limits the accessibility of telomerase to its DNA substrate. The existence of such inhibitory regulation of telomerase has been predicted from studies of the budding yeast *Kluyveromyces lactis*[29]. The Rap1 protein is a likely candidate for such an inhibitory protein in budding yeasts. The functional homology between Rap1p and Taz1p also extends to their effects on telomere-adjacent transcriptional repression. This conservation of function between proteins that do not share sequence homology, and that come from two evolutionarily distant organisms, suggests that proteins with similar functional repertoires will be found in other eukaryotes.

The homology displayed by Taz1p to the Myb DNA-binding domain of the human TRF protein suggests that these proteins are also related. The relative ease with which functions can be dissected in *S. pombe* may help illuminate the functions of this whole class of proteins. If hTRF plays a role similar to that of Taz1p in telomere-length regulation, it may provide one of the missing links in the interplay between telomerase activity, telomere length and tumorigenesis. □

Methods

***S. pombe* strains and manipulations.** The strains CF11 (*h*$^-$ *ade6-M210 leu1-32 ura4-D18*), CF14 (*h*$^+$ *ade-M216 leu1-32 ura4-D18*) and FY1778 (h^{90} *ura4-D18 his3-D1 ade6-M210 leu1-32 his3-telo*) were grown in YES or EMMG media[15] with required supplements. The construct for *taz1* gene disruption was made by ligating polymerase chain reaction (PCR) fragments corresponding to base pairs 43–768 and 1151–2143 of *taz1*$^+$ to either side of the *ura4*-containing *Eco*RI–*Bam*HI fragment of pSPORT-URA (provided by R. West). The construct for complete *taz1* deletion used a PCR fragment corresponding to base pairs −942 to +9 of the *taz1*$^+$ open reading frame as the upstream complementary sequence. Disruption and deletion of *taz1* were performed in CF11 × CD14 diploids and confirmed by PCR. Details of construction of the FY1778 *his*-telo strain will be described elsewhere (E.R.N. and R.C.A., unpublished data). Disruption of *taz1*$^+$ in FY1778 was as described above. Spore viability was measured by random spore analysis[15].

One-hybrid screen. A 32-mer of telomeric sequence (Fig. 1b, top sequence) was cloned into the unique *Bgl*II site of pB*gl-lacZ*[18], the resulting plasmid was linearized by digestion with *Stu*I and transformed into *S. cerevisiae* GGY1 (*MATalpha Δgal4 Δgal80 ura3 leu2 his3 ade2 tyr25*) to generate the reporter strain (both pB*gl-lacZ* and strain GGY1 were provided by J. Li). The *S. pombe* matchmaker cDNA library (Clontech; 1.3×10^7 independent clones with 90% containing inserts, average insert size 1.3 kb) was amplified on Luria broth plates and transformed into the reporter strain. All *S. cerevisiae* transformations were by dimethyl sulphoxide-enhanced whole-cell yeast transformation. After 3–4 days of growth on SC-leu plates, X-gal colony filter assays were performed as described in the Clontech manual. Dideoxy sequencing was performed according to standard procedures.

Measurements of telomere length. Genomic *S. pombe* DNA was prepared by a glass-bead lysis method, digested with *Apa*I, resolved on 1% agarose gels, transferred to nylon (Duralon) membranes and hybridized at 45°C to ^{32}P end-labelled telomeric oligonucleotides (Fig. 1b).

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