LETTERS

WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators

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Plants continuously maintain pools of totipotent stem cells in their apical meristems from which elaborate root and shoot systems are produced. In *Arabidopsis thaliana*, stem cell fate in the shoot apical meristem is controlled by a regulatory network that includes the CLAVATA (CLV) ligand–receptor system and the homeodomain protein WUSCHEL (WUS)[1,2]. Phytohormones such as auxin and cytokinin are also important for meristem regulation[3]. Here we show a mechanistic link between the CLV/WUS network and hormonal control. WUS, a positive regulator of stem cells, directly represses the transcription of several two-component *ARABIDOPSIS RESPONSE REGULATOR* genes (*ARR5*, *ARR6*, *ARR7* and *ARR15*), which act in the negative-feedback loop of cytokinin signalling[4,5]. These data indicate that *ARR* genes might negatively influence meristem size and that their repression by WUS might be necessary for proper meristem function. Consistent with this hypothesis is our observation that a mutant *ARR7* allele, which mimics the active, phosphorylated form, causes the formation of aberrant shoot apical meristems. Conversely, a loss-of-function mutation in a maize *ARR* homologue was recently shown to cause enlarged meristems[6].

Genetic analyses have led to the discovery of several essential regulators of stem cell fate in the shoot apical meristem of the reference plant *Arabidopsis thaliana*. Among them, the homeodomain transcription factors WUSCHEL (WUS) and SHOOTMERISTEM-LESS (STM) have positive functions[7,8], whereas the *CLAVATA* (*CLV*) genes negatively influence meristem size[9–11]. *WUS* is expressed in the organizing centre and induces stem cell fate in the overlaying cells[12] that in turn express CLV3, a small secreted peptide[13,14] that is thought to act as ligand for the CLV1–CLV2 heteromeric receptor complex[15,16]. Activation of the CLV1–CLV2 receptor leads to the suppression of *WUS* expression, creating a negative feedback loop that controls the size of the stem cell pool[1,2].

Despite the central role of the WUS transcription factor in the initiation and maintenance of stem cell fate, only a single direct target, the floral homoeotic gene *AGAMOUS* (*AG*), which represses the maintenance of stem cells in the flower, has been described[17]. To identify target genes of WUS and other meristem regulators, we performed a comparative microarray screen using plants with ethanol-inducible overexpression alleles[18] of *WUS* as well as *STM* and *LEAFY* (*LFY*), a floral regulator that interacts with *WUS*[17,19]. After 12 h of treatment with ethanol we harvested the shoot apex and surrounding tissue (Fig. 1a) and subjected it to expression profiling with Affymetrix Ath1 arrays. A combination of per-gene and common variance[20] filtering was used to identify 148 genes responsive to

