A Panel of Histone H3 Mutations to Investigate Centromere Maintenance and Gene Silencing

by

Madison L. Esposito

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APPROVED:

_____ _____

Michael Freitag, Biochemistry and Biophysics Date

_____ _____

Gary Merrill, Biochemistry and Biophysics Date

_____ _____

Katharine G. Field, BRR Director Date

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ABSTRACT

Modifications of histone tail amino acid residues are necessary for many reactions involving chromatin. These processes can produce transcriptionally active, euchromatic, or inactive, heterochromatic, segments of DNA. The formation and maintenance of facultative heterochromatin is critical for the proper growth and development of many eukaryotes. Euchromatin can be changed into heterochromatin by post-translational modifications of core histone tail residues, for example, addition of methyl or acetyl functional groups to lysines or phosphate groups to serines and threonines. In two fungi, *Neurospora crassa* and *Fusarium graminearum*, the distinction between facultative and constitutive heterochromatin is maintained by methylation of histone H3 on lysine 27 (H3K27) and lysine 9 (H3K9), respectively. The H3 tail residues surrounding K9 (TARK^{9}ST) and K27 (AARK^{27}SA) are identical except for the underlined residues, threonine in the K9 region and alanine in the K27 region. Switching these regions may alter activity of the two methylating enzymes, KMT1 and KMT6, respectively. A large set of histone alleles with point mutations was created by an improved “Quick Change” method and standard cloning, and integrated into wildtype or mutant *F. graminearum* and *N. crassa* strains by targeted gene replacement of the normal *hH3* gene. Verified mutants were screened for phenotypic changes and aberrant localization of the gene silencing machinery.

INTRODUCTION

The nucleus of a typical eukaryotic cell contains all information needed for cellular growth, development, propagation, and response to stimuli, encoded into DNA. Genes are the units of DNA identified as containing coding sequences that, when expressed, can produce proteins. Most genes are uniform in DNA sequence across all cells of an individual eukaryotic organism. However, their expression varies between tissues in complex multicellular organic lifeforms. When mutations occur in the genome, there is a chance that the normal pattern of expression and silencing of genes could be altered leading to sporadic diseases, such as heart disease and cancer. To avoid these diseases and ensure proper growth and development, normal maintenance of genetic expression and silencing is mandatory.

Eukaryotic cells control their gene expression across time and space through the maintenance of, or changes in, chromatin structure. Chromatin refers to complexes of DNA and proteins found within the nucleus. The DNA in chromatin is highly organized. The fundamental level of organization is that of nucleosomes. In nucleosomes, ~147 base pair (bp) lengths of DNA are associated with a positively charged octamer core comprised of a specialized group of proteins called histones [1]. Histone proteins are highly conserved throughout eukaryotic species, but do have slight variations [2,3]. Previous studies have shown that mutation, or in some cases deletion, of histone residues does not produce sweeping deleterious effects, meaning in depth studies can be conducted effectively on the structure and function of histones [4–8].

Histone tails are modified to encourage the compaction or relaxation of chromatin, forming heterochromatin or euchromatin, respectively [9,10]. Euchromatin

allows the transcription machinery to easily access DNA associated with the histones of a nucleosome. The subsequent messenger RNA (mRNA) to be translated into protein, or non-coding RNA to be used for other purposes, can then be quickly transcribed from the exposed DNA. Densely packed heterochromatin provides little space for the association of transcription factors with DNA, and so the genes sequestered in this formation of chromatin are not transcribed, and later translated into proteins, making them effectively silent [9,10].

The transition of euchromatin to heterochromatin, and vice-versa, occurs systematically. Typically, the first step of the process is the post-translational modification (PTM) of a specific histone tail amino acid residue, although DNA methylation also plays a role in some scenarios [11,12]. After the modification has been made a protein, or protein complex, can identify the modification and bind the histone. The bound protein then elicits a cellular response and ultimately produces a structural change in chromatin.

