Proteasome-dependent truncation of the negative heterochromatin regulator Epe1 mediate antifungal resistance

Intiyaz Yaseen1,2,5,7, Sharon A. White1,2,7, Sito Torres-Garcia1,2, Christos Spanos1,2, Marcel Lafos1,2,6, Elisabeth Gaberdiel1,2, Rebecca Yeboah1,2, Meriem El Karoui2,3, Juri Rappsilber1,4, Alison L. Pidoux1,2 and Robin C. Allshire1,2,3✉

Epe1 histone demethylase restricts H3K9-methylation-dependent heterochromatin, preventing it from spreading over, and silencing, gene-containing regions in fission yeast. External stress induces an adaptive response allowing heterochromatin island formation that confers resistance on surviving wild-type lineages. Here we investigate the mechanism by which Epe1 is regulated in response to stress. Exposure to caffeine or antifungals results in Epe1 ubiquitylation and proteasome-dependent removal of the N-terminal 150 residues from Epe1, generating truncated Epe1 (tEpe1) which accumulates in the cytoplasm. Constitutive tEpe1 expression increases H3K9 methylation over several chromosomal regions, reducing expression of underlying genes and enhancing resistance. Reciprocally, constitutive non-cleavable Epe1 expression decreases resistance. tEpe1-mediated resistance requires a functional JmjC demethylase domain. Moreover, caffeine-induced Epe1-to-tEpe1 cleavage is dependent on an intact cell integrity MAP kinase stress signaling pathway, mutations in which alter resistance. Thus, environmental changes elicit a mechanism that curtails the function of this key epigenetic modifier, allowing heterochromatin to reprogram gene expression, thereby restoring resistance to some cells within a population. H3K9me-heterochromatin components are conserved in human and crop-plant fungal pathogens for which a limited number of antifungals exist. Our findings reveal how transient heterochromatin-dependent antifungal resistant epimutations develop and thus inform on how they might be countered.

The overuse in agriculture of antifungal agents, related to those used in treating human fungal infections, has caused progressive increases in resistance in soil-borne fungi. Consequently, clinical treatment of people with aspergillosis (Aspergillus), candidiasis (Candida) or cryptococcosis (Cryptococcus) infection is challenging due to the limited number of effective antifungal drugs and the increasing prevalence of resistance2–4. A dilemma exists, as widespread use of antifungals in agriculture is required to combat major plant pathogens such as Magnaporthe oryzae (rice; blast fungus) and Zymoseptoria tritici (wheat; leaf blotch) in order to enhance crop yields. Optimized crop production is required to meet the nutritional needs of the human population, which is estimated to increase by approximately 2 billion in the next 30 years5. It is therefore important to understand the mechanisms that allow fungi to adapt and develop resistance to external insults, such as antifungals, in order to design more prudent interventions for clinical and agricultural settings.

Throughout their evolutionary history, fungi have been exposed to challenging environments6. Consequently, fungi have developed robust, intricate systems to sense and adapt to ‘new’ external insults, such as antifungal compounds. Altered environments or manipulations are known to allow transcriptional memory and epigenetic inheritance in single-celled organisms, such as some fungi7–11, and multicellular organisms12–17 so that resulting DNA and chromatin modifications can persist through cell division, long after the original stimulus has dissipated. Such ‘epigenetic memory’ encourages heterogeneity with variable persistence in otherwise genetically identical cell populations, providing a bet-hedging strategy that ensures adaptation and survival of a proportion of individual cells upon exposure to new environmental challenges. Thus, transient but metastable epigenetic states can confer a selective advantage to particular cell lineages within a clonal population, while allowing return to the initial normal state once external pressures are relaxed18,19.

The fission yeast Clr4 methyltransferase installs all methylation of histone H3 at K9, triggering heterochromatin formation and transcriptional repression at locations such as centromeres, telomeres, the mating-type locus18–21 and facultative heterochromatin islands22–26. Heterochromatin assembly is antagonized by the histone acetyltransferase Mst2, which acetylates H3, preventing H3K9 methylation, and the Junmoni C (JmJC) domain histone demethylase Epe1, which likely removes superfluous H3K9 methylation from euchromatic regions and islands22,27–30. Schizosaccharomyces pombe lacks DNA methylation31,32; thus, its heterochromatin-mediated epigenetic regulation relies entirely on histone modification.

In the absence of Epe1, synthetic heterochromatin induced to form at ectopic chromosomal locations can be transmitted through multiple cell divisions by a read-write mechanism following the
Articles
C-terminally TAP-tagged Lid2 JmjC H3K4 demethylase35 was with time and was undetectable after 9 h, when levels of normally cleavage.

Altered protein mobility can result from changes in transcription, protein synthesis is needed to restore Epe1-Myc to the normally migrating Epe1-Myc had recovered to those of untreated cells

Exposure Epe1 exhibits altered mobility upon exposure to stress. Exposure of fission yeast expressing N-terminally 3xFLAG-tagged Epe1 to caffeine results in reduced levels of this key negative regulator of heterochromatin13. However, caffeine treatment of cells expressing C-terminally 13xMyc-tagged Epe1 (Epe1-Myc) revealed a faster-migrating form (hereafter called tEpe1) and reduced levels of Myc-tagged Epe1 (Epe1-Myc) is dependent on signaling through the cell integrity MAP kinase stress pathway, which therefore modulates heterochromatin-mediated caffeine and antifungal resistance. Epe1-related histone demethylase proteins are broadly conserved, suggesting that similar mechanisms contribute to adaption to external insults and antifungal resistance in pathogenic fungi.

Results
Epe1 exhibits altered mobility upon exposure to stress. Exposure of fission yeast expressing N-terminally 3xFLAG-tagged Epe1 to caffeine results in reduced levels of this key negative regulator of heterochromatin13. However, caffeine treatment of cells expressing C-terminally 13xMyc-tagged Epe1 (Epe1-Myc) revealed a faster-migrating form (hereafter called tEpe1) and reduced levels of full-length (FL) Epe1-Myc (Fig. 1a,b). This effect was independent of the tag, as a faster-migrating form of carboxy-terminally green fluorescent protein (GFP)-tagged Epe1 (Epe1-GFP) was detected upon caffeine exposure (Extended Data Fig. 1a). tEpe1 appeared upon osmotic (1 M KCl) or prolonged oxidative (1 mM H2O2) stress (Fig. 1b). By comparison, neither amount nor mobility of C-terminally TAP-tagged Lid2 JmjC H3K4 demethylase16 was altered by caffeine exposure (Fig. 1c). Thus, the mobility of a proportion of Epe1-Myc increases in response to several external stresses.

The appearance of tEpe1 was dependent on time and concentration, with tEpe1 visible after 7 h of exposure to 14 mM caffeine and increasing steadily thereafter, and upon 16 h of treatment with 5–15 mM caffeine (Fig. 1d,e). Upon caffeine removal after 16 h of caffeine treatment (14 mM), the proportion of tEpe1 declined with time and was undetectable after 9 h, when levels of normally migrating Epe1-Myc had recovered to those of untreated cells (Fig. 1f,g). Cycloheximide prevented recovery, indicating that protein synthesis is needed to restore Epe1-Myc to the normally migrating form (Fig. 1f,h).

Cleavage and loss of the N-terminal region alters Epe1 mobility. Altered protein mobility can result from changes in transcription, translation initiation, post-translational modifications or protein cleavage. epe1 contains no introns, precluding altered splicing as an explanation (www.pombase.org/gene/SPCC622.16c). Quantitative reverse-transcription PCR (RT-qPCR) analysis indicated that steady-state levels of epe1 transcript measured either immediately upstream of the initiating AUG or within the coding sequence were unaffected by caffeine (Extended Data Fig. 1b). Thus, stress-induced tEpe1 is unlikely to result from a downstream transcriptional start site (TSS). Indeed, no change in epe1+ TSS usage under stress has previously been detected19.

Mass spectrometry analysis of affinity-selected Epe1-GFP indicated that several residues gained phosphorylation following caffeine treatment (Extended Data Fig. 1c). However, the mobility of neither normally migrating Epe1 nor tEpe1 was altered by lambda phosphatase treatment, suggesting that phosphorylation does not cause the stress-mediated increase in Epe1 mobility (Extended Data Fig. 1d).

To investigate whether exposure to caffeine results in post-translational cleavage of Epe1, a strain expressing Epe1 tagged at both the N terminus (3xMyc) and C terminus (GFP) from the endogenous locus was constructed (Myc-Epe1-GFP), Anti-GFP western analysis following caffeine treatment detected tEpe1-GFP, but only the normal, slower-migrating form was detected by anti-Myc western analysis, albeit at a reduced level (Fig. 2a). This observation suggests that, in response to stress, a region containing the N-terminal 3xMyc tag is cleaved away from Myc-Epe1-GFP to leave both FL-Epe1-GFP and tEpe1-GFP detectable.

Epe1 has a portable signal for stress-mediated processing. To investigate the requirements for stress-induced Epe1 cleavage, strains expressing a series of N-terminal truncations of Epe1-GFP were generated (Fig. 2b). Epe1-GFP with deletions of the first 50, 100, 150 or 200 residues from the N terminus (Epe1ΔN50-GFP, Epe1ΔN100-GFP, Epe1AN150-GFP and Epe1AN200-GFP) were expressed at similar levels to FL-Epe1-GFP. Following caffeine treatment, cells expressing Epe1-GFP, Epe1AN50-GFP and Epe1AN100-GFP exhibited faster-migrating forms. However, no caffeine-induced change in Epe1AN150-GFP or Epe1AN200-GFP mobility was observed (Fig. 2b), suggesting that a region required for caffeine-induced cleavage resides between Epe1 residues 100 and 150.

Further strains that expressed Epe1-GFP proteins lacking 10 or 20 residues between residues 100 and 210 (Fig. 2c and Extended Data Fig. 2a) were subjected to western analysis following caffeine treatment. Removal of residues 101–110 prevented stress-mediated cleavage of Epe1Δ101–110-GFP, whereas FL-Epe1-GFP, Epe1Δ111–120-GFP, Epe1Δ111–130-GFP, Epe1Δ130–150-GFP, Epe1Δ151–170-GFP, Epe1Δ171–190-GFP and Epe1Δ190–210-GFP all exhibited a faster-migrating form. These analyses suggest that residues 101–110 are required for Epe1 cleavage. Direct comparison of caffeine-induced tEpe1 migration with that of Epe1AN150-GFP and of Epe1AN200-GFP indicated that, although residues 101–110 are necessary for Epe1 processing, the new N terminus resides near residue 150 (Fig. 2d,e). A precise cleavage site in Epe1 could not be defined by mass spectrometry. However, after Epe1-Myc immunoprecipitation (three replicates), three peptides (residues: 52–57, 57–67, 83–96) from within the first 150 amino acids of FL-Epe1-Myc were identified in cells grown without caffeine with high confidence (1% false-discovery rate (FDR)) but were not detectable from tEpe1 following caffeine treatment. In contrast, 15 peptides beyond residue 150 were detected with high confidence (1% FDR) from both FL-Epe1-Myc and tEpe1-Myc (Extended Data Fig. 2b).

To determine whether the N-terminal region is sufficient to mediate caffeine-induced cleavage, the first 150 residues of Epe1 were fused to GFP (Epe11–150-GFP, Fig. 2e). Treatment with caffeine released an entity from Epe11–150-GFP that co-migrated with recombinant GFP (27 kDa, Fig. 2f). We conclude that the first 150 residues of Epe1 are necessary and sufficient to drive cleavage of a heterologous protein in response to external stress.