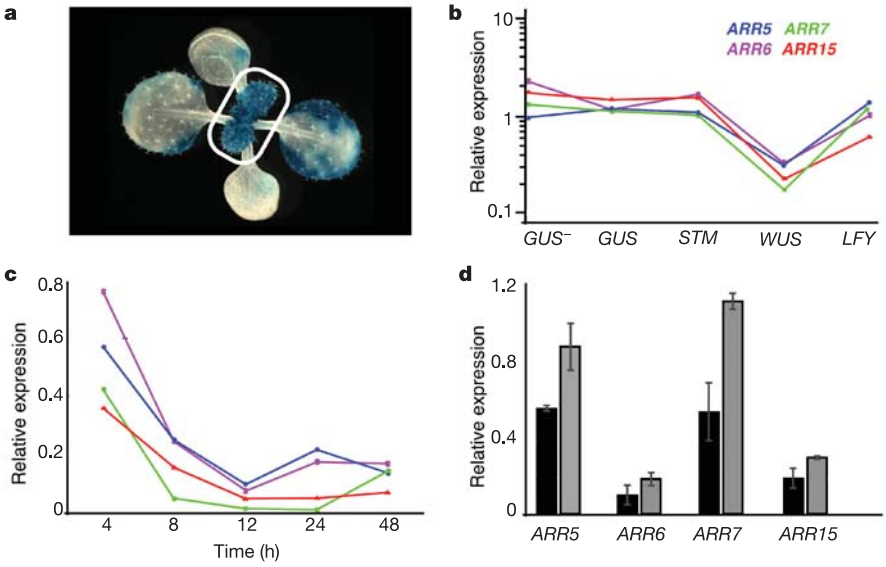

Figure 1 | Expression profiles of *ARR5*, *ARR6*, *ARR7* and *ARR15*. **a**, A 12-day-old seedling showing ectopic *AG::GUS* reporter gene activation in response to WUS induction. Tissue used for expression profiling is indicated. **b**, Expression of *ARR5* (blue), *ARR6* (purple), *ARR7* (green) and *ARR15* (red) is specifically repressed by WUS as detected by microarrays. **c**, Real-time qRT–PCR confirms rapid repression of *ARR* genes by WUS. Relative expression is normalized to induced *AlcA::GUS* controls. Line colours are as in **b**. **d**, *ARR* expression in response to downregulation of *WUS* by induction of *AlcA::CLV3* (grey bars). Black bars, *AlcA::GUS*. Relative expression measured by real-time qRT–PCR is normalized to *TUBULIN*. Error bars indicate s.e.m.

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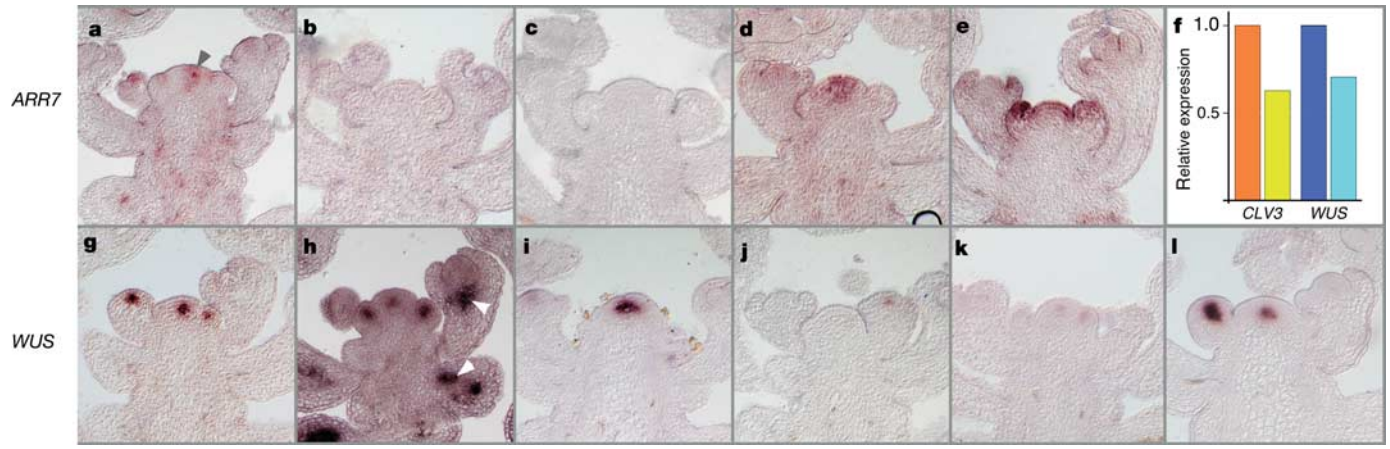

Figure 2 | Expression patterns of *ARR7* and *WUS* in response to meristematic signals. Upper panels show *in situ* hybridizations of *ARR7*; lower panels show *WUS*. **a**, **g**, Wild type. **b**, **h**, Induced *35S::AlcR AlcA::WUS*. *WUS* is moderately expressed in all cells with hot spots in more mature tissue (arrowheads). **c**, **i**, *clv3-7* mutant. **d**, **j**, Induced *35S::AlcR AlcA::CLV3*. **e**, 6-Benzylaminopurine-treated wild type. **f**, *CLV3* and *WUS* expression in inflorescence apices of wild-type and *arr3 arr4 arr5 arr6 arr7 arr8 arr9* septuple mutants as measured by qRT–PCR. Dark colours represent wild-type, light colours indicate mutant. **k**, *35S::ARR7*. **l**, *arr3 arr4 arr5 arr6 arr7 arr8 arr9* septuple mutant.