PTMs of histone tails are used to facilitate many methods of chromatin modification necessary to control chromatin and centromere maintenance within eukaryotic genomes. Commonly observed PTMs include methylation, phosphorylation, and acetylation [2,13]. These PTMs are highly involved in many fundamental cellular processes [2,13]. An established process of substituting residues of histones can be found in several publications and can be used to discern the importance of modifications of a particular histone amino acid residue [14]. Substitutions can be made that prevent the modification of the histone tail amino acid residues of interest [5–7,14].

For example, in this study, lysines were replaced with neutral glutamine or positively charged arginine to test the effects of these substitutions on histone activity.

The histone octamer consists of two copies of four core histones proteins. The core histones are H3, H2A, H2B, and H4. Methylation of lysine four on the histone H3 tail (H3K4me2/3) acts as an activating mark and encourages the relaxed and transcriptionally active form of chromatin, euchromatin [15,16]. Methylation of histone H3 lysine 27 (H3K27me3) or lysine 9 (H3K9me2/3) act as silencing marks and encourage the dense and transcriptionally inactive form of chromatin, heterochromatin [15,16]. . Previous research showed that the histone H3 tail was a central element in the methylation of DNA, specifically through H3K9me2/3 [14].

The canonical histone H3 isoform, H3.1, is integrated into nucleosomes during replication in many plants and animals [17]. Nucleosomes with a second isoform, H3.3, are inserted into repressed chromatin regions upon changes in transcriptional status [18]. H3.1 and H3.3 are nearly identical, only differing by a few residues, but can have different histone PTMs [19]. Fungi do not have replication-associated H3.1 isoform, thus one aim of this study was to replace the specific residues of the fungal H3.3 with those found in animal and plant H3.1 and record any potential changes in chromatin and silencing characteristics.

Specific amino acids are known to resemble modified forms of other amino acids [20]. These amino acids are referred to as genetic mimics and include arginine, seen as unmodified lysine; glutamine, seen as acetylated lysine; and methionine and leucine, both seen as methylated lysine [20]. The majority of amino acid residues which make up

the histone H3 tail are known to be covalently modified at some point in organismal growth and development (See Figure 1) [2,3,13]. An extensive set of substituted wildtype histones with mutated histones containing a genetic mimic were created in this study. Lysines and arginines that are seen as methylated in wild type (WT) were substituted with the genetic mimics leucine and methionine to simulate methylation. Lysines that are known to be acetylated in WT were substituted with the genetic mimics arginine and glutamine, to simulate hypoacetylated and acetylated states, respectively. Many residues were substituted with alanine to prevent modification or serine and threonine to allow for phosphorylation. Based on previous studies, we expect that these changes will produce genetically aberrant samples that will lack genetic silencing [14].

This study aimed to investigate mutations and epigenetic changes to gain insight to genetic pathologies of human disease. However, creating mutants of human histone genes, of which there are many, would be impractical and expensive. The model organisms *Neurospora crassa* and *Fusarium graminearum,* in contrast, have a manageable number of histone genes [21]. Since histones serve as the proteins responsible for the packaging of DNA into structures of chromatin, a process fundamentally necessary for proper growth and development, they are placed under extreme selection pressure and are four of the most highly conserved biological molecules across eukaryotes [2,3]. Because histone genes are highly conserved, studies within *F. graminearum* and *N. crassa* can be confidently applied to human biology. These two fungal models rely on H3K27me3 and H3K9me2/3 for the formation of facultative and constitutive heterochromatin, respectively [22]. DCDC (DIM-5/-7/-9/Cul4/DDB1 Complex), a complex containing a homologue of Lysine (K)

Methyltransferase-1 (KMT1), is responsible for creating a trimethyl-K (Kme3) on H3K9, leading to the formation of constitutive heterochromatin [22]. Facultative heterochromatin is maintained through methylation of H3K27 by the protein complex, Polycomb Repressive Complex-2 (PRC2). The exact method of targeting PRC2 to chromatin, however, is still unknown [16]. The use of *N. crassa* and *F. graminearum* as models in this project allowed for expedited experimentation and dissemination of results, while maintaining the integrity of resultant findings.