Caffeine-induced Epe1 cleavage is proteasome dependent. To gain insight into the mechanism of Epe1 stress-mediated cleavage, we affinity-selected Epe1-GFP from cells grown with or without 14 mM caffeine and applied proteomics to identify associated proteins. Notably, caffeine treatment resulted in increased association of 23 of 35 known proteasome subunits20, representing components of both the 20S core (9 subunits) and 19S regulatory (14 subunits) particles, with Epe1-GFP (Fig. 3a). Caffeine-induced cleavage of
Fig. 1 | Epe1 migrates faster after caffeine stress and requires protein synthesis for recovery. a, Schematic of cells expressing tagged Epe1 from endogenous promoter exposed to various stresses, followed by western analyses. b, C-terminally-tagged Epe1-13×Myc or α-tubulin western analysis from untreated (−) cells or cells treated (+) with 14 mM caffeine, 1 M KCl or 1 mM H2O2 for 16h. Western analysis was performed at least twice. c, Lid2-TAP or α-tubulin western analysis from untreated cells or cells treated with 14 mM caffeine. Western analysis was performed at least twice. d, Epe1-13×Myc or α-tubulin western analysis from cells incubated for the indicated times in 14 mM caffeine. e, Epe1-13×Myc or α-tubulin western analysis from cells incubated with indicated caffeine concentrations for 16 h. f, Cycloheximide block-recovery scheme performed on Epe1-13×Myc cells. g, Epe1-13×Myc or α-tubulin western analysis from untreated cells or cells treated with 14 mM caffeine. Western analysis was performed at least twice. h, As in g, but with recovery in 5mg/ml cycloheximide. Plots in d, g and h: FL-Epe1 and tEpe1 levels are relative to α-tubulin loading control. Data points are independent measurements from a minimum of three biological replicates and data are presented as mean values with error bars ± s.e.m.

3×Myc-Epe1-GFP should release an N-terminal product of approximately 180 residues with a predicted mass of ~20kDa (including 3×Myc). However, no such product was detected, suggesting that the N-terminal fragment is rapidly degraded by the proteasome following its initial cleavage (Extended Data Fig. 3a). To test proteasome involvement in stress-mediated post-translational processing of Epe1, we examined Epe1-13×Myc processing in cells harboring the mts2-1 mutation in the ATPase subunit Rpt2 of the 19S proteasome regulatory particle 39. Epe1 was generated in wild-type but not mts2-1 cells grown at a permissive temperature, indicating that proteasome function contributes to caffeine-dependent Epe1 cleavage (Fig. 3b). A shorter Mst2 histone acetyltransferase (HAT) isoform is made in response to stress by a distinct mechanism involving the use of a downstream TSS 31,36. Production of N-terminally-truncated Mst2-13×Myc in caffeine was unaffected by the mts2-1 mutant, indicating that proteasome-mediated processing is Epe1-specific and that mts2-1 does not prevent other changes in response to caffeine stress (Extended Data Fig. 3b).

The proteasome inhibitor bortezomib primarily inhibits the β5th peptidase of the 20S core particle 30. Addition of bortezomib
Although nuclear Epe1 levels were somewhat decreased by addition of caffeine to mts2-1 cells, substantial amounts remained. Thus, proteasome function, and presumably proteasome-dependent Epe1 processing, is required to deplete nuclear Epe1 in response to caffeine stress.

Following caffeine treatment, increased ubiquitin associated with affinity-purified Epe1-Myc and Epe1-Myc was enriched on ubiquitin-binding beads (Fig. 3e,f). Thus, ubiquitylation of Epe1 is associated with Epe1-to-tEpe1 caffeine-induced proteasome-dependent processing. The Cul4–Ddb1C142 E3 ubiquitin ligase targets Epe1 for degradation within major heterochromatin domains3; however, Ddb1C142 loss had little impact on Epe1-to-tEpe1 processing (Extended Data Fig. 3f), suggesting involvement of other E3 ligases. Our proteomics analysis of affinity-selected Epe1-GFP detected enrichment of the Hul5 and Pub3 E3 ubiquitin ligase targets Epe1 for degradation within major heterochromatin domains3; however, Ddb1C142 loss had little impact on Epe1-to-tEpe1 processing (Extended Data Fig. 3f), suggesting involvement of other E3 ligases. Our proteomics analysis of affinity-selected Epe1-GFP detected enrichment of the Hul5 and Pub3 E3 ubiquitin ligase targets Epe1 for degradation within major heterochromatin domains3; however, Ddb1C142 loss had little impact on Epe1-to-tEpe1 processing (Extended Data Fig. 3f), suggesting involvement of other E3 ligases.
decreased caffeine-induced Epe1-to-tEpe1 processing, whereas deletion of pub3Δ had little impact alone or in combination with hul5Δ. Thus Hul5, but not Pub3, E3 ubiquitin ligase contributes to proteasome-mediated Epe1 processing in response to caffeine (Fig. 3g). Consistent with decreased Epe1-to-tEpe1 processing in response to external stress, both hul5Δ and hul5Δpub3Δ cells, but not pub3Δ or ddb1Δ cells, exhibited a lower frequency of caffeine- and fluconazole-resistant colonies than wild-type cells (Fig. 3h). We conclude that Hul5 E3 ubiquitin ligase contributes to the removal of the N-terminal region from Epe1 to generate tEpe1 by promoting its ubiquitin-dependent proteasome-mediated processing; however, this does not rule out the possible involvement of other E3 ligases in Epe1-to-tEpe1 processing.

N-terminally-truncated Epe1 accumulates in the cytoplasm. To further investigate the cellular location of processed Epe1-GFP, we used constitutively expressed N-terminal truncations. Epe1ΔN100-GFP had a similar pattern and nuclear signal intensity to FL-Epe1-GFP. Removal of an additional 50 (Epe1ΔN200-GFP) residues resulted in the complete loss of nuclear GFP foci and reduced nuclear signal intensity, with a corresponding increase in cytoplasmic signal intensity (Fig. 4a,b). These analyses indicate that the N-terminal 150 residues of Epe1 are required to maintain normal nuclear Epe1 levels and its concentration at heterochromatin domains, and that their removal increases the cytoplasmic levels of tEpe1.

Lyssates from cells expressing Epe1-Myc grown with or without caffeine were fractionated into soluble cytosolic supernatants and insoluble chromatin-containing pellets (Fig. 4c,d). As expected, most α-tubulin was released into the supernatant, whereas chromatin (histone H3) was retained in the pellet. Without caffeine, most Epe1 stayed in the insoluble pellet, as expected for this chromatin-associated protein. Upon caffeine-induced processing, most tEpe1 was present in the soluble fraction, whereas unprocessed Epe1-Myc remained in the pellet. This finding indicates that caffeine-induced removal of Epe1’s N terminus results in its loss from chromatin. In agreement with this observation, constitutively truncated Epe1ΔN150-GFP was detected mainly in the soluble fraction and was unaffected by caffeine treatment. Moreover, comparison of FL-Epe1-GFP and Epe1ΔN150-GFP-associated proteomes revealed that they occupy distinct cellular compartments (Extended Data Fig. 4a,b). Epe1-GFP associated with several other chromatin-associated nuclear proteins, including histones, the chromodomain protein Swi6 and the bromodomain proteins Bdc1 and Bdf2. In contrast, constitutively truncated Epe1ΔN150-GFP (equivalent to the caffeine-induced tEpe1-GFP) was associated with cytoplasmic proteins, consistent with a more cytosolic location.

To minimize the deleterious effects of caffeine-induced stress on cell morphology and cytology, the location and the cytoplasmic and nuclear signal intensities of Epe1-GFP were assessed in the presence and absence of a lower caffeine concentration (7 mM). Under these less stressful conditions, less Epe1-to-tEpe1 processing occurs than at higher caffeine concentrations (Fig. 1e), and most Epe1-GFP remained in the nucleus; nevertheless, cytoplasmic Epe1-GFP signals were elevated (Fig. 4c,f). Moreover, employment of the significantly brighter Halo tag in place of GFP confirmed FL-Epe1-Halo to be primarily nuclear, whereas Epe1ΔN150-Halo was distributed throughout the nucleus and cytoplasm (Fig. 4g). Together, these data indicate that stress-mediated Epe1-to-tEpe1 processing, for which Epe1ΔN150-GFP and Epe1ΔN150-Halo provide constitutively expressed truncated proxies, results in the loss of tEpe1 from chromatin and its increased presence in the cytoplasm.

Epe1 N-terminal removal increases heterochromatin formation. Exposure of fission yeast to caffeine reduces Epe1 association with constitutive heterochromatin regions, alters the distribution of H3K9me-dependent heterochromatin and instigates caffeine resistance through the repression of underlying genes9. We next investigated whether caffeine-induced N-terminal Epe1 truncation results in changes to its chromatin distribution and that of heterochromatin. Silencing assays at the euchromatin–heterochromatin border on the left side of cent1 revealed that, as in epe1Δ cells, repression of the nearby inserted euchromatic urad4 marker gene occurred in cells expressing Epe1ΔN150 (growth on counter-selective FOA plates; Extended Data Fig. 5a). Quantitative chromatin immunoprecipitation (ChIP) showed that Epe1-GFP was enriched at centromere repeats (dg), a cent1-adjacent boundary region (IRCl-1), and a region near a telomere (tbr2), where Epe1 normally associates9 (Fig. 5a). In contrast, removal of Epe1’s N-terminal 150 residues (Epe1ΔN150-GFP) resulted in loss of association with these regions (Fig. 5a). As expected, epe1Δ cells exhibit higher levels of H3 dimethylated at K9 (H3K9me2) at known facultative heterochromatin islands9 (isl3, isl6; Fig. 5b). However, cells expressing only Epe1ΔN150-GFP exhibited greater H3K9me2 levels at several islands (isl3, isl6, isl9, isl9p). Importantly, cells expressing non-cleavable Epe1ΔN101–110-GFP exhibited no change in its association with major heterochromatin regions compared with Epe1-GFP before or after caffeine treatment (dg, irc, tel2R, Fig. 5a,c) or in H3K9me2 levels at dg centromere repeats or any island examined (Fig. 5b). Thus, removal of the Epe1 N-terminal region mimics caffeine treatment9 and Epe1 loss9 in increasing H3K9-methylation-dependent heterochromatin at various chromosomal locations.

ChIP–seq analysis confirmed that H3K9me2 levels increase at sub-telomeric and island regions in epe1Δ-null and Epe1ΔN150-GFP-expressing cells (Fig. 5d,e). Consistent with ChIP–qPCR analysis (Fig. 5b), ChIP–seq also showed that, at some locations, heterochromatin spreads further in Epe1ΔN150-GFP cells than in epe1Δ cells, indicating that Epe1ΔN150-GFP somehow promotes increased heterochromatin formation at the left telomere.