WUS but not to *STM* or *LFY* induction (Supplementary Table 1). Of these 148 genes, 44 were repressed, including *ARR5*, *ARR6*, *ARR7* and *ARR15*, which belong to the 10-member type-A *ARABIDOPSIS RESPONSE REGULATOR* gene family[21] (Fig. 1b). Type-A *ARR* proteins contain a phosphate-accepting receiver domain similar to bacterial two-component response regulators, but in contrast to type-B *ARR* proteins they lack a DNA-binding motif in their output domain[22]. Their expression is rapidly induced by cytokinin[21], which has been shown to be a potent inductor of cell proliferation when applied exogenously together with auxin and to induce shoot development when acting alone[23]. Type-A *ARR* proteins have been implicated in the negative feedback regulation of cytokinin signalling on the basis of the observation of decreased hormone sensitivity in plants overexpressing type-A *ARR* genes[4,24]. Furthermore, in *Arabidopsis*, type-A *arr* multiple mutants have increased cytokinin sensitivity. However, even in sextuple type-A *arr* mutants (*arr3 arr4 arr5 arr6 arr8 arr9*) morphological changes are minimal, indicating strong redundancy within the gene family[5]. *ARR5* and *ARR6*, as well as *ARR7* and *ARR15*, constitute closely related pairs within the gene family[21], and inspection of the AtGenExpress expression atlas[25] revealed co-expression of each pair, marked by widespread transcription with highest levels in meristematic tissue for *ARR7* and *ARR15*, and in roots for *ARR5* and *ARR6*.

By using quantitative real-time reverse transcriptase-mediated polymerase chain reaction (qRT–PCR), we found that 4 h after *WUS* induction by ethanol, RNA levels of *ARR5*, *ARR6*, *ARR7* and *ARR15* were already decreased, and after 12 h they reached a minimum at about 10% of control levels. Expression levels remained low for at least 48 h after treatment with ethanol (Fig. 1c). To test whether WUS is not only sufficient but also necessary for the repression of *ARR5*, *ARR6*, *ARR7* and *ARR15* in wild-type meristems, we used inducible *CLV3* to transiently repress *WUS*, because the morphology of *wus* mutants deviates strongly from the wild type even at very early stages of development[7]. Besides a strong reduction of *WUS* expression, we observed by qRT–PCR a moderate increase in expression of the *ARR* genes after 24 h of *CLV3* induction (Fig. 1d), which is consistent with the idea that *ARR* expression extended into the small *WUS* domain in these plants.

In situ hybridization on sections of inflorescence meristems demonstrated that *ARR7* RNA accumulates in a subdomain of the meristem consistent with a potential function in this tissue (Fig. 2a, and Supplementary Fig. 1). Reporter gene analysis confirmed this pattern and showed in addition that *ARR5*, *ARR6* and *ARR15* promoters are also active in the meristem (Supplementary Fig. 2).