The regions surrounding the targeted lysine in H3K9me2/3 (TARK^{9}ST) and H3K27me3 (AARK^{27}SA), may be important for targeting of PRC2 and DCDC to the specific Lysine, K27 or K9 respectively, to be methylated. One aim of this study was to exchange these underlined residues (T6A, T11A, A24T, and A29T) and record any changes in H3K9 and H3K27 PTMs.

We carried out systematic mutation of the *hH3* gene, specifically targeting sequences related to the histone tail and lysines targeted by proteins that modify chromatin, to reveal amino acid residues critical to centromere or chromatin maintenance and gene silencing. In a 2011 study, the methodical replacement of lysine and other important residues of the *N. crassa* histone H3 tail revealed several residues necessary for DNA methylation and viability [14]. This project extends the previous work by creating amino acid mutants of the entire H3 tail and replacing the wildtype gene with mutated versions in both *N. crassa* and *F. graminearum.*

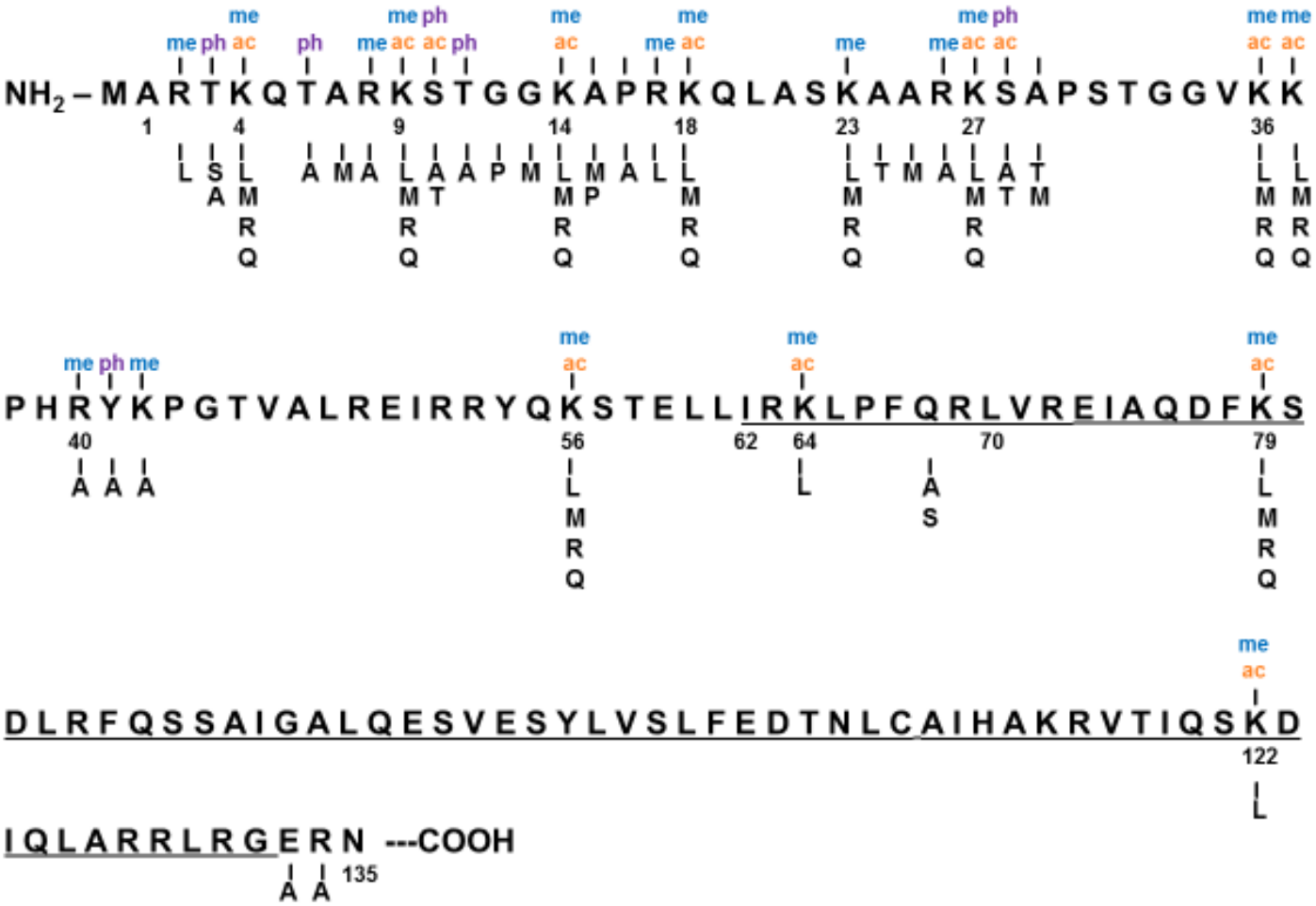

Figure 1. Substitutions for amino acids of interest in *hH3* were chosen based on the known covalent modifications. Sequence of *N. crassa* H3 full protein. Methylation (blue), Phosphorylation (purple), and acetylation (orange) are indicated above each amino acid of interest. The *hH3* alleles bearing indicate mutations are included below the residues in single letter code. The underlined section indicates the globular histone fold domain.