Fig. 3 | The Epe1 N-terminal region is sufficient for caffeine-induced cleavage that is dependent on proteasome function. a, Volcano plot showing proteins enriched with Epe1-GFP by proteomic analysis of extracts from untreated or treated cells grown with 14 mM caffeine for 16 h. Red dots: 265 proteasome components enriched with Epe1-GFP after caffeine treatment, named in the table (right). Blue dots: enriched E3 ubiquitin ligases. Full data are available in Supplementary Table 3. The Perseus software platform (1.6.2.1) was used to process label-free quantification (LFQ) intensities of the proteins generated by MaxQuant. LFQ intensities were transformed to log2, scale and filtered for proteins with at least two peptides in any sample. Samples were run in triplicate with three biological replicates. b, Epe1-GFP western from wild-type (WT) or proteasome-defective cells (mts2-1) that were untreated or treated with 14 mM caffeine. c, Epe1-Myc western from untreated or treated cells grown with 14 mM caffeine and/or the proteasome inhibitor bortezomib (BTZ). d, Western analysis detecting Epe1ΔN150-GFP following treatments as in c, or recombinant GFP. e, Western of immunoprecipitated Epe1ΔN3Myc probed with anti-Myc or anti-ubiquitin antibodies in the absence (-) or presence (+) of 14 mM caffeine. f, Western of Multidisk2 affinity resin enriched proteins probed with anti-Myc or anti-ubiquitin antibodies in the absence or presence of 14 mM caffeine. g, Western detecting Epe1-GFP or α-tubulin in wild-type, hul5Δ, pub3Δ or hul5Δpub3Δ cells that were untreated or treated with 14 mM caffeine for 16 h. h, Number of resistant colonies formed per 1×10^6 viable cells by wild-type, hul5Δ, pub3Δ or hul5Δpub3Δ cells plated on caffeine or fluconazole plates. Error bars are from independent measurements from three biological replicates. Data are presented as mean values ± s.d., with dots representing independent plate counts. The significance of the difference between samples was evaluated using a two-tailed Student’s t-test. *P<0.033; **P<0.002; ***P<0.0002; ****P<0.0001; n.s., not significant.

ARTICLES

NATURE STRUCTURAL & MOLECULAR BIOLOGY | VOL 29 | AUGUST 2022 | 745-758 | www.nature.com/nsmb

749
of chromosome 1 and at heterochromatin island 9 containing the mei4 gene (tel1L, isl9/mei4; Fig. 5d,e). This increased spreading is particularly prominent at sub-telomeric regions, and RNA-seq shows that many telomere-proximal genes display even greater repression in Epe1ΔN150-GFP cells than in epe1Δ cells (Extended Data Fig. 5b,c). Therefore, because Epe1ΔN150-GFP cells are not equivalent to epe1Δ cells, these data reveal that Epe1ΔN150-GFP, a surrogate for tEpe1, retains functions that can regulate heterochromatin distribution.

tEpe1 enhances resistance by forming more heterochromatin.

To further explore the influence of Epe1 processing on resistance of fission yeast to caffeine and antifungals, we measured the frequency of caffeine resistance among viable cells expressing truncated or

---

**ARTICLES**

---

**NATURe STRUCTuRAL & MoLECuLAR BiOLoGY**

---

**VOL 29 | AUGUST 2022 | 745–758 | www.nature.com/nsmb**

---

**750**
mutant forms of Epe1. Like epe1Δ, cells expressing Epe1ΔN150-GFP displayed an increased frequency of caffeine-resistant colonies relative to cells expressing wild-type Epe1-GFP or Epe1ΔN100-GFP (Fig. 6a). In contrast, cells expressing non-cleavable Epe1Δ101–110-GFP, which in response to caffeine remains enriched at major heterochromatic regions (dg, IRC-1L, tel2R; Fig. 5c), exhibited
a

b

c

d

e
greater sensitivity to caffeine wild-type cells (Fig. 6b). Removal of Clr4 H3K9 methyltransferase from epe1Δ, Epe1AN100-GFP or Epe1AN150-GFP cells reduced the frequency of caffeine-resistant colonies, demonstrating that heterochromatin is required to confer resistance41 (Fig. 6a). Thus, Epe1 N-terminal truncation to residue 150 results in increased cytoplasmic levels of Epe1 and phenocopies the H3K9me heterochromatin-dependent resistance phenotype of epe1Δ cells. Reciprocally, non- cleavable Epe1Δ101–110-GFP remains chromatin-associated in the presence of caffeine; thus, the reprogramming required to elicit resistance cannot take place, rendering these cells super-sensitive.

ChIP–qPCR performed on caffeine-resistant isolates from Epe1AN150-GFP cells revealed significantly higher H3K9me2 levels over the locus containing isl14 and the gene encoding ncRNA394 in eight of ten isolates analyzed (Fig. 6c). We have previously shown that caffeine resistance directly results from heterochromatin-associated gene repression over isl14/ncRNA394 (ref. 19). We therefore conclude that cells expressing constitutively truncated Epe1AN150-GFP exhibit increased caffeine resistance due to similar heterochromatin-mediated epimutations.

Caffeine-resistant epimutants have previously been shown to exhibit resistance to clinical and agricultural crop-plant antifungal5. As with caffeine and salt stress (Fig. 1b), treatment with a clinical antifungal (fluconazole, FLC) or crop-plant fungicides enilconazole (ENL) or tebuconazole (TEB), induced Epe1-to-tEpe1 processing (Fig. 6d). Moreover, N-terminally-truncated Epe1AN150-GFP, but not Epe1AN100-GFP, increased the frequency of colonies resistant to these antifungals to levels similar to those in epe1Δ cells (Fig. 6ef). Removal of Clr4 from epe1Δ, Epe1AN100-GFP or Epe1AN150-GFP cells reduced the frequency of fluconazole-resistant colonies, indicating that this increase in antifungal resistance requires H3K9me-dependent heterochromatin (Fig. 6g).

The phenotype of cells expressing constitutively truncated Epe1AN150-GFP appears similar to that of cells completely lacking Epe1 (epe1Δ), in that heterochromatin accumulates at particular chromosomal locations and both exhibit a Clr4-dependent increase in caffeine and antifungal resistance. However, insult-induced tEpe1 and Epe1AN150 retain an intact JmjC demethylase domain and accumulate in the cytoplasm (Fig. 4). Moreover, cells expressing Epe1AN150-GFP exhibit elevated H3K9me2 levels at certain locations (Fig. 5bd). To determine whether the JmjC domain of Epe1AN150 contributes to their resistance, we constructed cells expressing a predicted catalytically dead (cd) version of FL-Epe1-GFP and Epe1AN150-GFP bearing H297A and K314A substitutions to disrupt iron/Fe(II) and 2-oxoglutarate binding, respectively (Epe1-cd-GFP, Epe1AN150-cd-GFP). Both Epe1-cd-GFP and Epe1AN150-cd-GFP were expressed from the epe1 locus at similar levels to those in their catalytically active counterparts (Extended Data Fig. 6a). However, cells expressing Epe1-cd-GFP (naturally induced Epe1) or Epe1AN150-cd-GFP (constitutive tEpe1) displayed reduced frequencies of caffeine and fluconazole-resistant colonies relative to Epe1-GFP and Epe1AN150-GFP cells (Extended Data Fig. 6b). Since tEpe1 and Epe1AN150 are more cytoplasmically localized, their intact JmjC demethylase domains may promote heterochromatin-mediated resistance through other, perhaps cytoplasmic, substrates. Detailed future investigation will be required to identify potential additional Epe1 substrates and how they might contribute to resistance phenotypes.

A MAP kinase pathway regulates stress-induced Epe1 cleavage. TOR- and MAP-kinase-dependent signaling pathways sense and mediate adaptation of fission yeast to the external environment. TOR-serine/threonine-kinase-dependent pathways are major regulators of cellular metabolism, growth and the cell cycle in response to nutrient availability and stresses.42,43 TORC2

Fig. 6 | Clr4-dependent H3K9 methylation mediates caffeine and antifungal resistance and is enhanced by Epe1 truncation. a, Number of resistant colonies formed/1 × 104 viable cells plated by wild-type, epe1Δ, epe1Δchr4Δ, Epe1AN100-GFP, Epe1AN50-GFP clr4Δ, Epe1AN150-GFP and Epe1AN150-GFP clr4Δ on plates containing 16 mM caffeine (CAF). b, Number of resistant colonies formed/1 × 104 viable cells plated by wild-type, epe1Δ, Epe1AN101-110-GFP and Epe1AN111-120-GFP cells on plates containing 16 mM caffeine. c, ChIP–qPCR for H3K9me2 at the isl14/ncRNA394 or arg3 loci on ten caffeine-resistant and four control colonies (from no caffeine), formed by Epe1AN150-GFP cells. Signals were normalized to an octosporus spike-in control and significance calculated to control colony 1 (cross-hatched). Dots represent three biological replicates; bars show the mean and error bars are ± s.d. Significance of the difference between samples was evaluated using Student’s t-test. *P < 0.05; **P < 0.01; ***P < 0.001. d, Epe1Δ13xMyc or α-tubulin western analysis from untreated cells or cells treated with 0.5 mM fluconazole (FLC), 6 μM eniliconazole (ENL), 16 μM tebuconazole (TEB) or 14 mM caffeine. e, Serial dilution growth assay of cells with the indicated manipulation at epe1 on plates containing no antifungal (-), FLC, ENL or TEB. f, Number of resistant colonies formed/1 × 104 viable cells with indicated manipulation on epe1Δ on plates containing FLC, ENL or TEB. g, Number of resistant colonies formed/1 × 104 viable cells plated by wild-type, clr4Δ, epe1Δ, epe1Δclr4Δ, Epe1AN100-GFP, Epe1AN50-GFP clr4Δ, Epe1AN150-GFP and Epe1AN150-GFP clr4Δ on plates containing 0.5 mM fluconazole. For plots in a,b and g, error bars are from independent measurements from three biological replicates. Data are presented mean values ± s.d., with dots representing independent plate counts. The significance of the difference between samples was evaluated using a two-tailed Student’s t-test. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.
Fig. 7 | The cell integrity stress pathway regulates Epe1 processing through Pek1/MAPKK, Pmk1/MAPK and Pmp1/MAPK phosphatase. a, Diagram of the *S. pombe* stress-activated signaling pathway (SAP) end point with participating MAP Kinase (Wis1) and MAP Kinase Kinase (Sty1). b, Diagram of the *S. pombe* cell integrity signaling pathway (CIP) end point with participating MAP Kinase (Pek1), MAP Kinase Kinase (Pek1) and MAP Kinase Phosphatase (Pmp1). c, Epe1-GFP or α-tubulin western analysis from wild-type, *epe1Δ* or *pmk1Δ* cells that were untreated or treated with 14 mM caffeine. d, Epe1-GFP or α-tubulin western analysis from wild-type, *pek1Δ*, *pmk1Δ* or *pmp1Δ* cells that were untreated or treated with 14 mM caffeine. e, Number of resistant colonies formed/104 viable cells by wild-type, *epe1Δ*, *pek1Δ* or *pmp1Δ* cells on plates containing 0.5 mM fluconazole. Plots in g and h, error bars are from independent measurements from three biological replicates. Data are presented mean values ± s.d., with dots representing independent plate counts. The significance of the difference between samples was evaluated using a two-tailed Student’s t-test. *P < 0.033; **P < 0.002; ***P < 0.0002; ****P < 0.0001.

### Notes
- **Articles**
- **Nature Structural & Molecular Biology**
- **Vol 29 | August 2022 | 745-758 | www.nature.com/nsmb**

---

**Fig. 7 Legend**

**Fig. 7** | The cell integrity stress pathway regulates Epe1 processing through Pek1/MAPKK, Pmk1/MAPK and Pmp1/MAPK phosphatase.

**a.** Diagram of the *S. pombe* stress-activated signaling pathway (SAP) end point with participating MAP Kinase (Wis1) and MAP Kinase Kinase (Sty1).

**b.** Diagram of the *S. pombe* cell integrity signaling pathway (CIP) end point with participating MAP Kinase (Pek1), MAP Kinase Kinase (Pek1) and MAP Kinase Phosphatase (Pmp1).

