Epigenetic Control of Phenotypic Plasticity in the Filamentous Fungus *Neurospora crassa*

Ilkka Kronholm,*† Hanna Johannesson,* and Tarmo Ketola*

*Centre of Excellence in Biological Interactions, Department of Biological and Environmental Sciences, University of Jyväskylä, FI-40014, Finland and †Department of Organismal Biology, University of Uppsala, 752 36, Sweden

ORCID ID: 0000-0002-4126-0250 (I.K.)

ABSTRACT Phenotypic plasticity is the ability of a genotype to produce different phenotypes under different environmental or developmental conditions. Phenotypic plasticity is a ubiquitous feature of living organisms, and is typically based on variable patterns of gene expression. However, the mechanisms by which gene expression is influenced and regulated during plastic responses are poorly understood in most organisms. While modifications to DNA and histone proteins have been implicated as likely candidates for generating and regulating phenotypic plasticity, specific details of each modification and its mode of operation have remained largely unknown. In this study, we investigated how epigenetic mechanisms affect phenotypic plasticity in the filamentous fungus *Neurospora crassa*. By measuring reaction norms of strains that are deficient in one of several key physiological processes, we show that epigenetic mechanisms play a role in homeostasis and phenotypic plasticity of the fungus across a range of controlled environments. In general, effects on plasticity are specific to an environment and mechanism, indicating that epigenetic regulation is context dependent and is not governed by general plasticity genes. Specifically, we found that, in *Neurospora*, histone methylation at H3K36 affected plastic response to high temperatures, H3K4 methylation affected plastic response to pH, but H3K27 methylation had no effect. Similarly, DNA methylation had only a small effect in response to sucrose. Histone deacetylation mainly decreased reaction norm elevation, as did genes involved in histone demethylation and acetylation. In contrast, the RNA interference pathway was involved in plastic responses to multiple environments.

Natural environments are in a constant state of change. Organisms must cope with different environments and their dynamism by adjusting their development, behavior, and reproduction, while always maintaining physiological homeostasis. Phenotypic plasticity is defined as the ability of an organism to express different phenotypes in response to environmental changes (DeWitt and Scheiner 2004), where phenotype can be any morphological, physiological, or behavioral trait. In most cases, phenotypic plasticity is based on adjusting the patterns of gene expression (Nicotra et al. 2010). Phenotypic plasticity plays an important role in buffering fitness across a range of environments, and can help evolutionary adaptation to extreme environments (Lande 2009; Chevin et al. 2010; Draghi and Whitlock 2012). Specifically, plasticity can facilitate adaptation by increasing population size in the novel environment (Chevin et al. 2010), and by generating phenotypic variation that can be selected if it is heritable (Pål 1998). Heritable phenotypic variation could potentially be achieved with different mechanisms, one of them being plasticity mediated by epigenetic mechanisms. Interestingly, evolutionary models incorporating heritable genetic and epigenetic systems suggest that the latter play a significant role in adaptation (Day and Bonduriansky 2011; Klironomos et al. 2013; Kronholm and Collins 2016).

Transcriptomic studies have shown that environment has a large effect on gene expression profiles (Gibson 2008; Nicotra et al. 2010; Alvarez et al. 2015). The classical view is that those patterns are regulated by transcriptional activator and repressor proteins...
Furthermore, epigenetic mechanisms are involved in phenotypic plasticity (Slepecky and Starmer 2009; Bossdorf et al. 2010; Herrera et al. 2012; Baerwald et al. 2016) and transgenerational inheritance (Verhoeven et al. 2010; Verhoeven and van Gurp 2012; Luna and Ton 2012; Rasmann et al. 2012; Ou et al. 2012; Öst et al. 2014; Siklenka et al. 2015). While experiments that have artificially induced variation in DNA methylation have shown how this factor can contribute significantly to phenotypic variation (Cortijo et al. 2014) and plasticity (Kooke et al. 2015), the relative importance of other epigenetic modifications—even the extent to which their effects are environmentally dependent—remains unclear.

The filamentous fungus *Neurospora crassa* is a good model system with which to study epigenetics as it has nearly all the epigenetic mechanisms that higher eukaryotes have, but many of these mechanisms are not essential for viability (Allshire and Selker 2009). It is also easy to do controlled experiments in different environments as the fungus can be propagated axenically. As phenotypic plasticity in fungi has not been studied extensively (Slepecky and Starmer 2009), we need to first establish how *N. crassa* responds to different environments phenotypically, and ask what is the role of epigenetic mechanisms? This is necessary in order to study the possible transregenerative effects or the role of plasticity in adaptation in the future.

To explore the extent to which various epigenetic mechanisms are involved in phenotypic plasticity, we used an experimental set of 25 different mutant strains of *N. crassa*, each of which is deficient in a particular chromatin modification, affecting: DNA methylation (three mutants), histone methylation (five), histone deacetylation (eight), histone acetylation (two), histone demethylation (two), and RNA interference (five). We selected mutants that either had been previously characterized and were known to affect different epigenetic modifications, or based on their homology to genes known to modify chromatin in other organisms.

We measured the reaction norms—the phenotypic expression of a trait for a genotype across a range of environments—of each strain with respect to four different environmental variables: temperature, osmotic stress (NaCl), sucrose concentration, and pH, to investigate the potential effects of each epigenetic mechanism on phenotypic plasticity across a range of environments. A reaction norm is visualized by plotting the measurable performance (e.g., mycelial growth rate) of an organism scored at different values of an environmental parameter. Reaction norms can be described by their shape and elevation; shape refers to variation in phenotype that contributes to genotype by environment interaction, and elevation means variation in phenotype that contributes to the genotypic effect only. If epigenetic modifications play a role in phenotypic plasticity, we expect to see differences in reaction norm shapes for the mutant strain and wild type. Differences in reaction norm elevations indicate that the epigenetic modification is required for normal cellular function rather than a physiologically plastic response.

We show that epigenetic mechanisms are involved in plastic responses of *N. crassa*. These responses involve a specific epigenetic modification in a particular environment, e.g., histone modifications are important in the response to temperature and pH, and the RNA interference pathway also has notable effects. In contrast, lacking the ability to carry out DNA methylation had little effect on strain performance in any of the trial environments.

**MATERIALS AND METHODS**

**Neurospora strains**

We used 25 different strains from the *N. crassa* knockout collection (Colot et al. 2006) to investigate the role of epigenetic mechanisms in phenotypic plasticity. The mutants were generated by replacing the entire open reading frame of the target gene with an *hph* cassette, which confers resistance to the antibiotic Hygromycin B. Strains were obtained from the Fungal Genetics Stock Center (FGSC) (McCluskey et al. 2010); Supplemental Material, Table S1 shows the strains used in this study, and Table 1 shows the genotypes of those used in the experiments. We included strains that are viable in the homokaryotic state and for which we could confirm the gene deletion. Strain FGSC #4200 was used as a wild-type control for the reaction norm measurements. We grouped the strains into five categories based on the epigenetic mechanism in which they are deficient: DNA methylation, histone methylation, histone deacetylation, RNA interference, and “other,” which included two putative histone demethylases and two histone acetyl transferases (Table 1).

**Genotyping**

We confirmed that the mutant strains indeed have deletions, and verified their mating types by PCR. We relied on a rapid method of DNA extraction from conidia or asexual spores (Henderson et al. 2005). We grew a strain in an agar slant for 3–5 days until orange-colored spores were visible. Spores were then collected and suspended in water containing 0.01% Tween-80. Conidial boiling buffer was prepared by combining 100 parts of 50 mM Tris pH 8 and two parts of 0.5 M EDTA pH 8.5. We then distributed 10 μl of conidial boiling buffer across a 96-well PCR plate, and combined 40 μl of conidial suspension to each well. The loaded plate was boiled for 10 min at 98°C in a thermal cycler, and 2 μl of the resulting suspension was used as template in subsequent PCR reactions.