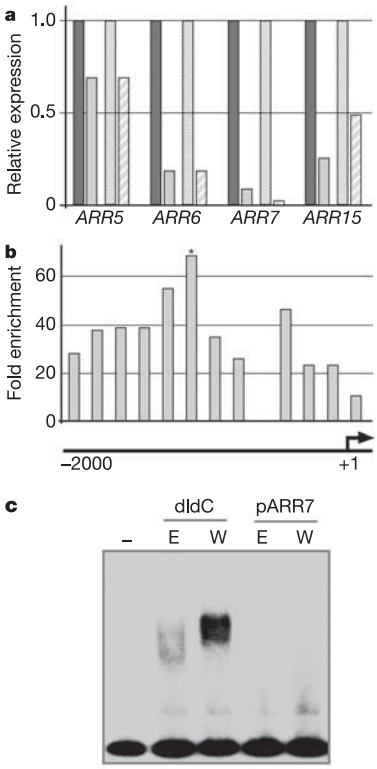

Figure 3 | Direct interaction of WUS with regulatory sequences of *ARR7*. **a**, Real-time qRT–PCR on *35S::WUS:GR* plants. Dark grey bars represent mock treatment, light grey bars induction with dexamethasone, cross-hatched bars mock treatment in the presence of cycloheximide, and hatched bars induction with dexamethasone in the presence of cycloheximide. Expression values are normalized to the respective mock treatment controls (see Supplementary Fig. 3a for alternative normalization). **b**, Detection of *ARR7* regulatory sequences by real-time qRT–PCR after ChIP with anti-WUS antiserum (see Supplementary Fig. 3b). Enrichment of overlapping genomic fragments upstream of the *ARR7* start codon is shown after normalization to unrelated control sequences (see also Supplementary Fig. 3c for alternative normalization). ChIP was performed on induced *35S::WUS:GR* tissue. Asterisk, promoter fragment used for gel shifts. **c**, EMSA using *ARR7* promoter sequences identified in **b**; –, free probe; E, control protein extract from yeast expressing LEAFY; W, protein extract from yeast expressing WUSCHEL; dIdC, poly(dIdC) used as unspecific competitor; pARR7, unlabelled probe used as specific competitor.

In plants with an inducible *WUS* transgene (Fig. 2h), *ARR7* RNA could no longer be detected 24 h after *WUS* induction (Fig. 2b), which is similar to the situation in *clv3* mutants (Fig. 2c), in which *WUS* expression is expanded (Fig. 2g, i). Conversely, after suppression of *WUS* by *CLV3* induction (Fig. 2j), we observed an expansion of the *ARR7* expression domain (Fig. 2d), confirming the qRT–PCR results. Activation of *ARR7* in cells outside the *WUS* domain might indicate a more direct effect of CLV3 on *ARR7* expression in parallel to its WUS dependent activity. Similarly to what has been observed for the maize homologue *ABPH1* (ref. 6), a 30-min treatment with the synthetic cytokinin 6-benzylaminopurine caused an expansion of *ARR7* expression in the wild type (Fig. 2e).

An additional level of regulation is provided by negative feedback of *ARR7* on *WUS*, because plants that overexpress *ARR7* from the constitutive *35S* promoter have lower *WUS* RNA levels (Fig. 2k). However, residual *WUS* activity in *35S::ARR7* plants is sufficient for correct function of the meristem, because *35S::ARR7* plants have no obvious defects in the shoot apical meristem, similar to induced *AlcA::CLV3* plants, which show a *wus* mutant phenotype only in flowers (data not shown).

Having established a regulatory interaction between *WUS* and *ARR7*, we next asked whether this interaction is direct. To this end, we first made use of an inducible form of WUS by means of a translational fusion to the ligand-binding domain of the rat glucocorticoid receptor (*WUS:GR*). Application of a steroid such as dexamethasone causes translocation of the fusion protein from the cytoplasm to the nucleus, allowing activation or repression of direct targets in the absence of protein synthesis[26,27]. After treatment of *35S::WUS:GR* plants with dexamethasone for 4 h, we observed robust repression of *ARR5*, *ARR6*, *ARR7* and *ARR15*. Repression of the *ARR* genes also occurred in the presence of the protein synthesis inhibitor cycloheximide (Fig. 3a, and Supplementary Fig. 3a), which is compatible with a direct interaction of WUS with the regulatory elements of the *ARR* genes. We then confirmed *in vivo* binding of WUS to *ARR7* promoter sequences by chromatin immunoprecipitation (ChIP) with a polyclonal anti-WUS antiserum (Fig. 3b, and Supplementary Fig. 3b, c). We observed a twofold enrichment of *ARR7* promoter DNA in wild-type inflorescences in comparison with leaves, in which *WUS* is not expressed, whereas in *WUS*-overexpressing tissue *ARR7* promoter DNA was enriched 68-fold. The ChIP results indicated binding of WUS to sequences located about 1,000 base pairs upstream of the start codon of *ARR7* in a region harbouring multiple TAAT elements, which have been shown to be the core binding sites for WUS (Fig. 3b)[17]. Subsequently, we were able to confirm sequence-specific binding of WUS protein to this promoter element by electrophoretic mobility-shift assays (EMSAs) (Fig. 3c).