**c.** Epe1-GFP or α-tubulin western analysis from wild-type, *epe1Δ* or *pmk1Δ* cells that were untreated or treated with 14 mM caffeine.

**d.** Epe1-GFP or α-tubulin western analysis from wild-type, *pek1Δ*, *pmk1Δ* or *pmp1Δ* cells that were untreated or treated with 14 mM caffeine.

**e.** Number of resistant colonies formed/104 viable cells by wild-type, *epe1Δ*, *pek1Δ* or *pmp1Δ* cells on plates containing 0.5 mM fluconazole.

**f.** Diagram of the *S. pombe* cell integrity stress pathway regulating Epe1 processing through Pek1/MAPKK, Pmk1/MAPK and Pmp1/MAPK phosphatase.

**g.** Number of resistant colonies formed/104 viable cells by wild-type, *epe1Δ*, *pek1Δ*, *Epe1ΔN150*, *Epe1ΔN150* or *pmp1Δ* cells plated on caffeine or fluconazole plates.

**h.** Number of resistant colonies formed/104 viable cells plated by wild-type, *epe1Δ*, *pek1Δ* and *pmp1Δ* on plates containing 0.5 mM fluconazole. Plots in **g** and **h**, error bars are from independent measurements from three biological replicates. Data are presented mean values ± s.d., with dots representing independent plate counts. The significance of the difference between samples was evaluated using a two-tailed Student’s t-test. *P < 0.033; **P < 0.002; ***P < 0.0002; ****P < 0.0001.
...signaling acts through the single downstream Gad8 effector kinase, which is chromatin-associated and known to influence heterochromatin integrity44,45 (Extended Data Fig. 7a). However, gua8Δ cells exhibited relatively normal Epe1-to-tEpe1 processing but a reduced frequency of caffeine-resistant colonies, suggesting that TORC2-mediated signaling may counteract the effects that Epe1 reduced frequency of caffeine-resistant colonies, suggesting that Epe1 truncation is required for stress-induced Epe1 truncation, whereas the counteracting MAPK phosphorylation Pmk1 acts to restrain tEpe1 generation. We conclude that activation of the CIP/MAPK pathway by external insults results in regulated N-terminal truncation of Epe1 processing, its loss from chromatin, and increased heterochromatin-mediated gene repression, which confers resistance41 (Fig. 8).

Discussion
We have uncovered an unusual mechanism by which external stresses alter the location of the pivotal heterochromatin regulator Epe1 by inducing its proteasome-mediated processing to a shorter form lacking the N-terminal 150 residues. As previously demonstrated41, epimutations arise due to increased heterochromatin formation over genes at various locations, allowing the survival of cells where reduced expression of underlying genes imparts beneficial resistance phenotypes in the face of external challenges. Such genes include hba1+, encoding a factor involved in nuclear export of the Pap1 stress-induced transcription factor, and cup1+, encoding a mitochondrial LYR domain protein.

Proteasome-mediated ubiquitin-dependent protein degradation is well known. However, it is less appreciated that some proteins are subject to proteasome-mediated processing in which only part of a target protein is degraded in a process termed RUP33,49–57. Mono-ubiquitylation of target proteins can be sufficient to stimulate such processing events46–50, which frequently terminate at nearby tightly folded structural domains51–55. Thus, elevated Epe1-Myc-associated ubiquitylation and enrichment of 23 proteasome subunits with Epe1-GFP upon caffeine treatment (Fig. 3a,c,f) suggest that the translocating activities of the 19S regulatory particle may stall at the JmjC domain. Subsequent cleavage and removal of the N-terminal 150 residues by peptidases within the 20S proteasome core would produce truncated tEpe1 (Fig. 8). Proteasome-dependent processing is known to result in ill-defined cleavage sites in substrate proteins due to bidirectional proteasome processivity following initiating cleavage events in the proteasome’s catalytic chamber56. Thus, our inability to detect a precise cleavage site is consistent with Epe1 cleavage being mediated by the proteasome. Such proteasome-mediated Epe1 processing may be relevant to the observation that 19S AAA-ATPase mutants that do not affect ubiquitin-dependent proteolyis exhibit an altered heterochromatin distribution41.

Fig. 8 | Model for induction of heterochromatin-mediated gene silencing and resulting resistance. External insults (that is, caffeine, antifungals) detected by the CIP MAP Kinase pathway result in increased Epe1 ubiquitylation (Ub) and proteasome-mediated processing of Epe1 to tEpe1. Loss of the Epe1 N-terminal region results in reduced Epe1 demethylase chromatin association, increased soluble and cytosolic Epe1 and accumulation of H3K9me-dependent heterochromatin over various locations, including facultative islands where resulting repression of embedded genes can confer resistance.

Epe1-to-tEpe1 processing requires an intact cell integrity signaling pathway (CIP/MAPK) to communicate the presence of external insults (Fig. 7). Both Pmk1–MAPK and Pek1–MAPKK are needed for stress-induced Epe1 truncation, whereas the counteracting MAPK phosphorylation Pmp1 acts to restrain tEpe1 generation. We conclude that activation of the CIP/MAPK pathway by external insults results in regulated N-terminal truncation of Epe1 by proteasome-mediated cleavage (Fig. 8). CIP/MAPK may directly phosphorylate Epe1, triggering its ubiquitylation. Alternatively, it might act equivalently to MAPK/Stt2p51, its Saccharomyces cerevisiae counterpart, which stimulates the assembly of complete 26S proteasomes in response to stress52.

Removal of residues 1–150 from Epe1 reduces its chromatin association and nuclear levels, increasing its presence in the cytoplasm, thereby permitting H3K9 methylation, and thus heterochromatin, to increase at known facultative heterochromatin islands and other locations (Fig. 5). Constitutive tEpe1 (Epe1ΔN150) expression increases the frequency of caffeine and antifungal resistance relative to that in wild-type cells. The majority of Epe1ΔN150

Cell integrity stress signalling pathway

Proteasome-mediated Epe1 cleavage

Epe1 lost from chromatin

Gene silencing for antifungal resistance

External insult

MAPKK

Pmk1

MAPK

MAPK

Epe1

tEpe1

Genes on

Cytosol

Genes off

Proteasome-dependent processing is known to result in ill-defined cleavage sites in substrate proteins due to bidirectional proteasome processivity following initiating cleavage events in the proteasome’s catalytic chamber. Our inability to detect a precise cleavage site is consistent with Epe1 cleavage being mediated by the proteasome. Such proteasome-mediated Epe1 processing may be relevant to the observation that 19S AAA-ATPase mutants that do not affect ubiquitin-dependent proteolyis exhibit an altered heterochromatin distribution.

Consistent with Pmk1 and Pmp1 promoting and hindering stress-induced Epe1 cleavage, respectively, Epe1-GFP association with major heterochromatic regions in response to caffeine decreased similarly in wild-type and pmp1Δ cells (more cleavage), but not pek1Δ cells (less cleavage) (cen dg, IRC1-L; Fig. 7f). Moreover, as in epe1Δ cells, pmp1Δ cells exhibited an increased frequency of caffeine- and fluconazole-resistant colonies compared with that of wild-type cells (Fig. 7c,h), consistent with increased Epe1-to-tEpe1 processing (Fig. 7d and Extended Data...
caffeine-resistant isolates examined exhibited high H3K9me2 levels at isl14/miRNA394 (Fig. 6), where reduced expression of the nearby cup1-encoded mitochondrial protein confers caffeine and antifungal resistance\(^1\). Mitochondrial dysfunction is known to confer antifungal resistance in fission yeast: resistance may result from increased intracellular oxidative stress and associated nuclear import of Pap1, a key stress-regulated transcription factor which elevates efflux pump expression\(^{2,3}\). Moreover, higher mitochondrial membrane potential in S. cerevisiae correlates with greater antifungal resistance, possibly due to efflux pump upregulation\(^4\). Thus, pathways that respond to mitochondrial-generated stress appear to confer resistance. Interestingly, the JmjC demethylase domain of constitutively truncated Epe1ΔN150 appears to contribute to resistance because mutants expected to disrupt its catalytic activity display reduced resistance (Extended Data Fig. 6b). Given the increased cytoplasmic presence of Epe1ΔN150 (Fig. 4a,d), it is possible that processed tEpe1 acts through unknown cytoplasmic substrates that perhaps alter metabolism and the levels of metabolites that contribute to heterochromatin regulation via chromatin modifiers, thereby influencing resistance\(^5\). Although the frequency of caffeine and fluconazole resistance was lower in cells expressing Epe1ΔN150-cd than in cells expressing Epe1ΔN150, it is possible that cytoplasmic tEpe1 is mostly non-functional and that its accumulation is mainly incidental, resulting from inefficient post-translational degradation. The identification of potential cytoplasmic tEpe1 substrates that contribute to resistance will be required to confirm a role for tEpe1 in mediating resistance phenotypes.

Constitutive Epe1 truncation causes H3K9me/heterochromatin to redistribute without any selection imposed by external insults. H3K9me2 increases over genes at various locations, including heterochromatin islands and especially telomeres, where gene expression is substantially decreased (Fig. 5b,d,e and Extended Data Fig. 5). We therefore propose that, in wild-type cells, continual exposure to insults triggers Epe1-to-tEpe1 proteasome-mediated processing and selects for the survival of cells where the resulting heterochromatin-imposed gene repression confers resistance through epimutations such as those at isl14/miRNA394 that reduce cup1 expression (Fig. 6c)\(^5\). Insults that trigger Epe1-to-tEpe1 processing include caffeine, salt, hydrogen peroxide and several clinical and agricultural azole-based antifungal compounds (Fig. 1b and Fig. 6d). Hence, fission yeast can transiently adapt to, and resist, external insults by altering the properties and location of Epe1.

The results presented identify a route by which wild-type fungal cells use chromatin-based epigenetic reprogramming to generate epimutations that survive environmental challenges such as those posed by antifungals. Unlike genetic mutants, such resistant epimutants remain wild-type and can thus return to the normal, baseline wild-type state upon removal of the triggering insult. However, long-term exposure to that insult will impose selection among surviving epimutants for irreversible genetic mutations that fix the resistant phenotype but may reduce overall fitness. Thus, insult-triggered proteasome-mediated Epe1 processing provides a bet-hedging strategy by which wild-type cells can develop transient resistance with only temporary fitness impacts. Such epigenetic regulation may explain in part the prevalence and variability of resistance in pathogenic plant and animal fungi and may contribute to the dearth of available effective antifungal treatments.

Azole-based antifungal overuse has resulted in increasing incidence of resistance in both clinical and agricultural settings. H3K9-methylation-dependent heterochromatin is conserved in human pathogenic fungi such as Cryptococcus\(^9\) and Aspergillus\(^9\), which threaten the health of immunocompromised individuals and in major crop pathogens such as Zymoseptoria tritici\(^10\) and Magnaporthe oryzae\(^3\), which regularly and significantly reduce global cereal grain yields. Our findings suggest that the development of fungal-specific inhibitors of processes that regulate or mediate heterochromatin formation, and that do not affect related host activities, could increase the sensitivity of pathogenic fungi to antifungal compounds. Such compounds would be expected to improve the prognosis of affected people and reduce crop destruction.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41594-022-00801-y.

Received: 26 November 2021; Accepted: 6 June 2022; Published online: 25 July 2022

**References**

1. Fisher, M. C., Hawkins, N. J., Sanglard, D. & Gurr, S. J. Worldwide emergence of resistance to antifungal drugs challenges human health and food security. *Science* 360, 739–742 (2018).