A strain’s mating type was determined by PCR in a single 10 μl reaction containing four primers, i.e., two pairs. These two primer pairs (Table S2) were designed using the *N. crassa* genome sequence to amplify a 200-bp fragment from the *mat a* locus, and a 400-bp fragment from the *mat A* locus. PCR products were resolved by electrophoresis on a 2–3% agarose gel. To confirm that our mutant strains indeed have deletions, we designed primers for each of the target genes that amplified a fragment of ~500-bp, and checked the absence of the deleted genes by PCR. We set up PCR reactions with the high-fidelity Phusion DNA polymerase (Thermo Scientific) according to the manufacturer’s instructions: the final PCR reactions contained 1× Phusion HF buffer, 0.2 mM each dNTP, 0.5 μM each primer, and 0.2 or 0.4 units of Phusion DNA polymerase for 10 or 20 μl reactions, respectively. All PCR reactions were run with the following thermal profile: initial denaturation of 98°C for 30 sec, then 35 cycles of 98°C for 5 sec, variable annealing for 10 sec, extension at 72°C for 20 sec, and a final extension at 72°C for 1 min. Primer sequences and their annealing temperatures are given in Table S2.

**Growth measurements**

In general, standard laboratory protocols for *Neurospora* (Davis and de Serres 1970) were followed. We grew *N. crassa* on Vogel’s growth medium (Metzenberg 2003) with 1.5% agar appropriately supplemented for the different environments listed below. To measure growth rate of the strains, we used the race tube method of Ryan et al. (1943), with tubes prepared following White and Woodward (1995). Briefly, we filled 25 ml plastic serological pipettes (Sarsted) with 10 ml of molten agar and placed them horizontally so that the agar solidified...
The table below shows the mutant strains used in this study.

| Gene ID | Gene ID | Epigenetic Mechanism | Modification | Function |
|---------|---------|----------------------|--------------|----------|
| NCU02247 | DNA methylation | DNA methyltransferase | DNA methyltransferase |
| NCU01554 | DNA methylation | Controls the spreading of DNA methylation from heterochromatic regions |
| NCU08289 | DNA methylation | Controls the spreading of DNA methylation from heterochromatic regions |
| NCU004402 | Histone methylation | H3K9me3 | H3-specific histone deacetylase; H3K9 is a mark for silent heterochromatin, guides DNA methylation |
| NCU01206 | Histone methylation | H3K4me3 | H3-specific histone deacetylase; H3K4 trimethylation affected |
| NCU00269 | Histone methylation | H3K36me | H3-specific histone deacetylase; H3K36; needed for correct transcriptional elongation |
| NCU07496 | Histone methylation | H3K27me3 | H3-specific histone deacetylase; H3K27; catalytic subunit of PRC2 |
| NCU06679 | Histone methylation | H3K27me3 | Homolog of Drosophila p55; part of chromatin remodeling complex |
| NCU04730 | RNA interference | NA | RNA-dependent RNA polymerase; initiation of the RNAi pathway |
| NCU04737 | RNA interference | NA | Argonaute; mutations abolish miRNA processing ability |
| NCU09120 | RNA interference | NA | Dicer ribonuclease; maturation of miRNAs |
| NCU10847 | RNA interference | NA | Histone demethylase; homolog of S. pombe lsd1 and Aspergillus HdmA |
| NCU01554 | DNA methylation | NA | Histone acetyl transferase; homolog of Aspergillus Hos2 |
| NCU08270 | RNA interference | NA | Histone acetyl transferase; homolog of S. pombe lid2 |
| NCU00076 | RNA interference | NA | Histone acetyl transferase; acetylation of H3K14 |

Gene ID is based on the N. crassa genome assembly NC12. Epigenetic mechanism is the classification for the strains studied here. Modification is the chromatin modification affected by the mutation, and function describes what is known about the biochemical activity of the protein. NA, not applicable; HDAC, histone deacetylase; miRNA, microRNA-like RNA.

at the bottom of the pipettes. The tip of the pipette was snapped off, and that end was inoculated with conidia and subsequently sealed with Parafilm. The other end of the pipette contained a cotton plug. Growth of the mycelial front in the tube was measured by marking its position against the distance the mycelial front had grown in a race tube. The first marking was made when the mycelial front was clearly visible, and growth rate data were collected from this point on. Growth immediately following inoculation until the mycelial front was first visible was not included. This effectively corrects for possible differences in initial growth rate due to inoculum size. We extracted the slope of the regression line for each growth assay to obtain the mycelial growth rate as mm/hr. Growth rates were used as a dependent variable in subsequent analyses.

**Reaction norms**

As a measure of phenotypic plasticity, we measured reaction norms of the different strains with respect to four different environmental parameters: temperature, osmotic stress (NaCl), sucrose concentration, and pH. We used six different settings within each parameter, 26 different genotypes with five replicate growth rate measurements in each treatment combination, yielding 3120 growth rate measurements in total. The different parameter settings were: 15, 20, 25, 30, 35, and 40°C for the temperature; 0, 0.2, 0.4, 0.8, 1.2, and 1.6 M of NaCl added for osmotic stress; 0.015, 0.15, 1.5, 5, 15, and 30% (w/v) sucrose added for sucrose concentration; or pH adjusted to 4.0, 5.0, 5.8, 7.0, 8.0, and 9.0. Except for temperature, which was controlled by the growth chamber, the standard growth medium was manipulated by either adding NaCl, varying the sucrose level, or adjusting pH with either HCl or NaOH. We did not control for any changes in nutrient availability in the medium that may result from pH changes, and allowed the environmental changes to be complex.

Normal growth conditions were 25°C, 0 M NaCl, 1.5% sucrose, pH 5.8, and constant darkness. Independent measurements were collected during all experiments under these “control” conditions. The reaction norm experiment was performed in growth chambers, and replicate measurements were blocked in time by replicates, such that each strain and environmental setting ran simultaneously, and the growth tubes were randomized in the growth chamber. However, since the temperature treatment had to be applied to the entire growth chamber, for the temperature treatment, we used two growth chambers of the same model (Lab companion ILP-02/12; Jeio Tech, South Korea), where we always switched the identity of the growth chamber between replicate measurements. For example, for replicate 1, chamber A was set to 25°C and chamber B to 30°C; for replicate 2, chamber A was set to 30°C and chamber B to 25°C; and so on. This allowed us to check for any possible effect of either growth chamber independent of temperature.
Backcrossing and validation

To control for possible genetic background effects between the mutants and the control strain 4200, we performed a validation experiment with strains where we had backcrossed the mutant strain five times into FGSC # 2489 background using standard crossing techniques (Davis and de Serres 1970). The pedigree of the wild type strains 4200 and 2489 is known (Myllyk et al. 1974; Newmayer et al. 1987; Perkins 2004). They are nearly isogenic, expect for the mating type locus, and the theoretical expectation from the number of backcrosses is that they share > 99.99% of their genetic background. The deletion mutants were generated by transforming the strain 4200 (Colot et al. 2006). Theoretically, after five backcrosses, the backcrossed strains should share 98.44% of their genetic background with 2489, but as 4200 and 2489 were already nearly isogenic, they share more of their genetic background than would be expected from five backcrosses.

The sexual cycle can be induced by growing N. crassa in a low-nitrogen medium. The fungus has two different mating types: mat A and mat a. When the two types meet, a conidial or mycelial cell of one type fertilizes the protoperitheciun, the “female” organ, of the other type, and the different nuclei fuse and undergo meiosis to yield haploid spores. We used crossing medium (Davis and de Serres 1970) containing 0.2% sucrose, but, instead of agar, we used 5 ml of liquid medium in a large 20 × 150 mm test tube with a 40 × 90 mm piece of vertically folded filter paper that wicks the medium by capillary action. The filter paper was inoculated with conidia from the opposite mating types. We germinated ascospores on plates with sorbose to induce colonial growth and 200 μg/ml of Hygromycin B, where appropriate, to select for mutant-strain progeny. We checked mating type of the progeny, and controlling for possible genetic background effects between the mutants and the control strain 4200, we performed a validation experiment with 2489 as a control.

and assays were replicated 12 times in the validation experiment, each including strain 2489 as a control.