It has recently been shown that maize mutants defective for *ABPH1*, a type-A *ARR* homologue, have defects in phyllotaxis and meristem size regulation[6]. In contrast, neither *Arabidopsis* plants lacking individual type-A *ARR* genes nor plants overexpressing *ARR5*, *ARR6*, *ARR7* or *ARR15* have obvious phenotypes (data not shown, and refs. 4, 5). We therefore constructed *arr7 arr15* double mutants, because they are closely related and both are expressed in meristematic tissue. However, the double mutant combination caused female gametophytic lethality, precluding analysis of the progeny. To reduce redundancy outside the *ARR7*/*ARR15* pair, we then extended our analysis to *arr3 arr4 arr5 arr6 arr7 arr8 arr9* septuple mutants. These plants were viable, although they had defects in phyllotaxis and organ initiation (Fig. 4a, b), indicating that the redundant function of *ARR7* and *ARR15* might be sufficient for meristem maintenance. *WUS* expression in the inflorescence meristem of septuple mutants was decreased (Fig. 2f, l), indicating that, in addition to the negative regulatory activity of *ARR7* on *WUS*, there might be positive effects on *WUS* expression by other type-A *ARR* genes.

As an alternative to exploring *ARR7* function, we constructed alleles that either mimic the active, phosphorylated state or the inactive non-phosphorylated state of ARR7 by mutating aspartate 85 to glutamate or asparagine, respectively[28]. Whereas ubiquitous overexpression of the dominant-negative form (Asp 85 → Asn) did not cause any morphological defects, the constitutively active form (Asp 85 → Glu) had severe effects on the function of the shoot apical meristem. In some of the transgenic seedlings meristems were arrested for several days after expansion of the cotyledons, resulting in an almost complete block of organ formation, very similar to that