METHODS

Quick Change PCR

Point mutations were synthesized according to an improved “Quick Change” method [23]. For each mutation, staggered primers containing the point mutation of interest (Table S1) were allowed to replicate against wildtype *hH3* gene within plasmids that contained either *N. crassa* (pSF6; provided by Steve Friedman) or *F. graminearum* (pRG9; provided by Rodrigo Gonçalves) flanking regions (Figure 2). These flanking regions are critical for the later integration of the mutated histone gene into its respective fungal host. The primers were designed to contain the point mutation at the 3’ end of the primer to allow for substantial annealing. PCR product was digested with *Dpn*I to remove parental methylated and hemi-methylated DNA. All enzymes used in this and subsequent protocols were obtained from New England Biolabs (NEB Ipswich, Massachusetts).

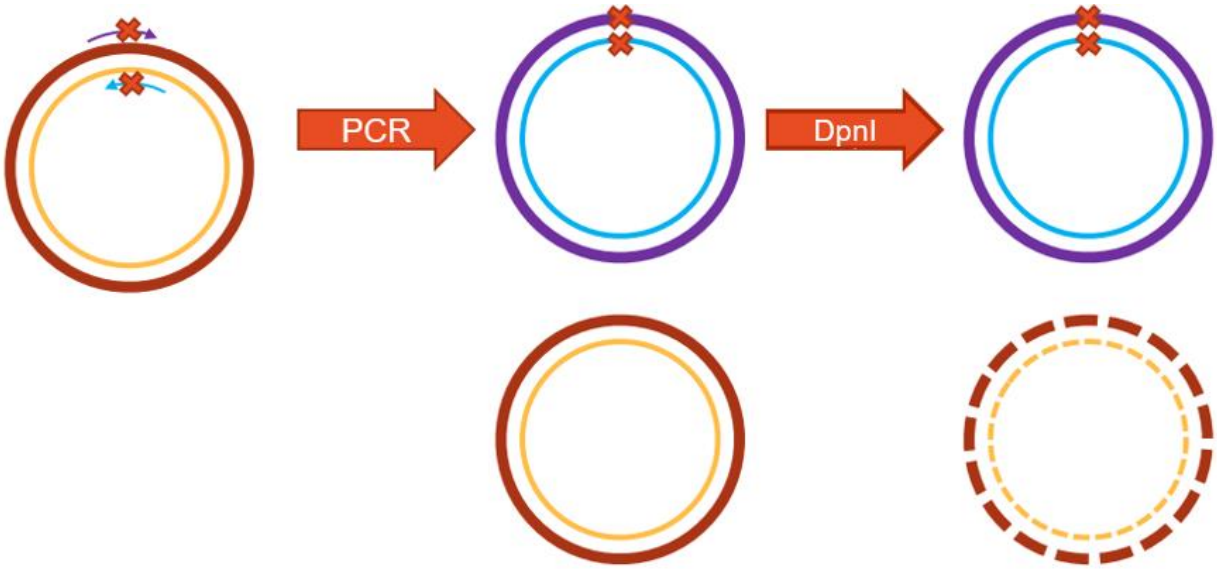

Figure 2. Histone alleles with point mutations were created by an improved “Quick Change PCR” method using staggered primer schemes. Staggered primers (blue and purple arrows) containing the point mutation of interest (red “x”) were allowed to replicate against the parental plasmid (red and orange). Quick Change plasmids progressed to Sanger sequencing for conformation before transformation. Parental plasmids were digested using *Dpn*I.