Data analysis

We used an ANOVA to investigate whether different epigenetic mechanisms have different effects, and whether they are specific to particular environments. Because the reaction norms were nonlinear, we encoded the different parameter settings as continuous variables; we used splines because some of the strains showed reaction norm shapes that made fitting the same regression model to each of the genotypes inappropriate. The drawback of using splines is that we cannot estimate the critical thresholds when growth rate approaches zero, as natural splines do not allow extrapolation outside of the data range.

Bayesian estimation of differences between the control and mutant strains

To test for differences between the control and mutant strains in specific environments, we used a Bayesian model analogous to a one-way ANOVA. The model specification followed Gelman (2006) and Kruschke (2011):
We calculated contrasts between the control and mutant strains using the posterior distributions for $\beta_j$ as $\beta_{\text{mutant}} - \beta_{\text{control}}$, and we also rescaled the $\beta_j$ values to their original scale. If a strain is growing slower than the control, its difference will be negative. We considered growth rates of the control and the mutant strains to be significantly different if the 95% highest posterior density (HPD) interval for the difference did not include zero.

Data availability

Strains are available upon request. File S1 contains all phenotypic data. The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

RESULTS

Growth rates

We found growth of *N. crassa* in our race tubes to be linear: the 95% quantiles for $R^2$ values of a linear fit across all measurements were 0.952 – 0.999 with a median of 0.998. The only genotypes exhibiting deviations from a strict linear pattern were *dim-5*, *nfg-1*, and *npf*, with the latter showing the lowest $R^2$ in the whole dataset (0.622). This deviation from linear growth was observed particularly in environments where growth was very slow. Because these cases represent only a small portion of the entire dataset, we also used a linear model for the growth of these genotypes. We found that, in the control environment, our control genotype 4200 grew at a rate of 3.29±0.24 (95% CI) mm/hr, in line with previous reports (Ryan *et al.* 1943).

In some tubes where no growth had occurred during the growth assay, we could observe growing mycelium after an extended amount of time (e.g., *npf* in high osmotic stress, and *dim-5* at low temperature). We assigned a growth rate of zero to those measurements.

Reaction norm experiment

In the reaction norm experiment, data were missing for 19 out of 3120 measurements. Most of these were probably due to failed inoculations, as, in many cases, we were able to distinguish between missing data and no growth in a particular trial. Reaction norms were visualized by plotting growth rate against the different environmental parameters (Figure 1). Visual inspection revealed that nearly all reaction norms were nonlinear, as even in the osmotic stress reaction norms there was some indication of curvature. Even in the osmotic stress environment, the optimum is at zero and growth rate decreases as the salt concentration increases (Figure 1). First, we performed ANOVA to investigate whether the different epigenetic mechanisms have different effects on phenotypic plasticity (Table 2). We did not observe a significant main effect of epigenetic mechanism type, but we did find a significant interaction between epigenetic mechanism and parameter setting nested within stress type, $F_{68,400.17} = 1.397$, $p = 0.021$ (Table 2). This result indicates that different epigenetic mechanisms have different effects on different environmental parameters. Thus, subsequently, we analyzed the data by stress type.

When analyzing the environmental parameters separately, we did not observe a significant main effect of epigenetic mechanism in any of them, and the interaction between mechanism and parameter setting was significant only in the pH trial $F_{60,100.061} = 2.271$, $p = 0.004$. However, the main effect of genotype, and the interaction between genotype and parameter setting, is significant for each parameter (Table 3). These results suggest that epigenetic mechanisms in general contribute to phenotypic plasticity. Among the genotypes, there were differences in both elevation and shape of the reaction norms. We did not see a general effect of particular epigenetic modification type, e.g., all mutant strains with nonfunctional histone methylation have a characteristic reaction norm. On the contrary, we noticed that the reaction norms were specific to a given mutant strain in a particular environment. We also performed the previous analyses excluding genotypes *nfg-1* and *dim-5* as these two genotypes grew much slower in general than the rest, but this did not change any of our conclusions. For the temperature trial, we also investigated whether the two growth chambers used had any different effects on growth. We included growth chamber identity as a fixed factor in the mixed model for the temperature trial, but this term was not significant $F_{1,593.15} = 0.545$, $P = 0.461$, and the growth chamber term was dropped from the final model. We compared the individual mutants to the control with a pairwise ANOVA. We observed that the genotype × environment interaction was significant for many mutants, and these values are reported in Table S3.

We also observed that there were changes in reaction norm optima among the mutant strains, as calculated from the natural spline fits. Plotting the distribution for the optimal environment of each genotype shows that osmotic stress is the only parameter where the optimum remains constant at 0 M NaCl for all genotypes (Figure 2), except for *dim-5*, where the reaction norm is rather flat (Figure S2). In the other environments, some genotypes have a different environmental optimum than the control (Figure 2).

Having established that there are statistically significant differences among the mutant strains in the different environments, we now present the results according to each epigenetic mechanism, and by parameter, highlighting interesting mutants and their effects. Reaction norms for all the different classes separately can be found in Figure S1, Figure S2, Figure S3, Figure S4, Figure S5, and Figure S6. Figure 3 shows differences of the mutants to the control, for simplicity, in selected environmental settings. These environmental settings were selected because they contain many cases where mutants differ from the control. A summary of the results for each mutant can be found in Table 4.

Effects of DNA methylation

So far, no apparent phenotype other than the lack of methylation has been detected for the DNA methyltransferase mutant *dim-2*. In our experiment, *dim-2* failed to demonstrate any observable phenotypic effects in the pH, salt, or sucrose trials. In the temperature trial, *dim-2* had no effect up to 35°C, but grew slower than the control at 40°C, with a difference of $-0.57$ (−1.14 to −0.02, 95% HPD) mm/hr, and we sought to validate this suggestive difference. In the validation experiment, we observed that *dim-2* grew at the same rate as the control with a difference of 0.01 (−0.17 to 0.19, 95% HPD) mm/hr. Thus, we conclude that lacking a DNA methylation system has no effect at 40°C. In the 30°C sucrose setting, we first observed a nonsignificant tendency of *dim-2* to grow faster than the control (Figure S1), and we confirmed this finding in a validation experiment, where we observed a small but significant effect; with a difference of 0.07 (0.03 to 0.12) mm/hr.

The *dmm-1* and *dmm-2* mutants control the propagation of DNA methylation from heterochromatic regions (Honda *et al.* 2010). We observed that while *dmm-1* had the same optimum sucrose concentration as the control, it grew slower at lower sucrose concentrations (Figure S1); with a difference of $-0.21$ (−0.38 to −0.05) mm/hr at 0.015% sucrose—a reduction of 8%. It also grew slower at higher pH, and the reaction norm had a lower elevation in the salt stress trial. Generally, *dmm-2* had a slightly lower elevation for all reaction norms (Figure S1). In the pH trial, its reaction norm had a different shape compared to the control, and the optimal setting of *dmm-2* was 5.0 compared to 5.8 of the control, and the difference in growth rates at pH 4 was $-0.36$ (−0.61 to −0.11) mm/hr. We validated the growth of *dmm-2* in pH 4 and pH 9; at pH 4 the difference in growth was...
20:43 (20:50 to 20:36) mm/hr, and at pH 9 the difference was 20:61 (20:70 to 20:51) mm/hr, confirming the phenotypic response to different pH for dmm-2. Thus, the dmm mutant strains had different phenotypic responses. The results suggest that (possibly silenced) genes adjacent to heterochromatic regions control the response to several environmental parameters.

Overall, our data suggest that DNA methylation does not play a very important role in phenotypic plasticity, but spurious DNA methylation has the potential to affect phenotypic plasticity.