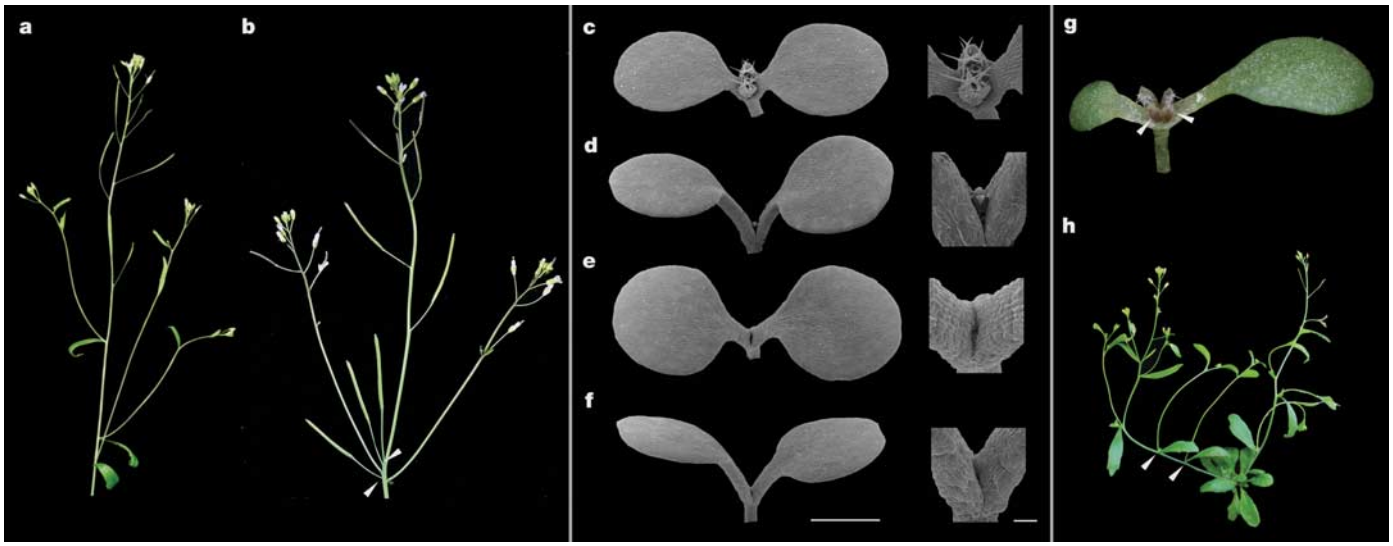

Figure 4 | Phenotypes of type-A *ARR* mutant plants. **a**, Wild type. **b**, *arr3 arr4 arr5 arr6 arr7 arr8 arr9* septuple mutant; note irregular organ positioning indicated by arrowheads. **c**–**f**, Activity of the shoot apical meristem is arrested in *35S::ARR7* (Asp 85 → Glu) plants similar to *wus* mutants 5 days after sowing. Scale bars, 1 mm for seedlings and 100 μm for meristem insets unless otherwise noted. **c**, *35S::ARR7* (Asp 85 → Glu) plant with wild-type morphology. Scale bar, 200 μm. **d**, *35S::ARR7* (Asp 85 → Glu) plant with intermediate phenotype. **e**, *35S::ARR7* (Asp 85 → Glu) plant with strong phenotype. **f**, *wus* mutant seedling. **g**, *35S::ARR7* (Asp 85 → Glu) seedling shortly after recovery of meristematic activity. Arrowheads indicate duplicated meristems. **h**, Phenotype of an adult *35S::ARR7* (Asp 85 → Glu) plant after recovery. Note duplicated rosettes. Arrowheads indicate irregular side-shoot positions.

observed in *wus* mutants (Fig. 4c–f). Subsequently, shoot apical meristems recovered proliferative activity, but often split into two or three independent meristems (Fig. 4g), giving rise to multiple primary shoots. Similarly to the *abph1* mutant of maize[6], these shoots had defects in phyllotaxis (Fig. 4h) and flower formation; in addition they did not produce seeds.

Our results show that direct interaction between the CLV/WUS network and the cytokinin signalling circuitry is required for proper meristem function. Together with the recently uncovered role of the type-A response regulator *ABPH1* in maize[6], our findings are a first step towards understanding how global hormonal signals are integrated with local transcriptional inputs in the regulation of cell behaviour at the shoot apical meristem.

METHODS

Plant material and treatments. Plants were of Columbia background and grown at 23 °C in continuous light. Inductions with ethanol were performed at 20 °C by watering with 1% ethanol. For inductions with dexamethasone, tissue was incubated in 15 μM dexamethasone and 0.015% Silwet L-77. Cycloheximide was used at 10 μM. For 6-benzylaminopurine treatments, tissue was incubated in 1 μM 6-benzylaminopurine and 0.1% DMSO. The Columbia *wus* allele corresponds to *wus-4* (provided by Martin Hobe and Rüdiger Simon); details on the *arr3 arr4 arr5 arr6 arr7 arr8 arr9* septuple mutant are available in Supplementary Information.

Microarray experiments. Affymetrix Ath1 microarrays were hybridized as described[29] in duplicates using RNA from pools of 20 plants for each replicate. Expression estimates were calculated by gcRMA (ref. 30) and statistical testing for differential expression was performed with LogitT (ref. 20).

Quantitative real-time RT–PCR. qRT–PCR was performed as described[29] with the use of either SYBR-green or Taq-Man probes (Fig. 1d). Experiments were performed in triplicates from RNA of pooled tissue. Amplification of *TUBULIN* served as control. Oligonucleotides are listed in Supplementary Table 2.

***In situ* hybridization.** *In situ* hybridization was performed in accordance with standard protocols, with the addition of 10% poly(vinyl alcohol) (molecular mass 70–100 kDa) to the staining solution.

ChIP. Genomic fragments were analysed by real-time qRT–PCR in triplicates. Unrelated sequences in the experimental tissue and *ARR7* sequences in leaves, where WUS should not be present, served as controls. A detailed protocol is available as Supplementary Information.

EMSA. EMSA was performed as described in ref. 17.

Transgenes. Complementary DNAs flanked by the *AlcA* promoter and the *OCS* terminator were inserted into a pMLBART-derived binary vector, which harbours a *35S::AlcR* cassette[18]. Constitutive overexpression constructs were made in pMLBART or pART27 binary vectors using a *35S* promoter and an *OCS* terminator.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions A.L. performed *in situ* hybridizations and qRT–PCRs, constructed reporter genes, the mutated alleles of *ARR7* and performed electron microscopy; J.P.C.T. and J.J.K. generated and analysed the *arr* double and septuple mutants; W.B. performed the ChIP experiments; S.S. generated constructs and transgenic lines of *ARR* genes; A.K. generated *AlcA::CLV3* plants; M.D. performed qRT–PCRs; and J.U.L. carried out the microarray experiment and analysis, performed gel-shifts and wrote the paper. All authors discussed the results and commented on the manuscript.

Author Information Microarray data have been deposited at ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MEXP-432. Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to J.U.L. (jlohmann@tuebingen.mpg.de).