Both pRG9 and pSF6 contain a selectable gene marker, *hph*, coding for hygromycin phosphotransferase, which confers resistance to the antibiotic Hygromycin B [24]. Prior to integration of engineered plasmids containing mutant histone H3 into the fungal genomes, the *hH3* allele on each plasmid was confirmed by Sanger sequencing.

Standard Cloning

hH3 gene point mutants carried on smaller plasmids (pEB series) were located and used as a source of fragments for standard cloning by digestion with restriction endonuclease, isolation and ligation of fragments, and transformation into *Escherichia coli*. pEB was not suitable for integration into *F. graminearum,* and so mutated *hH3* fragments from pEB plasmids were isolated and ligated into the pRG9 backbone. Enzymes *Age*I and *Blp*I were used to isolate *hH3* gene fragments of interest and pRG9 plasmid backbone. The gene fragments and plasmid backbone were run out on a 0.8% agarose gel and isolated by Qiagen Gel Extraction kit, following the manufacturer's protocol (Qiagen Germantown, Maryland). Gene fragments and pRG9 backbones were ligated using T4 DNA ligase. Successfully ligated plasmids were used in *F. graminearum* transformations.

E. coli Transformation

Plasmids created by Quick Change PCR were used to transform *E. coli* by standard protocols [25]. Chemically competent *E. coli* cells generated by following a previously published protocol were used for the transformation [26].

Media and Conditions for *F. graminearum* Growth

Fusarium graminearum strains were grown in liquid yeast extract-peptone-dextrose (YPD) to generate vegetative tissue, and carboxymethylcellulose (CMC) was used to generate asexual macroconidia [16]. Both fungal vegetative tissue and macroconidia were grown, collected, and stored as described previously [16].

Generation of “split marker” genes

The 5’ and 3’ flanks for pRG9 plasmids containing confirmed mutant *hH3* were amplified from genomic DNA. 5’ flanks were amplified from each confirmed plasmid with primer OMF 4691 (5’-CGCGGTGGCGGCCGCTCTAGAATACTGAGCATGTACTACC-3’) and *hph* split marker primer OMF 4069 (5’-AGAAGATGTTGGCGACCTCG-3’). 3’ fragments were amplified from unmodified pRG9 using *hph* split marker primer OMF 4068 (5’-GTGCTTTCAGCTTCGATGTAGG-3’) and primer OMF 4391 (5’-CTTGGAGGTTTACGGTGTCG-3’). PCR-amplified fragments were gel-purified using a Qiaquick gel purification kit (Qiagen). The approach has been previously described [23].

Protoplasting and Transforming *F. graminearum* wildtype strain PH-1

PH-1 protoplasts were generated from mycelia. Split marker fragments synthesized from pRG9 plasmids with confirmed *hH3* mutations were used to transform protoplasts as described elsewhere [16]. Resistant transformant colonies were picked and strains were screened for targeted gene replacements or fusions by PCR and Southern blot analyses [16].

F. graminearum Crosses to Produced Double Recombinants

Fusarium graminearum is a self-fertile species; the two mating type idiomorphs are localized immediately next to each other on Chromosome 2. Self-crosses of transformants were set on carrot agar to determine if transformed strains would produce structures associated with fertility, as well as ascospores; procedures have been described [16]. Out-crosses were performed between histone mutant strains and FMF 651 (*eed*$^{+}$*gfp*$^{+}$), which lacks one of the mating type idiomorphs and thus requires a partner to generate fertile crosses. Crosses were performed on carrot agar at room temperature (RT, about 25° C), taking usually ~10 days.