**Effects of histone methylation**

Histone methylation has multiple functions depending on which residues are methylated (Rothbart and Strahl 2014). For the set-7 mutant, which lacks H3K27me3, we did not observe any growth responses among the environmental parameters and settings we tested (Figure S2). The npf mutant also lacks H3K27me3, but is also impaired in other functions (Jamieson et al. 2013). Generally, npf grew much slower than the control; in the sucrose and pH trials, the differences were seen in reaction norm elevation rather than shape (Figure S2), but, in the temperature and salt stress trials, npf presents a different shape. In particular, npf seems to be sensitive to high temperatures and osmotic stress, as its growth rate collapses at 40°C and in high salt (Figure S2). However, these changes are not related to H3K27me3 as set-7 does not show them.

The set-1 mutant lacks H3K4me3, and shows a lower elevation of its reaction norms and also shape changes (Figure S2). In the sucrose and pH trials, growth of set-1 slows down more than the control when the pH is changed from 5.8 to pH 4.0, a difference of 0.19 (−0.05 to 0.43)

---

**Figure 1** Overview of reaction norms according to each environmental parameter: temperature, osmotic stress, sucrose concentration, and pH (from top left to bottom right). Black reaction norms are the control, and dashed gray lines are the different mutant strains. Nearly all reaction norms are nonlinear. See Figure S1, Figure S2, Figure S3, Figure S4, Figure S5, and Figure S6 for detailed pictures of the different epigenetic mechanisms.
For histone deacetylation, we used two different classes of mutants: observable phenotypic effect, H3K4 trimethylation and H3K36 methylation previously noted (Tamaru and Selker 2001). Thus, H3K9me plays a noticeable role in the pH trial, where growth was drastically reduced at high pH 4.5 for the control: pH 4.0 for the mutant, which encodes an exonuclease involved in siRNA maturation, we saw a phenotype effect only in the 40°C environment, where the difference from the control was −0.08 (−0.28 to 0.12) mm/hr. As H3K4me3 has been implicated in transcriptional activation (Pokholok et al. 2005; Raduwan et al. 2013), some genes may not activate correctly in these environments.

H3K36me is believed to be required for efficient transcriptional elongation (Morris et al. 2005). For the set-2 mutant, which lacks H3K36me, we observed a generally lower elevation for reaction norms (Figure S2), but not to the same extent as set-1. However, set-2 has a markedly different response to temperature, as its optimal setting from the control the difference is only 0.09 (−0.15 to 0.32) mm/hr, although this is only marginally significant. This can also be observed when going from 1.5 to 0.15% sucrose; for the control the difference is only −0.08 (−0.28 to 0.12) mm/hr. As H3K4me3 has been implicated in transcriptional activation (Pokholok et al. 2005; Raduwan et al. 2013), some genes may not activate correctly in these environments.

The final histone modification we investigated was H3K9me; the mutant dim-5 that lacks this modification (Tamaru and Selker 2001; Tamaru et al. 2003) grew very poorly in all environments (Figure S2), as noted previously (Tamaru and Selker 2001). Thus, H3K9me plays a central role in essential cellular processes.

Taken together, our results show that H3K27 trimethylation has no observable phenotypic effect, H3K4 trimethylation and H3K36 methylation have some effects on elevation of reaction norms but also on their shapes, while H3K9 methylation is needed for normal cellular function.

**Effects of histone deacetylation**

For histone deacetylation, we used two different classes of mutants: hda-1, hda-2, and hda-4, and type III (NAD⁺ dependent) histone deacytases nst-1, nst-2, nst-4, nst-6, and nst-7. We observed that, for the hda mutants, reaction norm elevations were reduced (Figure S3), except in the salt stress trial, where hda-1 and hda-2 grew faster than the control (Figure 3). The difference in growth between hda-1 and the control in 0.8 M NaCl was −0.19 (0.07 to 0.31), an increase of 0.29 to 0.34 mm/hr— an increase of 22%. An increase in growth rate compared to the control was also observed in the 30% sucrose environment, a difference of 0.34 (0.21 to 0.46) mm/hr. We confirmed this result in a validation experiment where we observed a difference of 0.33 (0.28 to 0.38) mm/hr. The growth rate of qde-2 also had a tendency to increase in the 0.015% sucrose environment, and although this effect was not significant in our first experiment, we observed a significant increase in the validation experiment; a difference of 0.10 (0.05 to 0.16) mm/hr. Based on natural spline fit, the qde-2 mutant strain also had a lower optimal temperature of 31.9°C (vs. 33.7°C), and we validated this result by measuring backcrossed qde-2 at 30 and 35°C. We observed that the shape of the qde-2 reaction norm did indeed change, and the growth rate of qde-2 increased by 0.29 (0.18 to 0.41) mm/hr compared to an increase of 0.68 (0.57 to 0.70) mm/hr when the temperature increased from 30 to 35°C. This indicates that small RNA molecules that are QDE-2-dependent (Lee et al. 2010) are involved in the response to high temperatures. For the pH trial, elevation of the qde-2 reaction norm was...
generally lower, but, at pH 9, a qde-2 shape change was indicated (Figure S5). We validated the growth difference between the control and qde-2 at pH 9, and found a difference of 20.69 to 20.49 mm/hr.

In summary, for the RNA interference pathway, only qde-2 showed a different reaction norm; other mutant strains did not show any effects.

Effects of histone demethylation and acetylation

The viable histone acetyltransferase mutant ngf-1 grew poorly (Figure S6), indicating the critical role played by this gene in normal cellular function. The other mutant, elp3, presented reaction norms with slightly lower elevations in all trials. We observed that the elp3 mutant grew slower at high temperatures. We also observed that its pH optimum dropped from 5.6 to 4.9.

The two putative histone demethylases (lid2 and aof2) had lower elevation in their reaction norms for all environments (Figure S6). However, the reaction norm shape of lid2 changed as the temperature decreased to an optimum of 32.0°C, but this result could not be validated as there was no significant difference in the change in growth rate between control and lid2 when the temperature increased from 30 to 35°C. In contrast, aof2 had a growth rate that was indistinguishable from the control at 35°C, but its growth rate dropped dramatically when the temperature increased to 40°C (Figure 3 and Figure S6). The difference in growth rate between the control and aof2 in 40°C was 21.85 to 20.66 mm/hr, a drop of 38%. We validated this result and observed that aof2 grew slower than the control in the validation experiment at 40°C as well; with a difference of 0.38 (0.57 to 0.18) mm/hr. However, this growth response was less obvious in the validation experiment, where the change in growth rate was not significantly

Table 3 ANOVA of overall differences in reaction norms separately for each environmental parameter

| Environmental parameter | Fixed effects | Random effects | p-value |
|-------------------------|---------------|----------------|---------|
| Salt Stress             | Df, Df2, F-value, p-value | Df, Df2, F-value, p-value | Df, Df2, F-value, p-value | Df, Df2, F-value, p-value |
| Temperature             | M, E, G, M x E | M, E, G, M x E | M, E, G, M x E | M, E, G, M x E |
| Salt                    | 4, 20.049     | 4, 10,020.24  | 0.236   | 1, 100.13 |
| Temperature             | 5, 10.235     | 4, 100.23    | 0.340   | 1, 100.14 |
| Sucrose                 | 5, 20.348     | 4, 100.21    | 0.355   | 1, 100.16 |
| pH                      | 5, 30.457     | 4, 100.08    | 0.059   | 1, 100.06 |

Fixed effects were tested with F-tests with Satterthwaite approximation for degrees of freedom and random effects were tested with χ²-test. For fixed effects: Df, numerator degrees of freedom; Df2, denominator degrees of freedom. The main effect of genotype and the interaction between genotype and environmental setting is significant for each environmental stress type. This suggests that in general epigenetic mechanisms contribute to phenotypic plasticity.
different from the control. Therefore, we conclude that *aof2* and *lid2* are not important to the temperature-dependent responses of *N. crassa*.

In conclusion, the effects of histone acetylation on reaction norms were mainly on reaction norm elevation, and presented only slight changes to shape, while histone demethylation affected mainly the elevation of reaction norms.