2. Perlis, D. S., Rautemaa-Richardson, R. & Alastruey-Izquierdo, A. The global problem of antifungal resistance: prevalence, mechanisms, and management. *Lancet Infect. Dis.* 17, e383–e392 (2017).

3. Robbins, N., Caplan, T. & Cowen, L. E. Molecular evolution of antifungal drug resistance. *Annu. Rev. Microbiol.* 74, 753–775 (2017).

4. Stop neglecting fungi. *Nat. Microbiol.* 2, 17120 (2017).

5. Godfray, H. C. J. et al. Food security: the challenge of feeding 9 billion people. *Science* 327, 812–818 (2010).

6. Fisher, M. C. et al. Threats posed by the fungal kingdom to humans, wildlife, and agriculture. *mBio* 11, e00449-20 (2020).

7. Audergon, P. N. C. B. et al. Restricted epigenetic inheritance of H3K9 methylation. *Science* 348, 132–135 (2015).

8. Ragunathan, K., Jih, G. & Moazed, D. Epigenetic inheritance uncoupled from sequence-specific recruitment. *Science* 348, 1258699 (2015).

9. Cerulus, B., New, A. M., Pougach, K. & Verstrepen, K. J. Noise and epigenetic inheritance of single-cell division times influence population fitness. *Curr. Biol.* 26, 1138–1147 (2016).

10. Hall, I. M. et al. Establishment and maintenance of a heterochromatin domain. *Science* 297, 2232–2237 (2002).

11. Calo, S. et al. Antifungal drug resistance evoked via RNA-dependent epimutations. *Nature* 513, 555–558 (2014).

12. Bheda, P. et al. Single-cell tracing dissects regulation of maintenance and inheritance of transcriptional reinduction memory. *Mol. Cell* 79, 905–923 (2020).

13. Torres-Garcia, S. et al. Epigenetic gene silencing by heterochromatin primes fungal resistance. *Nature* 585, 453–458 (2020).

14. Heard, E. & Martienssen, R. A. Transgenerational epigenetic inheritance: myths and mechanisms. *Cell* 157, 95–109 (2014).

15. Whittaker, C. & Dean, C. The FLC locus: a platform for discoveries in epigenetics and adaptation. *Annu. Rev. Cell Dev. Biol.* 33, 555–575 (2017).

16. Miska, E. A. & Ferguson-Smith, A. C. Transgenerational inheritance: models and mechanisms of non-DNA sequence-based inheritance. *Science* 354, 59–63 (2016).

17. Duempelmann, L., Skribbe, M. & Bühler, M. Small RNAs in the transgenerational inheritance of epigenetic information. *Trends Genet.* 36, 203–214 (2020).

18. Allshire, R. C., Nimmo, E. R., Ekwall, K., Javerzat, J. P. & Cranston, G. Mechanisms of non-DNA sequence-based inheritance. *Curr. Opin. Genet. Dev.* 33, 555–575 (2017).

19. Nakayama, J., Rice, J. C., Strahl, B. D., Allis, C. D. & Grewal, S. I. S. Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. *Science* 292, 110–113 (2001).

20. Wang, J. et al. The proper connection between shelterin components is required for telomeric heterochromatin assembly. *Genes Dev.* 30, 827–839 (2016).

21. Kanoh, J., Sadae, M., Urano, T. & Ishikawa, F. Telomere binding protein Tat1 establishes Suv39 heterochromatin independently of RNAi at telomeres. *Curr. Biol.* 15, 1808–1819 (2005).

22. Zoffoli, M. et al. RNA elimination machinery targeting meiotic mRNAs promotes facultative heterochromatin formation. *Science* 335, 96–100 (2012).

23. Sugiyama, T. et al. Enhancer of rudimentary cooperates with conserved RNA-processing factors to promote meiotic mRNA decay and facultative heterochromatin assembly. *Mol. Cell* 61, 747–759 (2016).

24. Yamakane, S. et al. RNAi triggered by specialized machinery silences developmental genes and retrotransposons. *Nature* 493, 557–560 (2013).
25. Zofall, M., Smith, D. R., Mizuguchi, T., Dhakshnamoorthy, J. & Grewal, S. I. S. Taz1-shelterin promotes facultative heterochromatin assembly at chromosome-internal sites containing late replication origins. Mol. Cell 62, 862–874 (2016).

26. Gallagher, P. S. et al. Iron homeostasis regulates facultative heterochromatin assembly in adaptive genome control. Nat. Struct. Mol. Biol. 25, 372–383 (2018).

27. Wang, J., Reddy, B. D. & Jia, S. Rapid epigenetic adaptation to uncontrolled heterochromatin spreading. eLife 2015, e06179 (2015).

28. Treweck, S. C., Minc, E., Antonelli, R., Urano, T. & Allshire, R. C. The JmjC domain protein Epe1 prevents unregulated assembly and disassembly of heterochromatin. EMBO J. 26, 4670–4682 (2007).

29. Roguey, A. et al. Conservation and rewiring of functional modules revealed by an epistasis map in fission yeast. Science 322, 405–410 (2008).

30. Wang, J., Lawry, S. T., Cohen, A. L. & Jia, S. Chromosome boundary elements and regulation of heterochromatin spreading. Cell. Mol. Life Sci. 71, 4841–4852 (2014).

31. Antequera, F., Tamame, M., Villanueva, J. R. & Santos, T. DNA methylation in the fungi. J. Biol. Chem. 259, 8033–8036 (1984).

32. Capuano, F., Müller, M., Sok, R., Blom, H. J. & Ralser, M. Cytosine DNA methylation is found in Drosophila melanogaster but absent in saccharomyces cerevisiae, schizosaccharomyces pombe, and other yeast species. Anal. Chem. 86, 3697–3702 (2014).

33. Rape, M. & Jentsch, S. Productive RPTure: activation of transcription factors by proteasomal processing. Biochim. Biophys. Acta 1605, 209–213 (2004).

34. Tian, L. & Matouschek, A. Where to start and when to stop. J. Biol. Chem. 273, 10385–10387 (1998).

35. Li, F. et al. Lid2 is required for coordinating H3K4 and H3K9 methylation of chromosome-internal sites containing late replication origins. Mol. Cell 39, 1045–1053 (2010).

36. Seo, H. D. et al. The 19S proteasome is directly involved in the regulation of heterochromatin spreading in fission yeast. J. Biol. Chem. 293, 4670–4682 (2017).

37. Zofall, M., Smith, D. R., Mizuguchi, T., Dhakshnamoorthy, J. & Grewal, S. I. S. SWI6/HP1 recruits a JmjC domain protein to facilitate transcription of heterochromatic repeats. Mol. Cell 33, 681–692 (2009).

38. Brown, S. et al. The Cul4-Ddb1 Cdt2 ubiquitin ligase inhibits invasion of a metastatic ovarian cancer cell line. Mol. Cell 62, 862–874 (2016).

39. Zofall, M. & Grewal, S. I. S. SWI6/HP1 Recruits a JmjC domain protein to facilitate transcription of heterochromatic repeats. Mol. Cell 33, 681–692 (2009).

40. Tian, L., Holmgren, R. A. & Matouschek, A. A conserved processing mechanism regulates the activity of transcription factors Cubitus interruptus and NF-kB. Nat. Struct. Mol. Biol. 12, 1045–1053 (2005).

41. Pan, Y., Bai, C. B., Joyner, A. L. & Wang, B. Sonic hedgehog signaling regulates gli2 transcriptional activity by suppressing its processing and degradation. Mol. Cell. Biol. 26, 3365–3377 (2006).

42. Sato, K., Ito, H., Yokoyama, A., Toba, G. & Yamamoto, D. Partial proteasomal degradation of Lola triggers the male-to-female switch of a dimorphic courtship circuit. Nat. Commun. 10, 166 (2019).

43. Heusch, M., Lin, L., Geleziunas, R. & Greene, W. C. The generation of nfkβ1 precursor protein and the activation of NF-kB. Cell 78, 773–785 (1994).

44. Cohen, A. et al. TOR complex 2 in fission yeast is required for cell wall biosynthesis and cell integrity signaling. Cell Surf. 4, 1–9 (2018).

45. Oya, E. et al. Expression of the F-box/WD40-repeat protein Slimb shows an essential role in the proteasome homeostasis. Cell 154, 493–496 (1998).

46. Cohen, A. et al. Proteasomal processing of the p105 NF-κB precursor. Mol. Cell 33, 496–504 (2009).

47. Wieczorek, A., Bertolotti, A. An evolutionarily conserved pathway controls proteasome homeostasis. Nature 536, 184–189 (2016).

48. Liu, Q. et al. Dysfunction of prohibitin 2 results in reduced susceptibility to multiple antifungal drugs via activation of the oxidative stress-responsive transcription factor Pap1 in fission yeast. Antimicrob. Agents Chemother. 62, e00860-18 (2018).

49. Yang, Y. et al. A genomewide screen in Schizosaccharomyces pombe for genes affecting the sensitivity of antifungal drugs that target ergosterol biosynthesis. Antimicrob. Agents Chemother. 56, 1499–1509 (2012).

50. Calvo, I. A., García, P., Ayté, J. & Hidalgo, E. The transcription factors Pap1 and Prr1 collaborate to activate antioxidant, but not drug tolerance, genes in response to H2O2. Nucleic Acids Res. 40, 4816–4824 (2012).

51. Dhar, R., Missarova, A. M., Lehner, B. & Carey, L. B. Single cell functional genomics reveals the importance of mitochondria in cell-to-cell phenotypic variation. eLife, e38904 (2019).

52. Kaelin, W. G. & McKnight, S. L. Influence of metabolism on epigenetics and disease. Cell 153, 56–69 (2013).

53. Dumesic, P. A. et al. Product binding enhances the genomic specificity of a yeast Polycomb repressive complex. Cell 160, 204–218 (2015).

54. Liu, Q. et al. Dysfunction of prohibitin 2 results in reduced susceptibility to multiple antifungal drugs via activation of the oxidative stress-responsive transcription factor Pap1 in fission yeast. Antimicrob. Agents Chemother. 56, 1499–1509 (2012).

55. Lin, L., Geleziunas, R. & Greene, W. C. The generation of nfkβ1 precursor protein and the activation of NF-kB. Cell 78, 773–785 (1994).

56. Palombella, V. J., Rando, O. J., Goldberg, A. L. & Maniatis, T. The ubiquitinproteasome pathway is required for processing the NF-kB precursor protein and the activation of NF-kB. Cell 78, 773–785 (1994).

57. Rape, M. & Jentsch, S. Taking a bite: proteasomal protein processing. Nat. Cell Biol. 4, E113–E116 (2002).

58.normal text

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s), under exclusive licence to Springer Nature America, Inc. 2022
Methods

Yeast strains and manipulations. S. pombe strains used in this study are described in Supplementary Table 1. Oligonucleotide sequences are listed in Supplementary Table 2. N-terminus-truncated Epe1 mutants, 3xMyc-Epe1 and epe1 deletion strains were constructed in CRISPR-Cas9 mediated genome editing using the SpDIDT system 72; available on Addgene (no. 166698, no. 166699, no. 166700) with oligonucleotides listed in Supplementary Table 2. Epe1-GFP and Epe1-13xMyc strains and deletion mutants were generated by using the Kahler tagging and deletion method 72.