**DISCUSSION**

While epigenetic mechanisms have been shown to be important in certain plastic responses (Slepecky and Starmer 2009; Bossdorf et al. 2010; Herrera et al. 2012; Baerwald et al. 2016), the extent to which they contribute to phenotypic plasticity, or how they maintain homeostasis in organisms facing changing environments has been largely unexplored. By exposing a set of deletion mutants of the filamentous fungus *N. crassa* to a spectrum of controlled environmental parameters, we showed that certain epigenetic modifications have strong effects on plasticity, while others do not. In our experiment, epigenetic modifications affected the sensitivity to environmental change, and, to a lesser extent, growth of the mutant strain. There remains the theoretical possibility that some phenotypic changes could be due to changes in genetic background, as we did not test backcrossed strains of all mutants, but this possibility is remote as the genetic background of the control and the mutants is nearly isogenic as described in Materials and Methods. Modification types did not have a consistent pattern in their effects on phenotype. However, it may be that our classification of epigenetic mechanism was too coarse, and this may be why we did not observe a consistent effect. For instance, histone methylation at different residues are known to have different effects (Greer and Shi 2012), and it is also possible that there is redundancy between different epigenetic mechanisms. Thus, it may be that no general effects exists for a certain type of modification as a group. Instead, phenotypic effects were specific to the epigenetic modification in a given environment.

Epigenetic mechanisms clearly played a role in the phenotypic plasticity of growth according to several environmental variables, corroborating recent suggestions concerning the epigenetic control of phenotypic plasticity (Schlichting and Wund 2014). One of the main findings of this study was that epigenetic modifications were more important for plasticity than average growth rate, *i.e.*, genotypes...
H3K36me is present in the active regions of transcribed genes (Pokholok et al. 2003; Reinberg 2003). H3K36me seems essential for normal cellular function, as H3K9me lacking H3K36me in genome integrity (Lewis et al. 2010a). It also grew more slowly than the wild type in most environments, but especially so at high temperatures. The optimum growth rate of set-2 is at 25°, while the wild type has an optimum at 35°. This indicates that H3K36 methylation is required for the correct expression of genes required at temperatures above 25°. In other organisms, H3K36 methylation has been associated with transcriptional elongation (Morris et al. 2005; Hampsey and Reinberg 2003); H3K36me is present in the active regions of eukaryotic genomes, and its function seems to be to keep the chromatin of actively transcribed genes open (Venkatesh et al. 2012). It may be that genes expressed under certain environmental circumstances need to be kept in open conformation by H3K36me, and the nonfunctional strain clearly had a problem at high temperatures. The gene responsible for H3K4 trimethylation, set-1, is important for the response to pH. Previously, it has been reported that H3K4me3 is needed for correct expression of the circadian clock gene frq in N. crassa (Raduwan et al. 2013). In general, H3K4me3 is associated with the 5’-regions of actively transcribed genes (Pokholok et al. 2005; Ardehali et al. 2011). In N. crassa, set-1 has a growth phenotype, suggesting that H3K4me3 is needed for normal cellular metabolism as well as a specific response to acidic pH. In contrast to set-1 and set-2, the set-7 mutant strain did not present any phenotypic effect. The gene set-7 is responsible for H3K27 trimethylation, and genes marked with H3K27me3 are silent in N. crassa (Jamieson et al. 2013). Genes marked with H3K27me3 tend to be less conserved, suggesting that they are needed only in certain environmental conditions. Therefore, it is surprising to observe that the set-7 mutant strain performed as well as the wild type in our trials. It may be that a lack of repression by H3K27me3 (which allows genes to be expressed) does not prevent a plastic response. The nufg mutant also lacks H3K27me3 (Jamieson et al. 2013); however, it has a very different phenotype from that of set-7, and severe growth defects, which cannot be attributed to lack of H3K27me3. Furthermore, H3K9 methylation seems essential for normal cellular function, as H3K9me lacking dim-5 mutant (Tamaru and Selker 2001; Tamaru et al. 2003) had a severe growth defect in all trials. This phenotype is possibly due to the role of H3K9me in genome integrity (Lewis et al. 2010a).

**Histone modifications**

Our results suggest that histone modifications play an important role in how N. crassa responds to environmental perturbation. Histone modifications H3K36me and H3K4me3 are important in plastic responses to temperature and pH, respectively. Set-2 is responsible for H3K36 methylation, and the strain lacking a functional form of this gene suffered some developmental deficiencies, i.e., female sterility, and production of few conidia (Adhvaryu et al. 2005). It also grew more slowly than the wild type in most environments, but especially so at high temperatures. The optimum growth rate of set-2 is at 25°, while the wild type has an optimum at 35°. This indicates that H3K36 methylation is required for the correct expression of genes required at temperatures above 25°. In other organisms, H3K36 methylation has been associated with transcriptional elongation (Morris et al. 2005; Hampsey and Reinberg 2003); H3K36me is present in the active regions of eukaryotic genomes, and its function seems to be to keep the chromatin of actively transcribed genes open (Venkatesh et al. 2012). It may be that genes expressed under certain environmental circumstances need to be kept in open conformation by H3K36me, and the nonfunctional strain clearly had a problem at high temperatures. The gene responsible for H3K4 trimethylation, set-1, is important for the response to pH. Previously, it has been reported that H3K4me3 is needed for correct expression of the circadian clock gene frq in N. crassa (Raduwan et al. 2013). In general, H3K4me3 is associated with the 5’-regions of actively transcribed genes (Pokholok et al. 2005; Ardehali et al. 2011). In N. crassa, set-1 has a growth phenotype, suggesting that H3K4me3 is needed for normal cellular metabolism as well as a specific response to acidic pH. In contrast to set-1 and set-2, the set-7 mutant strain did not present any phenotypic effect. The gene set-7 is responsible for H3K27 trimethylation, and genes marked with H3K27me3 are silent in N. crassa (Jamieson et al. 2013). Genes marked with H3K27me3 tend to be less conserved, suggesting that they are needed only in certain environmental conditions. Therefore, it is surprising to observe that the set-7 mutant strain performed as well as the wild type in our trials. It may be that a lack of repression by H3K27me3 (which allows genes to be expressed) does not prevent a plastic response. The nufg mutant also lacks H3K27me3 (Jamieson et al. 2013); however, it has a very different phenotype from that of set-7, and severe growth defects, which cannot be attributed to lack of H3K27me3. Furthermore, H3K9 methylation seems essential for normal cellular function, as H3K9me lacking dim-5 mutant (Tamaru and Selker 2001; Tamaru et al. 2003) had a severe growth defect in all trials. This phenotype is possibly due to the role of H3K9me in genome integrity (Lewis et al. 2010a).
DNA methylation
DNA methylation in Neurospora is directed at regions where histone 3 lysine 9 methylation (H3K9me) is present. H3K9me is required for DNA methylation as dim-5 lacks both H3K9me and the ability to perform DNA methylation (Tamaru and Selker 2001; Tamaru et al. 2003). DNA methylation can cause gene silencing in Neurospora as growth defects of dmm mutants were alleviated after the removal of DNA methylation (Honda et al. 2010), and DNA methylation can also cause silencing of antibiotic resistance genes (Lewis et al. 2010b) but is not required for all gene silencing (Honda et al. 2012). However, a complete lack of DNA methylation in the dim-2 mutant was associated with only a slight response in the 30% sucrose environment. This is in stark contrast to land plants and vertebrates, where DNA methylation appears to be indispensable (Li et al. 1992; Rai et al. 2006; Xiao et al. 2006; Yaaś et al. 2015). We observed lower elevation of the reaction norms for dmm-1 and dmm-2 mutants in all environments, and a change in reaction norm shape for dmm-2 in response to pH (i.e., poor growth at pH 4), suggesting that genes required for this response are silenced as DNA methylation spreads from heterochromatic regions in these mutant strains (Honda et al. 2010).