Protein extraction and western analysis. Cells were treated with 14 mM caffeine, 1 M KC1 and 1 mM H2O2 for 16 h, with fresh H2O2 added every 2 h to compensate for its decomposition. Protein samples were prepared essentially as previously detailed 73. Briefly, a 10-mL culture of log-phase cells was collected, resuspended in 1 mL of cold water and transferred to an Eppendorf tube. Cells were pelleted and resuspended in 750 µL of water. Then, 150 µL 1.85 M NaOH and β-mercaptoethanol 7.5% were added, and the samples incubated on ice for 15 min. Next, 150 µL 55% TCA was added and incubated for a further 10 min on ice. Samples were centrifuged at 16,800 g for 10 min, and pellets were resuspended in HU-buffer (8 µM urea, 5% SDS, 200 mM Tris pH 6.8, 1 mM EDTA, with bromo-phenol blue, 1.5 DTT).

Samples were run on a 4–12% NuPAGE Bis-Tris gel (Thermo Fisher) and blotted using the MiniBlot Module (Life Technologies) 20 V for 1 h. Western analysis detection was performed using anti-myc 9B11 (Cell Signaling) 1:1,000, anti-GFP (Roche) 1:1,000 and anti-mouse IgG (whole molecule)–peroxidase antibody 1:4000. Bands were visualized using ChemiDoc imaging system (BioRad) and analyzed with ImageJ. Where quantified, levels of FL-Epe1 and Epe1 were normalized to no treatment and adjusted relative to the α-tubulin loading control. Resulting numbers are an average of three biological replicates.

Ubiquitin assay. Collected cells were washed with PBS and frozen in liquid nitrogen. Frozen cell pellets were ground using a Retsch MM400 mill. Ground cells were resuspended in lysis buffer (10 mM Tris pH7.4, 5 mM CaCl2, 5 mM MgCl2, 50 mM NaCl, 0.1% IGEPEAL-CA630, 20µM MgG132, 50µM Pr-619) supplemented with protease inhibitor (Merck Life Sciences) and thawed for 30 min on ice. After centrifugation at high speed, supernatants were subjected to immunoprecipitation with anti-Myc antibody crosslinked to Protein G Dynabeads beads (Thermo Fisher) for 2 h. After three washes with lysis buffer, beads were resuspended in NuPage LDS sample buffer (Thermo Fisher), separated on 4–12% NuPAGE Bis-Tris gel (Thermo Fisher) and probed with anti-Myc (9B11; Cell Signaling) and anti-ubiquitin (BML-PW8810; Enzo) antibodies.

MultiDsk2 pull-down assay. MultiDsk2 pull-down assays were performed as previously described with slight modifications 74. Ground cell power was resuspended in D-buffer (150 mM Tris-Acetate pH 7.4, 100 mM potassium acetate, 1 mM EDTA, 0.1% Triton X-100, 10% glycerol, 20 µM MgG132 and 50 µM Pr-619) supplemented with protease inhibitor (Merck Life Sciences) and incubated for 30 min on ice. After centrifugation, 5 mg of protein from each sample was incubated for 2 h with purified MultiDsk2 recombinant protein bound to glutathione agarose magnetic beads (Sigma G9024). After incubation, beads were washed four times with D-buffer and resuspended in NuPage LDS sample buffer (Thermo Fisher) and subjected to western analysis, as described above.

Serial dilution and colony-count assays. Equal amounts of cells were grown in yeast extract supplemented (YES) medium without agents then serially diluted81. Peptide and protein identifications were filtered to 1% FDR. The quadrupole was 1.4 Thomson. Only ions with charge between 2 and 7 were acquired. A maximum of 200 ions were included for fragmentation, with the threshold set to 500. A fragmentation C isolation width width of 10% was set, and a trypsin peptide tolerance of 20ppm was enforced. Label-free quantitation analysis was performed by employing the MaxLFQ algorithm as described previously82. Absolute protein quantification was performed as described previously72. Peptide and protein identification filters were set to 1% FDR.

Cytology. Cells were fixed with 3.7% formaldehyde (Sigma) for 10 min at room temperature. Immunolocalization staining was performed as described previously72. The following antibodies were used: anti-CENP-A (λ) (sheep in-house, 1:3,000), anti-GFP (Thermo Fisher, 1:200), Alexa Fluor 594- and 488-labeled secondary antibodies at 1:1,000 dilution (Life Technologies). Images were acquired with Zeiss Axiosplan, using a ×100/1.4 NA Plan-Apochromat Oil DIC M27 objective lens and processed using Metamorph acquisition and processing software (Zeiss MicroImaging) or FIJI.

For imaging cells with Halo-tagged proteins, 250 to 500 µL of HaloTag ligand JF464 dye was added to 400 µL of log-phase cells and incubated at 32°C for 3 h. Cells were washed with PMG and plated on a PMG agarose pad and were dried before addition of a coverslip. Images were taken with an inverted microscope (Nikon, 60×) equipped with an ORCA Camera (T. Leiden). Images were processed using Fiji v1.8.0, or Miro Suite (Nikon, 10×).
were taken to cover the entire depth of the cells. Live cells were kept at 32°C during imaging. All image analysis was performed in Fiji as follows: a region of interest was manually selected (nuclear/cytoplasmic spot). Background intensity for each cell was subtracted from the mean intensity measurement. Significance of the difference between samples was evaluated using Student’s t-test. *P < 0.002; **P < 0.0002; ***P < 0.0001.

qRT–PCR. Total RNA was extracted using 5× 10^4 cells and Monarch Total RNA Miniprep Kit (NEB) as per manufacturer’s instructions. Samples were treated with Turbo DNase (Ambion) to remove contaminating DNA. Reverse transcription was performed using LunaScript RT SuperMix Kit (NEB). Oligonucleotides used for qRT–PCR are listed in Supplementary Table 1. qRT–PCR histograms represent 3 technical replicates; error bars correspond to standard error of the mean.

Fractionation. Cells were grown in 4xYES and harvested by centrifugation at 3,500g, washed twice with water and flash frozen in liquid nitrogen. Frozen cell pellets were ground using Retsch MM400 mill. Ground cells were resuspended in lysis buffer (10 mM Tris pH 7.4, 5 mM CaCl_2, 5 mM MgCl_2, 50 mM NaCl, 0.1% IGEPAL-CA630) and supplemented with protease inhibitor (P8215, Sigma) and 2 mM PMSF. After thawing on ice for 30 min, samples were vortexed 15 times for 30 s, with 1 min on ice between each wash. After centrifugation at 5,000g, supernatant was collected as soluble fraction, pellet was washed twice with the same lysis buffer and then collected as insoluble fraction.

ChiP. ChiP experiments were performed as previously described using anti-H3K9me2 (S1.1, a kind gift from T. Urano) or anti-GFP (Life Technologies). Immunoprecipitated DNA was recovered with Chelex-100 resin. qChIPs were analyzed by real-time PCR using Lightcycler 480 SYBR Green (Roche) Oligonucleotides are listed in Supplementary Table 1. ChiP enrichments were calculated as percentage DNA immunoprecipitated at the locus of interest relative to the corresponding input samples. For H3K9me2 ChiP, formaldehyde-fixed S. octoporum cells were added at the cell-lysis stage for spike-in control. For Epe1-GFP ChiP, formaldehyde-fixed Sgo1-GFP S. cerevisiae cells were added at the cell-lysis stage for spike-in control. Histograms represent data averaged from three biological replicates; error bars represent standard deviations. Significance of the difference between samples was evaluated using Student’s t-test. *P < 0.003; **P < 0.002; ***P < 0.0001.

ChiP–seq, RNA-seq and bioinformatic analyses. Samples were prepared as previously described. ChiP–seq and RNA-seq samples were compared using DESeq2 statistics of read counts per feature, accessed by HTSeq-count and genomic 1-kb bins for ChiP–seq or by Salmon-quant and annotated genes for RNA-seq analyses. Features with adjusted P < 0.1 were selected as significantly changed. Features with log((fold change) value) > 1 (RNA-seq) or > 2 (ChiP–seq) are presented in figures.

Statistics and Reproducibility. No sample size calculation was performed, the sample sizes used are considered standard in the field and were selected to ensure robust and statistically significant comparisons. Unless otherwise stated, the experiments presented were all repeated as three independent biological replicates. There were no data exclusions, and findings were reliably reproducible. Samples were not randomized, and no blinding was employed.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The publicly accessible PomBase database (https://www.pombase.org) was used to obtain genome sequence and annotation for the Schizosaccharomyces pombe (fission yeast) genome. All raw and processed reads from sequancing experiments (ChiP–seq and RNA-Seq) are available at the GEO under accession number GSE190267. All raw mass spectrometry data are available at PRIDE using project accession number PXD030205. Source data are provided with this paper.

References
72. Torres-Garcia, S. et al. SpEDIT: a fast and efficient CRISPR/Cas9 method for fission yeast. Wellcome Open Res. 5, 274 (2020).
73. Sato, M., Dhiu, S. & Toda, T. New drug-resistant cassettes for gene disruption and epitope tagging in Schizosaccharomyces pombe. Yeast 22, 583–591 (2005).
74. Wilson, M. D., Saponaro, M., Leidl, M. A. & Svejstrup, J. Q. Multi-Drug: a ubiquitin-specific affinity resin. PLoS ONE 7, e46398 (2012).
75. Singh, P. P. et al. Hap2-Ino80 facilitated transcription promotes de novo establishment of CENPA chromatin. Genes Dev. 34, 226–238 (2020).
76. Rappisiber, J., Iihihama, Y. & Mann, M. Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelcoelectrospray, and LC/MS sample pretreatment in proteomics. Anal. Chem. 75, 663–670 (2003).
77. Olsen, J. V. et al. Higher-energy C-trap dissociation for peptide modification analysis. Nat. Methods 4, 799–712 (2007).
78. Cox, J. & Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat. Biotechnol. 26, 1367–1372 (2008).
79. Cox, J. et al. Andromeda: a peptide search engine integrated into the MaxQuant environment. J. Proteome Res. 10, 1794–1805 (2011).
80. Cox, J. et al. Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. Mol. Cell. Proteom. 13, 2513–2526 (2014).
81. Schwähnensser, B. et al. Global quantification of mammalian gene expression control. Nature 473, 337–342 (2011).
82. Shukla, M. et al. Centromere DNA destabilizes H3 nucleosomes to promote CENPA deposition during the cell cycle. Curr. Biol. 28, 3924–3936 (2018).
83. Tong, P. et al. Interspecies conservation of organisation and function between nonhomologous region centromeres. Nat. Commun. 10, 2343 (2019).
84. Nerushева, O. O., Galiander, S., Fernius, J., Kelly, D. & Marston, A. L. Tension-dependent removal of pericentromeric shugoshin is an indicator of sister chromosome biorientation. Genes Dev. 28, 1291–1309 (2014).

Acknowledgements
We thank D. Kelly (WCW, Edinburgh) for microscopy and instrument support; members of the Allshire Lab for valuable discussions and input; T. Urano for the S1.1 (H3K9me2) antibody; A. Fellas for GFP expressing and cI1A strains; K. Gull for α-tubulin antibody; K. Swain for Mts2 antibody; A. L. Marston for the Sgo1-GFP S. cerevisiae strain; J. Svejstrup for provision of the MultiDsk2 expression construct; M. D. Wilson for comments on the manuscript; and colleagues at WCW for support and encouragement during a difficult 2020–21. This research was supported by award of an EMBO Long Term Fellowship to I. Y. (EMBO ALTF 130–2018), Darwin Trust of Edinburgh PhD studentships to S. T.-G. and R. Y., a Wellcome 4 Year iCM program PhD studentship to E. G. (218470), a Wellcome Instrument grant to J. R. (108504), a Wellcome Investigator award to M. E. K. (205068), a Wellcome Principal Research Fellowship to R. C. A. (208885; 224358) and core funding for the Wellcome Centre for Cell Biology (203149). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript. For the purpose of Open Access, the author has applied a CC-BY public copyright licence to any author-accepted manuscript version arising from this submission.