Histone deacetylation
We examined two different classes of histone deacetylase genes: the Class I histone deacetylases, and NAD+ dependent Class III histone deacetylases. Class I includes the hda genes: hda-1, hda-2, and hda-4. Although it has been reported that histone H2B is the main target of HDA-1, it can also deacetylate H3, and is involved in controlling DNA methylation (Smith et al. 2010; Honda et al. 2012). HDA-4 broadly increases acetylation of histones H3 and H4, and no marked effects were reported for the hda-2 mutant (Smith et al. 2010). Therefore, it is striking that the phenotypes of hda-1 and hda-2 are very similar. These knockout strains presented reaction norms with a similar shape to the wild type in all environmental trials other than osmotic stress (where they grew faster), but with a lower elevation, indicating that normal cellular functioning is impaired. Enhanced growth in osmotic stress is surprising, and one interpretation is that there is some cost associated with expressing these genes in this environment. On the other hand, hda-4 was no different from the control, indicating that it is not involved in phenotypic plasticity in the environments tested here. Class III histone deacetylases include the nst genes: nst-1, nst-2, nst-4, nst-6, and nst-7; these are homologous to the Sir2 family of histone deacetylases (Blander and Guarante 2004). In N. crassa, it has been shown that nst-1 and nst-2 are involved in telomeric silencing (4, 6, and 7 were not examined), and that NST-1 deacetylates H4K16 (Smith and Kruglyak 2008). In other eukaryotes, siruin proteins can have targets other than histones (Blander and Guarante 2004), so, for nst-4, nst-6, and nst-7, we cannot be certain of their functions. In trials, nst-1, nst-2, and nst-4 did not present any growth effect in the environments tested. However, the reaction norms of nst-6 and nst-7 had pronounced shape changes in sucrose concentration and pH trials. Moreover, the reaction norms of these two mutants were very similar, suggesting that they may work in a similar way.

RNA interference pathway
RNA interference in Neurospora may not be strictly an epigenetic mechanism. The canonical RNA interference pathway is not required for DNA methylation (Freitag et al. 2004), and it is not known if RNA-directed epigenetic modifications, such as the plant RNA-directed methylation pathway (Matzke and Mosher 2014), exist or if RNA molecules can mediate epigenetic inheritance like in animals (Rassoulzadegan et al. 2006; Rechavi et al. 2011; Ashe et al. 2012) or other fungi (Qutob et al. 2013; Calo et al. 2014). However, a class of small RNAs called disiRNAs may be involved in controlling DNA methylation at specific loci (Dang et al. 2013). Therefore, the possibility of RNA-mediated epigenetic effects exists, so we included appropriate genes in our examination of the system. We observed that the N. crassa ARGONAUTE (Meister 2013) homolog QDE-2 was involved in multiple responses to environmental stress, while the two Dicer protein homologs (DCL-1 and DCL-2), QDE-1, and QIP were not. In N. crassa, there are several pathways that generate different kinds of small RNAs: the biogenesis of siRNAs is dependent on Dicer proteins, QDE-2, and QIP (Mařík et al. 2007); qirRNAs are Dicer- and QDE-1-dependent, and are involved in the DNA damage response (Lee et al. 2009); disiRNAs are Dicer-independent small interfering RNAs that are generated from loci that have overlapping sense and antisense transcripts (Lee et al. 2010; Deng et al. 2013); and microRNA-like RNAs (miRNAs) that can silence genes and are generated by multiple different mechanisms (Lee et al. 2010). The biogenesis of some miRNAs requires QDE-2 (Lee et al. 2010), other miRNAs are Dicer-dependent and QDE-2-independent, and some require QIP while others do not (Lee et al. 2010). While it remains possible that some Dicer-dependent small RNAs are produced by dcl-1 and dcl-2, as these genes are, at least partially, redundant (Catalano et al. 2004), our results suggest that those small RNAs that are QDE-2-dependent are involved primarily in plastic responses to the environment.

Histone demethylation and acetylation
Of the remaining genes, ngf-1 and elp3 are believed to encode histone acetyltransferases based on their similarity to those genes in yeast (Wittschieben et al. 1999; Brenna et al. 2012). In N. crassa, Brenna et al. (2012) showed that NGF-1 is involved in transducing environmental signals, but we found that the ngf-1 mutant grew very slowly in all environments, indicating that key cellular processes are impaired. The elp3 mutant grows slower and its reaction norms have generally lower elevation, but there was no indication of a shape change. Previously, it has been reported that yeast elp3 has a temperature-sensitive phenotype (Wittschieben et al. 1999). Indeed, we observed that differences in growth between elp3 and the wild type were largest at 40°. However, elp3 still has the same temperature optimum (35°) as the wild type, suggesting that rather than a temperature response itself, a more fundamental biological process is impaired in the elp3 mutant strain. The genes aof2 and lid2 are inferred to encode histone demethylases. In fission yeast, the N. crassa AOF2 protein homolog LSD1 acts as a histone demethylase that demethylates H3K4me and H3K9me (Lan et al. 2007). We observed aof2 reaction norms with lower elevation in salt stress, sucrose concentration, and pH trials. This suggests that certain cellular processes are not functioning normally. The yeast homolog of LID2 also acts as a H3K4 demethylase, and interacts with the H3K9 methylation complex (Li et al. 2008). The phenotype of the lid2 mutant is similar to aof2 in that it had lowered reaction norm elevation in all trials, although its reaction norm shape appears similar to wild type.

Conclusions
In terms of the different environmental parameters tested, we observed that epigenetic mechanisms in N. crassa play a much greater role in the response to temperature and pH changes than they do in the response to shifts in sucrose concentration and osmotic stress. This can be explained by the ecology of Neurospora, and considering that it is a saprotrophic fungus found in dead plant matter (Jacobson et al. 2006), or can act as an endophyte under certain conditions (Kuo et al. 2014).
Temperature changes are the most common environmental variable that organisms experience, and pH changes are likely to occur as the fungus encounters different substrates in nature. It may be that *N. crassa* rarely encounters elevated NaCl levels in a terrestrial environment, and has not evolved a plastic response to it. In the sucrose concentration trial, we examined how the level of available nutrients and osmotic stress affect the growth of *N. crassa*, and it would be interesting to investigate how the fungus responds to different types of carbon sources, and whether those responses are under epigenetic control.

Another question that requires investigation is whether the plastic responses we have detected are heritable. It has been observed that maternal or transgenerational effects can be mediated mechanistically by epigenetic changes. In plants, DNA methylation and RNA-directed methylation, in particular, have been implicated in transgenerational inheritance (Luna et al. 2012; Luna and Ton 2012). In fruit flies, histone modifications have also been linked to transgenerational inheritance, where H3K27 and H3K9 methylation regulate offspring lipid content in response to paternal diet (Öst et al. 2014).

Our results show that epigenetic mechanisms are involved in plastic responses of *N. crassa*, and that histone methylation is likely to be the main mechanism, along with small RNAs that are dependent on QDE-2. We suggest that epigenetic mechanisms are likely to be important mediators of plastic responses. Epigenetic mechanisms may also facilitate evolutionary adaptation via phenotypic plasticity, as suggested by models (Lande 2009; Chevin et al. 2010; Draggi and Whitlock 2012) and experiments (Schaum and Collins 2014; Lind et al. 2015). The role of epigenetic mechanism remains unknown until we have determined whether, and how often, plasticity occurs across generations.

**ACKNOWLEDGMENTS**

We acknowledge the Fungal Genetics Stock Center (Manhattan, Kansas) for providing the *Neurospora* strains, and Mr. Juha Ahonen for constructing the race tube racks. We would like to thank Eric Selker, Jouni Laakso, and anonymous reviewers for comments that improved the manuscript. Michael Hardman of Lucidia checked the English. This research is supported by the Academy of Finland (grants no. 274769 to I.K. and no. 278751 to T.K.), and the Centre of Excellence in Biological Interactions of University of Jyväskylä.

**LITERATURE CITED**

Adhvaryu, K. K., S. A. Morris, B. D. Strahl, and E. U. Selker, 2005 Methylation of histone H3 lysine 36 is required for normal development in *Neurospora crassa*. Eukaryot. Cell 4: 1455–1464.

Allshire, R. C., and E. U. Selker, 2009 Fungal models for epigenetic research: *Schizosaccharomyces pombe* and *Neurospora crassa*, in Epigenetics, edited by C. D. Allis T. Jenuwein, D. Reinberg, and M.-L. Catalánotto, C., M. Pallotta, P. ReFalo, M. S. Sachs, L. Vaysse et al., 2014 Antifungal drug resistance evoked via RNAi-dependent epimutations. Nature 513: 555–558.

Catalanotto, C., M. Pallotta, P. ReFalo, M. S. Sachs, L. Vaysse et al., 2014 Redundancy of the two dicer genes in transgene-induced post-transcriptional gene silencing in *Neurospora crassa*. Mol. Cell. Biol. 24: 2536–2545.

Chevin, L.-M., R. Lande, and G. M. Mace, 2010 Adaptation, plasticity, and extinction in a changing environment: towards a predictive theory. PLoS Biol. 8: e1000357.

Colot, H. V., G. Park, G. E. Turner, C. Ringelberg, C. M. Crew et al., 2006 A high-throughput gene knockout procedure for *Neurospora* reveals functions for multiple transcription factors. Proc. Natl. Acad. Sci. USA 103: 10352–10357.

Cortijo, S., R. Wardenaar, M. Colomé-Tatché, A. Gilly, M. Etcheverry et al., 2014 Mapping the epigenetic basis of complex traits. Science 343: 1145–1148.

Dang, Y., L. Li, W. Guo, Z. Yue, and Y. Liu, 2013 Conversion transcript induces dynamic DNA methylation at disiRNA loci. PLoS Genet. 9: 1–10.

Davidson, E. H., 2006 The Regulatory Genome: Gene Regulatory Networks in Development and Evolution. Academic Press, New York.

Draggi, R. H., and F. J. de Serres, 1970 Genetic and microbiological research techniques for *Neurospora crassa*. Methods Enzymol. 17: 79–143.

Day, T., and R. Bonduriansky, 2011 A unified approach to evolutionary consequences of genetic and nongenetic inheritance. Am. Nat. 178: E18–E36.

de la Paz Sanchez, M., P. Aceves-García, E. Petrone, S. Steckenborn, R. Vega-Léon et al., 2015 The impact of Polycumb group (PcG) and Trithorax group (TrxG) epigenetic factors in plant plasticity. New PhytoL 208: 684–694.

DeWitt, T. J., and S. M. Scheiner, 2004 Phenotypic variation from single genotypes: a primer, in Phenotypic Plasticity: Functional and Conceptual Approaches, edited by T. J. DeWitt and S. M. Scheiner, Oxford University Press, Inc., New York.

Draghi, J. A., and M. C. Whitlock, 2012 Phenotypic plasticity facilitates mutational variance, genetic variance and evolvability along the major axis of environmental variation. Evolution 66: 2891–2902.

Freitag, M., D. W. Lee, G. O. Kothe, R. J. Pratt, R. Aramayo et al., 2004 DNA methylation is independent of RNA interference in *Neurospora*. Science 304: 1939.

Gelman, A., 2006 Prior distributions for variance parameters in hierarchical models. Bayesian Anal. 1: 515–533.

Gelman, A., J. Hill, and M. Yajima, 2012 Why we (usually) don’t have to worry about multiple comparisons. J. Res. Educ. Eff. 5: 189–211.

Gibson, G., 2008 The environmental contribution to gene expression profiles. Nat. Rev. Genet. 9: 575–581.

Greer, E. L., and Y. Shi, 2012 Histone methylation: a dynamic mark in health, disease and inheritance. Nat. Rev. Genet. 13: 343–357.

Hampsey, M., and D. Reinberg, 2003 Tails of intrigue: phosphorylation of RNA polymerase II mediates histone methylation. Cell 113: 429–440.

Henderson, S. T., G. A. Eariss, and D. E. A. Catcheside, 2005 Reliable PCR amplification from *Neurospora crassa* genomic DNA obtained from conidia. Fungal Genet. NewsL 52: 24.

Herrera, C. M., M. I. Pozo, and P. Bagaza, 2012 Jack of all nectars, master of most: DNA methylation and the epigenetic basis of niche width in a flower living yeast. Mol. Ecol. 21: 2602–2616.

Holm, S., 1979 A simple sequentially rejective multiple test procedure. Scand. J. Stat. 6: 65–70.
Honda, S., Z. A. Lewis, M. Huarte, L. Y. Cho, L. D. David et al., 2010 The DMM complex prevents spreading of DNA methylation from transposons to nearby genes in *Neurospora crassa*. Genes Dev. 24: 443–454.

Honda, S., Z. A. Lewis, K. Shimada, W. Fischle, R. Sack et al., 2012 Heterochromatin protein 1 forms distinct complexes to direct histone deacetylation and DNA methylation. Nat. Struct. Mol. Biol. 19: 471–477.

Jacobson, D. J., J. R. Dettman, R. I. Adams, C. Boesl, S. Sultana et al., 2006 New findings of *Neurospora* in Europe and comparisons of diversity in temperate climates on continental scales. Mycologia 98: 550–559.

Jamieson, K. M. R. Rountree, Z. A. Lewis, J. E. Stajich, and E. U. Selker, 2013 Regional control of histone H3 lysine 27 methylation in *Neurospora*. Proc. Natl. Acad. Sci. USA 110: 6027–6032.

Klironomos, F., J. Berg, and S. Collins, 2013 How epigenetic mutations can affect genetic evolution: model and mechanism. BioEssays 35: 571–578.

Kooke, R., F. Johannes, R. Wardenaar, F. Becker, M. Etcheverry et al., 2015 Epigenetic basis of morphological variation and phenotypic plasticity in *Arabidopsis thaliana*. Plant Cell 27: 337–348.

Kronholm, L., and S. Collins, 2016 Epigenetic mutations can both help and hinder adaptive evolution. Mol. Ecol. 25: 1856–1868.

Kruschke, J. K., 2011 *Doing Bayesian Data Analysis with R and BUGS*. Academic Press, New York.

Kuo, H.-C., S. Hui, Y. Choi, F. O. Asiegbu, J. P. T. Volkonen et al., 2014 Secret lifestyles of *Neurospora crassa*. Sci. Rep. 4: 5135.

Kuznetsova, A., P. B. Brockhoff, and R. H. B. Christensen, 2015 *lmerTest: Tests in Linear Mixed Effects Models*. R package version 2.0-9. https://cran.r-project.org/web/packages/lmerTest/. (Accessed: October 12, 2016).

Lee, H.-C., and J. Khaspeyrov, 2010 *Plasticity, memory and the adaptive landscape of the genome*. Proc. Biol. Sci. 265: 1319–1323.

Perkins, D. D., 2004 Wild type *Neurospora* strains preferred to use as standards. Fungal Genet. News. 51: 7–8.

Plummer, M., 2003 *JAGS: a program for analysis of Bayesian graphical models using Gibbs sampling*. Proceedings of the 3rd International Workshop on Distributed Statistical Computing. https://www.r-project.org/conferences/DSC-2003/Proceedings/Plummer.pdf.

Plummer, M., 2014 *rjags: Bayesian graphical models using MCMC*. R package version 3-14. https://cran.r-project.org/web/packages/rjags/rjags.pdf. (Accessed: October 12, 2016).

Qutob, D., B. Patrick Chapman, and M. Gijzen, 2013 *Transgenerational gene silencing causes gain of virulence in a plant pathogen*. Nat. Commun. 4: 1349.

R Core Team, 2013 *R: A Language and Environment for Statistical Computing*. http://www.R-project.org.

Rothbart, S. B., and B. D. Strahl, 2014 Interpreting the language of histone modifications. Biochim. Biophys. Acta 1839: 627–643.

Rybak, F. J., G. W. Beadle, and E. L. Tatum, 1943 The tube method of measuring the growth rate of *Neurospora*. Am. J. Bot. 30: 784–799.

Schaum, C. E., and S. Collins, 2014 Plasticity predicts evolution in a marine alga. Proc. Biol. Sci. 281: 20141486.