Author contributions
I. Y. and S. A. W. designed and performed experiments. S. T.-G. contributed to ChiP, ChiP–seq and RNA-seq experiments and CRISPR design. M. L. carried out ChiP–seq and RNA-seq analyses and data visualization. E. G. performed Halo-tag experiments with advice on microscopy and experimental design from M.E.K. R. Y. contributed to antifungal-resistance experiments. C. S. ran samples and performed mass spectrometry analysis with J. R. A. L. P. advised on experimental design, performed ChiP and constructed strains. I. Y., S. A. W., A. L. P. and R. C. A. prepared figures and wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Extended data is available for this paper at https://doi.org/10.1038/s41594-022-00801-y.
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41594-022-00801-y.
Correspondence and requests for materials should be addressed to Robin C. Allshire.
Peer review information Nature Structural and Molecular Biology thanks Gary Karpen and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Primary Handling Editor: Carolina Perdigoto, in collaboration with the Nature Structural & Molecular Biology team.

Reprints and permissions information is available at www.nature.com/reprints.
Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Caffeine-induced alteration in Epe1-GFP mobility does not result from transcriptional changes in 5’ region of epe1+ gene or Epe1-Myc phosphorylation.  

a. Epe1-GFP or α-tubulin western from cells untreated (-)/treated (+) with 14 mM caffeine for 16 h. 

b. qRT-PCR measurement of steady-state Epe1 transcript levels in cells untreated (-)/treated (+) with 14 mM caffeine for 16 h. Locations of primers used indicated. Data shown represent 3 technical replicates +/- SEM. 

c. Scheme to detect caffeine-induced Epe1-GFP phosphorylation by mass spectrometry (top). Representation of phosphorylated residues detected on Epe1-GFP from cells untreated or treated with 14 mM caffeine for 16 h (middle). Spectra showing detection of phosphorylated serine S791, S795 and S803 residues after caffeine treatment (bottom). Full data, Supplementary Table 4. 

d. Epe1-Myc and Mto2 westerns from cells treated with 14 mM caffeine for 16 h before (-)/after (+) lambda protein phosphatase addition. Mto2 mobility is known to be Lambda Phosphatase sensitive.
Extended Data Fig. 2 | Deletions beyond residue 150 in Epe1-GFP do not hinder caffeine-induced processing and peptides within the first 100 residues are not detected following caffeine treatment. a. Schematic of indicated 20 residue deletion mutants in the N-terminal coding region of the endogenous epe1 gene expressed as GFP fusions (Epe1\(\Delta\)150–170-GFP, Epe1\(\Delta\)171–190-GFP, Epe1\(\Delta\)191–210-GFP; left). Western detecting indicated mutant Epe1-GFP fusion proteins or \(\alpha\)-tubulin from cells untreated (-) or treated (+) with 14 mM caffeine; right). b. Epe1-Myc peptides detected following immunoprecipitation from cells untreated (-) or treated (+) with 14 mM caffeine and analysis by mass spectrometry. Top: schematic showing position of peptides detected relative to Epe1 (residues 1–948). Bottom: Epe1 peptides detected in Epe1-Myc immunoprecipitates from treated (+) or untreated (-) with 14 mM caffeine. Of the eighteen peptides detected from Epe1-Myc in untreated (-) samples three were not detected (n.d.; red) in the caffeine-treated sample. The three peptides not detected in the presence of caffeine are derived from within the first 100 residues on the N-terminus. Analysis was performed on three independent immunoprecipitates. Full data, Supplementary Table 5.
Extended Data Fig. 3 | A caffeine-induced N-terminal processing product is not detectable but Epe1-associated ubiquitination increases and Epe1 processing is sensitive to loss of specific E3 ligases which influence resistance. a, Anti-Myc western of cells untreated (-)/treated (+) with 14 mM caffeine showing that a predicted N-terminal 20 kDa Myc-Epe1₁₁–₁₅₀ processing product is not detectable. b, Western showing detection of both isoforms of the histone acetyltransferase Mst2-Myc or α-tubulin from wild-type or mst2-1 proteasome defective cells untreated (-)/treated (+) with 14 mM caffeine for 16 h. This control demonstrates that other known stress-induced changes still occur in mst2-1 cells. c, Western showing detection of both isoforms of the histone acetyltransferase Mst2-Myc or α-tubulin from wild-type cells incubated without (-)/with (+) with the 26S proteasome inhibitor Bortezomib (BTZ) prior to no caffeine (-)/14 mM caffeine (+) treatment for 16 h. This control demonstrates that other known stress-induced changes still occur in the presence of BTZ. d, Immunolocalization of Epe1-GFP in wild-type or mts2-1 cells untreated (-)/treated (+) with 14 mM/16 h caffeine. No tag, negative control. e, Quantification of anti-GFP/Epe1-GFP nuclear signals of cells in d. Data are represented as individual measurements from ≥ 20 cells per sample. Bars represent mean, with error bars +/− SD. The significance of the difference between samples was evaluated using a two-tailed Student’s t-test. (*) P < 0.033, (**) P < 0.002; (***) P < 0.0002; (****) P < 0.0001; (n.s) not significant. f, Western detecting Epe1-GFP or α-tubulin in wild-type or ddb1Δ cells untreated (-)/treated (+) with 14 mM/16 h caffeine. Numbers below tracks: levels of full-length FL-Epe1 and truncated tEpe1 normalised to no treatment and adjusted relative to α-tubulin loading control, measurement average of 2 biological replicates. g, Number of resistant colonies formed/1 × 10⁴ viable cells by wild-type or ddb1Δ cells plated on caffeine or fluconazole plates. Error bars are from independent measurements from 3 biological replicates. Data are presented mean values +/− SD, with dots representing independent plate counts. The significance of the difference between samples was evaluated using a two-tailed Student’s t-test. (*) P < 0.033, (**) P < 0.002; (***) P < 0.0002; (****) P < 0.0001; (n.s) not significant.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Chromatin-associated nuclear proteins are enriched with full length Epe1 whereas more cytoplasmic proteins are enriched with constitutively truncated Epe1ΔN150-GFP. a. Volcano plot of proteins enriched with full-length Epe1-GFP extracted from cells detected by proteomic analysis (top), specific proteins labelled (red dots). Table with names and functions of proteins enriched (bottom). Full data, Supplementary Table 6. b. Volcano plot of proteins enriched with constitutively processed Epe1ΔN150-GFP extracted from cells and detected by proteomic analysis (top), specific proteins labelled (red dots). Table with names and functions of proteins enriched (bottom). Full data, Supplementary Table 7.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Altered silencing and gene expression in epe1Δ and Epe1-ΔN150-GFP cells. a. Wild-type, epe1Δ, Epe1-ΔN150, Epe1-GFP/wt and Epe1-ΔN150-GFP cells harbouring *ura4*<sup>+</sup> marker gene insertions at either site 1 or site 2 as indicated (top) were serially diluted and plated on non-selective (N/S), selective (-URA) or counter-selective (FOA) plates. b. Heat-map generated from processed data of RNA-seq two biological replicates from Epe1ΔN150-GFP or epe1Δ cells compared with that of wild-type Epe1-GFP cells. Genes with higher (turquoise) or lower (brown) expression relative to wild-type Epe1-GFP cells are shown as a Log<sub>2</sub> scale. The position of specific affected genes along *S. pombe* chromosomes Chr I, II, III are shown with arrowheads indicating telomeres on Chr I and ChrII. The annotated *S. pombe* genome shows only rDNA arrays (rectangles) at both ends of Chr III. c. Heat-map showing that RNA-seq data (used in b.) resulting from two Epe1ΔN150-GFP and epe1Δ biological replicates are more similar to each other than they are to wild-type Epe1-GFP cells.
Extended Data Fig. 6 | Cells expressing predicted catalytically inactive Epe1-GFP and Epe1ΔN150-GFP exhibit decreased resistance. a. Schematic showing position of H297A and K314A mutations predicted to reduce association of the essential iron/Fe(2) and 2-Oxoglutarate (α-ketoglutarate) cofactors, respectively, with the JmjC demethylase domain of Epe1 (Top). Westerns detecting Epe1-GFP, Epe1-cd-GFP, Epe1ΔN150-GFP and Epe1ΔN150-cd-GFP (cd; catalytically dead) or α-tubulin (bottom). b. Number of resistant colonies formed/1×10⁴ viable cells by Epe1-GFP, Epe1-cd-GFP, Epe1ΔN150-GFP and Epe1ΔN150-cd-GFP cells plated on caffeine or fluconazole plates Error bars are from independent measurements from 3 biological replicates. Data are presented mean values +/−SD, with dots representing independent plate counts. The significance of the difference between samples was evaluated using a two-tailed Student’s t-test. (*) P < 0.033, (**) P < 0.002; (*** ) P < 0.0002; (****) P < 0.0001; (n.s). not significant.
Extended Data Fig. 7 | The cell integrity/Pmk1 but not the TORC2/Gad8 stress signalling pathway regulates Epe1 processing and resistance. a, Diagram of part the S. pombe TORC2/Tor1-dependent signalling pathway. b, Western detecting Epe1-GFP or α-tubulin from wild-type or gad8Δ cells untreated (−)/ treated (+) with 14 mM/16 h caffeine. c, Western detecting Epe1-Myc or α-tubulin from wild-type, pek1Δ, pmk1Δ, atf1Δ or pmp1Δ cells untreated (−)/ treated (+) with 0.5 mM/16 h fluconazole (FLC). d, Number of resistant colonies formed/1×10⁴ viable cells plated by wild-type, epe1Δ, sty1Δ, atf1Δ and gad8Δ on plates containing 16 mM caffeine (CAF). e, Number of resistant colonies formed/1×10⁴ viable cells plated by wild-type, epe1Δ, sty1Δ, atf1Δ and gad8Δ on plates containing 0.5 mM fluconazole (FLC). Plots d and e; Error bars are from independent measurements from 3 biological replicates. Data are presented mean values ±/− SD, with dots representing independent plate counts. The significance of the difference between samples was evaluated using a two-tailed Student’s t-test. (*) P < 0.033, (**) P < 0.002; (***) P < 0.0002; (****) P < 0.0001; (n.s), not significant.
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a  Confirmed

☐ ☑ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
☐ ☑ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐ ☑ The statistical test(s) used AND whether they are one- or two-sided
  Only common tests should be described solely by name; describe more complex techniques in the Methods section.
☐ ☐ A description of all covariates tested
☐ ☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐ ☑ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐ ☑ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  Give P values as exact values whenever suitable.
☐ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☐ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐ ☑ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

- Epson Scan 2 Perfection V600 (6.4.99) software [Epson] for serial dilution assay picture acquisition and western blotting image acquisition.
- Metamorph software (v7) (Universal Imaging Corporation) for cytology acquisition.
Data analysis
- Fiji Image J (2.0)
- Snapgene 5.2
- FastQC (v0.11.8)
- FastQscreen (v0.14.1)
- Trimmomatic (v0.39)
- Cutadapt (v2.8)
- Bowtie2 (v2.3.5.1)
- Samtools (v1.10)
- STAR (v2.7.3)
- Salmon (v0.12.0)
- deepTools (v3.5.0)
- MACS2 (v2.2.7.1)
- IGV (v2.8.10)
- Bioconductor (R v4.1.1):
  - Sushi (v1.28.0)
  - DESeq2 (v1.30.1)
  - Metamorph (v7)
  - MaxQuant (1.6.1.0)

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g., GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Data Availability
The publicly accessible PomBase database https://www.pombase.org was used to obtain genome sequence and annotation for the Schizosaccharomyces pombe (fission yeast) genome. All raw and processed reads from sequencing experiments (ChIP-seq and RNA-Seq) are available at GEO with accession number GSE190267. All raw mass spectrometry data are available at PRIDE using project accession number PXD030205.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- [x] Life sciences
- [ ] Behavioural & social sciences
- [ ] Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-fi.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
No sample size calculation was performed, the samples sizes used are considered standard in the field and were selected to ensure robust and statistically significant comparisons.

For ChIP-qPCR and RT-qPCR experiments, biological triplicates (samples independently cultured for the experiment) were used for each strain analysed.

For RNA-seq experiments two biological replicates (samples independently cultured for the experiment) were performed for each strain analysed.

For ChIP-seq three biological replicates were performed for each strain and results were confirmed by ChIP-qPCR.

For experiments performed to test whether specific genetic mutant strains form more caffeine-resistant or fluconazole-resistant colonies than wild-type cells, 3 biological replicates were used to derive statistics. Equal volumes from a culture from each strain were plated on 8-10 (indicated in figure legend) caffeine/fluconazole-containing media plates. After 7 days, the number of resistant colonies on each plate was counted. Sample sizes are provided in the figure legends.

For quantification of cytological samples by microscopy, 100 cells for each sample was assayed.

Data exclusions
There were no data exclusions.

Replication
Findings were reliably reproduced.
For ChIP-qPCR and RT-qPCR, data are mean +/- standard deviation from 3 biological replicates.
ChiP-seq experiments were performed three times and results were confirmed by ChiP-qPCR. RNA-seq experiments were performed two times using two biological replicates. Serial dilution growth assays were repeated at least twice on different days (biological replicates) with similar results. Resistance frequency measurements were performed 3 times on distinct starting cultures (biological replicates).

Randomization
No randomization was required because the results of physical measurements of biomolecules, phenotypic analysis (e.g., drug resistance test) or sequencing of nucleic acid libraries are not affected by sample randomization.

Blinding
No blinding was required because the results of physical measurements of biomolecules, phenotypic analysis (e.g., drug resistance test), or sequencing of nucleic acid libraries are not affected by the researchers' knowledge of sample identities.

---

**Reporting for specific materials, systems and methods**

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | Antibodies            |
| ☑   | Eukaryotic cell lines |
| ☑   | Palaeontology         |
|     | Animals and other organisms |
|     | Human research participants |
|     | Clinical data         |

### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | ChiP-seq              |
|     | Flow cytometry        |
|     | MRT-based neuroimaging |

### Antibodies

**Antibodies used**

- Anti-Myc - Cell Signalling - 9811 - for western analysis. 1:1000.
- Anti-FLAG M2 - Merck - F1804 - for western analysis. 1:4000.
- Anti-H3K9me2 - Mouse monoclonal S1.1 - for H3K9me2. ChiP-qPCR. Kindly provided by Takeshi Urano. 1 ug per ChiP-qPCR.
- Anti-GFP - Life Technologies - A1122 - for GFP ChiP-qPCR and cytology. 2 ug per ChiP-qPCR. 1:200 for cytology.
- Anti-GFP - Merck - 1181446000 - for immunoprecipitation. 20 ug per IP. for western analysis 1:750
- Anti-alpha-tubulin - for western analysis. Kindly provided by Keith Gull. 1:15000.
- Anti-Bip1 - for western analysis. Lab stock. 1:1000.
- Anti-Chpl - for cytology. Lab stock 1:3000.
- Peroxidase anti-peroxidase - Merck - P1291 - for western analysis. 1:1000.
- Anti-Mito2 - for western analysis. Kindly provided by Ken Sawin. 1:1000.
- Chicken anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 - for cytology. Life Technologies - A21441. 1:1000.
- Donkey anti-Sheep IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 - for cytology. Life Technologies - A11016. 1:1000.
- Peroxidase conjugate goat anti-rabbit IgG - for western analysis - Merck - A6154. 1:10000
- Peroxidase conjugate goat anti-mouse IgG - for western analysis - Merck - A4416. 1:10000
- Anti-ubiquitin mouse monoclonal - for western analysis - Enzo - BML-PW8810. 1:1000.

### Validation

- Mouse mAb S1.1: Raised in Urano lab, validated in Nakagawachi et al (2003) Oncogene 22, 8835. Additionally, this antibody has been validated in our lab using a strain lacking the H3K9 methyltransferase.
- Anti-alpha-tubulin raised in Gull lab, validated in Woods et al [1989] J. Cell. Sci. 93 (Pt 3), 491–500.
- Anti-Bip1 raised and validated in Pidoux & Armstrong [1993] J. Cell. Sci. 105 (Pt 4), 1115–1120.
- All other antibodies have been extensively used for ChiP, western and cytology analyses in our laboratory and have been validated using no tag (GFP/FLAG/Myc) controls. For previous studies where these antibodies have been used see Tong et al. (2019) Nat. Commun and Bayne et al. (2010) Cell.

---

**Eukaryotic cell lines**

Policy information about cell lines.

**Cell line source(s)**

All Schizosaccharomyces pombe strains used in this study are derivatives of 972 h− or other commonly used lab strains. Detailed genotypes are listed in Supplementary Table 1.

**Name Genotype Source**

972 h− wild type lab stock
A5688 h− his2-TAP:ura4− ura4-D18 leu1-32 ade6-210 his3D Parental FL201. Gift from Fei Li [Cande lab]
B2835 h− epe1-GFP-KAN Torres-Garcia, 2020
B3111 S. octosporus wild type Lab stock
B3204 h− epe1-13Myc-NAT This study
B3250 S. cerevisiae Sgo1-GFP Lab stock, gift from Adele Marston
B3826 h- 3xFLAG-epe1-GFP-KAN Torres-Garcia, 2020
B3827 h- 3xMyc-epe1-GFP-KAN This study
B3828 h- 100 amino acids deletion from N-terminus of epe1-GFP-KAN This study
B3829 h- 150 amino acids deletion from N-terminus of epe1-GFP-KAN This study
B3830 h- 200 amino acids deletion from N-terminus of epe1-GFP-KAN This study
B4001 h? Ade65-SGFP-Ura4-Terminator Lab stock
B4038 h? epe1-GFP-KAN mts2-1 TS This study
B4039 h? epe1-13Myc-NAT mts2-1 TS This study
B4040 h? sty1A::NAT, epe1-GFP-KAN This study
B4288 h? gpd8A::NAT epe1-GFP-KAN This study
B4289 h? pmk1A::NAT, epe1-GFP-KAN This study
B4292 h? alf1A::NAT, epe1-GFP-KAN This study
B4621 h- epe1 deletion by Crispr Torres-Garcia, 2020
B4782 h- 50 amino acids deletion from N-terminus of epe1-GFP-KAN This study
B4789 h? pek1A::NAT This study
B4940 h- 101-110 amino acids deletion from epe1-GFP-KAN This study
B4942 h- 111-120 amino acids deletion from epe1-GFP-KAN This study
B4944 h- 121-130 amino acids deletion from epe1-GFP-KAN This study
B5048 h? pmpl1A::HYG, epe1-GFP-KAN This study
B5090 h N-ter 150 amino acids of Epe1 with GFP This study
B5623 h- 151-170 amino acids deletion from epe1-GFP-KAN This study
B5625 h- 171-190 amino acids deletion from epe1-GFP-KAN This study
B5627 h- 190-210 amino acids deletion from epe1-GFP-KAN This study
B4057 h? cfr4A::NAT This study
B5641 h- 100 amino acids deletion from N-terminus of epe1-GFP-KAN, cfr4A::NAT This study
B5645 h- 150 amino acids deletion from N-terminus of epe1-GFP-KAN, cfr4A::NAT This study
B5650 h? epe1 deletion by Crispr, cfr4A::NAT This study
B5654 h? epe1-GFP-KAN, cfr4A::NAT This study
B5775 h? hul5A::NAT This study
B5776 h? pub3A::NAT This study
B6243 h? Epe1-13Myc NAT, dbb1A::HYG This study
B6453 h? Epe1-cd-GFP This study
B6455 h? Epe1-cd-GFP This study
B6469 h? hul5A::HYG, pub3A::NAT This study
B6483 h? tepe1-GFP resistant colony-1 This study
B6484 h? tepe1-GFP resistant colony-2 This study
B6485 h? tepe1-GFP resistant colony-3 This study
B6486 h? tepe1-GFP resistant colony-4 This study
B6487 h? tepe1-GFP resistant colony-5 This study
B6488 h? tepe1-GFP resistant colony-6 This study
B6489 h? tepe1-GFP resistant colony-7 This study
B6490 h? tepe1-GFP resistant colony-8 This study
B6491 h? tepe1-GFP resistant colony-9 This study
B6492 h? tepe1-GFP resistant colony-10 This study
B6493 h? tepe1-GFP untreated colony-1 This study
B6494 h? tepe1-GFP untreated colony-2 This study
B6495 h? tepe1-GFP untreated colony-3 This study
B6496 h? tepe1-GFP untreated colony-4 This study

Authentication
All strains were generated by transformation and combined using genetic crosses. Strains were verified by the presence or absence of marker genes allowing growth on selective medium and/or by PCR to determine the presence of the desired genetic alteration and/or by DNA sequencing and/or western blotting to confirm the presence of epitope tags, as appropriate.

Mycoplasma contamination
Not applicable - Yeast strains do not suffer from mycoplasma contamination, but all cultures were observed by microscopy to be free of bacterial contamination.

Commonly misidentified lines
No commonly misidentified lines were used. This study used the yeast Schizosaccharomyces pombe.

ChIP-seq

Data deposition
☒ Confirm that both raw and final processed data have been deposited in a public database such as GEO.
☒ Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links
May remain private before publication.
For "Initial submission" or "Revised version" documents, provide reviewer access links. For your "Final submission" document, provide a link to the deposited data.

Files in database submission
epel1D_input_rep1_R1.fastq.gz
epel1D_input_rep2_R1.fastq.gz
epel1D_input_rep3_R1.fastq.gz
Genome browser session
(e.g. UCSC)
Not applicable. Visualized data using IGV.

### Methodology

#### Replicates

3 ChIP-seq replicates were performed for each strain. Results were confirmed by ChIP-qPCR.

#### Sequencing depth

All libraries were sequenced by 75 bp paired-end reads. We did not calculate number of uniquely mapped reads, since Bowtie2 default options do not do so. Heterochromatin regions/sequences are repetitive and the number of uniquely mapped reads is not informative.

#### Antibodies

Mouse mAb 5.1.1 anti-H3K9me2 for H3K9me2 ChIP-seq

#### Peak calling parameters

macs2 callpeak -f BAMPE -t sample.bam -c sample.bam --broad -g 14e6 --broad-cutoff 0.05 --n sample

#### Data quality

MACS2 was used to call peaks from paired-end ChIP-seq reads.

#### Software

- FastQC (v0.11.9)
- FastqScreen (v0.14.1)
- Trimomatic (v0.39)
- Cutadapt (v2.8)
- Bowtie2 (v2.3.5.1)
- Samtools (v1.10)
- STAR (v2.7.3)
- Salmon (v0.12.0)
- deepTools (v3.5.0)
- MACS2 (v2.2.7.1)
- IGV (v2.8.10)
- Bioconductor (R v4.1.1):
  - Sushi (v1.28.0)
  - DESeq2 (v1.30.1)