McCluskey, K., A. West, and M. Plamann, 2010 The fungal genetics stock center: a repository for 50 years of fungal genetics research. J. Biosci. 35: 119–126.

Meister, G., 2013 Argonaute proteins: functional insights and emerging roles. Nat. Rev. Genet. 14: 447–459.

Metzenberg, R. L., 2003 Vogel’s medium N salts: avoiding the need for ammonium nitrate. Fungal Genet. News. 50: 14.

Morris, S. A., Y. Shibata, K.-i. Noma, Y. Tsukamoto, E. Warren et al., 2005 Histone H3 K36 methylation is associated with transcription elongation in *Schizosaccharomyces pombe*. Eurkaryot. Cell 4: 1446–1454.

Mykyt, O. M., E. G. Barry, and D. R. Galeazzi, 1974 New isogenic wild types in *N. crassa*. Neurospora Newsletter 21: 24.

Newmayer, D. D., D. D. Perkins, and E. G. Barry, 1987 An annotated pedigree of *Neurospora crassa* laboratory wild types, showing probable origin of the nucleolar satellite and showing that certain stocks are not authentic. Fungal Genet. News. 34: 46–52.

Nicotra, A. B., O. K. Atkin, S. P. Bonser, A. M. Davidson, E. F. Finnegnan et al., 2010 Plant phenotypic plasticity in a changing climate. Trends Plant Sci. 15: 684–692.

Öst, A., A. Lempradl, E. Casas, M. Weigt, T. Tiiko et al., 2014 Paternal diet defines offspring chromatin state and intergenerational aging. Cell 159: 1352–1364.

Ou, X., Y. Zhang, C. Xu, X. Lin, Q. Zang et al., 2012 Transgenerational inheritance of modified DNA methylation patterns and enhanced tolerance induced by heavy metal stress in rice (*Oryza sativa* L.). PLoS One 7: e41143.

Pál, C., 1998 Plasticity, memory and the adaptive landscape of the genome. Proc. Biol. Sci. 265: 1319–1323.

Perkins, D. D., 2004 Wild type *Neurospora* strains preferred to use as standards. Fungal Genet. News. 51: 7–8.

Plummer, M., 2003 *JAGS: a program for analysis of Bayesian graphical models using Gibbs sampling*. Proceedings of the 3rd International Workshop on Distributed Statistical Computing. https://www.r-project.org/conferences/DSC-2003/Proceedings/Plummer.pdf.

Plummer, M., 2014 *rjags: Bayesian graphical models using MCMC*. R package version 3-14. https://cran.r-project.org/web/packages/rjags/rjags.pdf. (Accessed: October 12, 2016).

Pisk, C., 1998 Plasticity, memory and the adaptive landscape of the genome. Proc. Biol. Sci. 265: 1319–1323.

Perkins, D. D., 2004 Wild type *Neurospora* strains preferred to use as standards. Fungal Genet. News. 51: 7–8.

Plummer, M., 2003 *JAGS: a program for analysis of Bayesian graphical models using Gibbs sampling*. Proceedings of the 3rd International Workshop on Distributed Statistical Computing. https://www.r-project.org/conferences/DSC-2003/Proceedings/Plummer.pdf.

Plummer, M., 2014 *rjags: Bayesian graphical models using MCMC*. R package version 3-14. https://cran.r-project.org/web/packages/rjags/rjags.pdf. (Accessed: October 12, 2016).

Qutob, D., B. Patrick Chapman, and M. Gijzen, 2013 *Transgenerational gene silencing causes gain of virulence in a plant pathogen*. Nat. Commun. 4: 1349.

R Core Team, 2013 *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

Ragunathan, H. L. Isola, and W. J. Belden, 2013 Methylation of histone H3 lysine 4 by the lysine methyltransferase SET1 protein is needed for normal clock gene expression. J. Biol. Chem. 288: 8380–8390.

Rai, K., L. D. Nauda, S. Chidester, E. J. Manos, S. R. James et al., 2006 Zebra fish Dnmt1 and Suv39h1 regulate organ-specific terminal differentiation during development. Mol. Cell. Biol. 26: 7077–7085.

Rasmann, S., M. De Vos, C. L. Casteel, D. Tian, R. Halitschke et al., 2012 Herbivory in the previous generation primes plants for enhanced insect resistance. Plant Physiol. 158: 854–863.

Rassoulzadegan, M., V. Grandjean, P. Gouon, S. Vincent, I. Gillot et al., 2006 RNA-mediated non-Mendelian inheritance of an epigenetic change in the mouse. Nature 441: 469–474.

Rechavi, O., G. Minevich, and O. Hober, 2011 Transgenerational inheritance of an acquired small RNA-based antiviral response in *C. elegans*. Cell 147: 1248–1256.

Rothbart, S. B., and B. D. Strahl, 2014 Interpreting the language of histone and DNA modifications. Biochim. Biophys. Acta 1839: 627–643.
Schlichting, C. D., and M. A. Wund. 2014. Phenotypic plasticity and epigenetic marking: an assessment of evidence for genetic accommodation. Evolution 68: 656–672.

Siklenka, K., S. Erkek, M. Godmann, R. Lambrot, S. McGraw et al., 2015. Disruption of histone methylation in developing sperm impairs offspring health transgenerationally. Science 350: aab2006.

Slepecky, R. A., and W. T. Starmer. 2009. Phenotypic plasticity in fungi: a review with observations on Aureobasidium pullulans. Mycologia 101: 823–832.

Smith, E. N., and L. Kruglyak. 2008. Gene-environment interaction in yeast gene expression. PLoS Biol. 6: e83.

Smith, K. M., J. R. Dobovy, J. E. Reifsnyder, M. R. Rountree, D. C. Anderson et al., 2010. H2B- and H3-specific histone deacetylases are required for DNA methylation in Neurospora crassa. Genetics 186: 1207–1216.

Tamaru, H., and E. U. Selker. 2001. A histone H3 methyltransferase controls DNA methylation in Neurospora crassa. Nature 414: 277–283.

Tamaru, H., X. Zhang, D. McMillen, P. B. Singh, J.-i. Nakayama et al., 2003. Trimethylated lysine 9 of histone H3 is a mark for DNA methylation in Neurospora crassa. Nat. Genet. 34: 75–79.

Venables, W. N., and B. D. Ripley. 2002. Modern Applied Statistics with S. Ed. 4. Springer, New York.

Venkatesh, S., M. Smolle, H. Li, M. M. Gogol, M. Saint et al., 2012. Set2 methylation of histone H3 lysine 36 suppresses histone exchange on transcribed genes. Nature 489: 452–455.

Verhoeven, K. J., and T. P. van Gorp. 2012. Transgenerational effects of stress exposure on offspring phenotypes in apomictic dandelion. PLoS One 7: e38605.

Verhoeven, K. J. F., J. J. Jansen, P. J. van Dijk, and A. Biere. 2010. Stress-induced DNA methylation changes and their heritability in asexual dandelions. New Phytol. 185: 1108–1118.

White, B., and D. Woodward. 1995. A simple method for making disposable race tubes. Fungal Genet. News. 42: 79.

Wittschieben, B. O., G. Otero, T. de Bizemont, J. Fellows, H. Erdjument-Bromage et al., 1999. A novel histone acetyltransferase is an integral subunit of elongating RNA polymerase II holoenzyme. Mol. Cell 4: 123–128.

Xiao, W., K. D. Custard, R. C. Brown, B. E. Lemmon, J. J. Harada et al., 2006. DNA methylation is critical for Arabidopsis embryogenesis and seed viability. Plant Cell 18: 805–814.

Yaari, R., C. Noy-Malka, G. Wiedemann, N. Auerbach Gershovitz, R. Reski et al., 2015. DNA methyltransferase 1 is involved in (m)CG and (m)CCG DNA methylation and is essential for sporophyte development in Physcomitrella patens. Plant Mol. Biol. 88: 387–400.

Communicating editor: M. S. Sachs