Conidia of each cross were grown in 50 ml CMC shaking at 200rpm at RT for four days. Conidia were harvested by filtration through cheesecloth, spun down to a pellet, and quantified using a hemocytometer. 200, 300, and 600 conidia were spread on separate YPD + Hygromycin (Hyg) agar plates to isolate single spores that retained a resistance to Hyg. Single spores were transferred to single wells within a 48-well plate; 24 single spores were picked from each cross.

N. crassa Transformation

Plasmids containing *N. crassa* flanks were linearized using *Kpn*I and integrated into xSF9.1 (*fghH3::bar; mus51*::*bar; his-3 mata*) using standard electroporation [27]. Transformants were inoculated on FGS plates supplemented with 1X Hygromycin (Hyg) and left to grow for 7 days. Hyg^{+} colonies were transferred to baby slants. Spores from

baby slants were inoculated in tubes with 3 mL of Vogel's minimal medium + 2%sucrose + Hyg and histidine to produce tissue for genomic DNA isolation.

N. crassa Crosses to Produce Double Recombinants

Spore samples were taken from successful transformants and crossed with NMF426 (*NcCenH3*::*mCherry-NcCenH3-hph; matA*). Crosses were performed on solid synthetic crossing (SC) medium at RT, taking usually ~14 days.

PCR Confirmation of Targeting

Genomic DNA was isolated from transformants according to a previously published method [28]. The 5' end of *hH3* was amplified from the isolated genomic DNA with primers OMF 4404 (5'-GCGCTAGAGCCACCCGCACCTGG-3') and OMF 4693 (5'-CTGGGTAGCGACGCGTTAGC-3').

Southern Analyses

Genomic DNA was isolated according to a previously published method [28]. *F. graminearum* DNA was digested with *Hin*dIII or *Sac*I. *N. crassa* DNA was digested with *Sac*I. The blotting process is described elsewhere [29].

Screening for Phenotypic Differences in *F. graminearum*

Transformant strains verified by Southern blot analysis or PCR and successfully crossed were compared to PH-1 to observe any differences in appearance. Samples which were slow growing, bright orange, and wrinkled were identified as exhibiting the

“sick” phenotype, similar to *F. graminearum* Δ*kmt6* [16]. Samples which were fast growing, white, and fluffy were identified as retaining a wild type phenotype. The difference of growth rate was measured for samples showing the sick phenotype. Conidia of targeted strains were examined using a fluorescent microscope to identify the presence of GFP, confirming double recombination, and localization of GFP in the nucleus.

RESULTS

Transformant Strains were verified by Southern Blotting, PCR, and Microscopy

Not all transformations yielded colonies (See Tables S2 and S3). The *F. graminearum* histone transformations for residues R2L, K4L, A7M, K9M, K9Q, K14L, K18M, K23L, K36R, K37L, K37M, K37Q, R40A, Y41A, K42A, K56M, K56R, and K122L did not produce any transformants. This was most likely due to low DNA concentrations in the transformations. The transformations to create *F. graminearum* histone mutants K14M, K18L, A24T, A25M, and K64L were successful, and *N. crassa* histone mutants K18L and K23L were also created in this study. The resulting colonies were picked, selected on Hyg-enriched YPD agar, and screened for targeted gene insertion.

Figure 3 includes the Southern blots for *N.crassa* histone mutants. When the *hph* gene was used as the probe, WT was expected to show no bound probe (empty lane) and mutant strains were expected to have a fragment at 2.2 kb when digested with *Sac*I. Southern blots for *F.graminearum* histone mutants are shown in Figure 3**.** When probed with an *hph* probe*,* strains digested with *Sac*I were expected to have a fragment at 2.26 kb and strains digested with *Hin*dIII were expected to have a fragment at 6.9 kb. WT was expected to show no bound probe when using the *hph* probe.

PCR results of the 5’ flank of *hH3* are shown in Figure 3. Mutations with correct targeting were expected to show bands at 1.2 kb, WT samples were expected to have no product (empty lane).

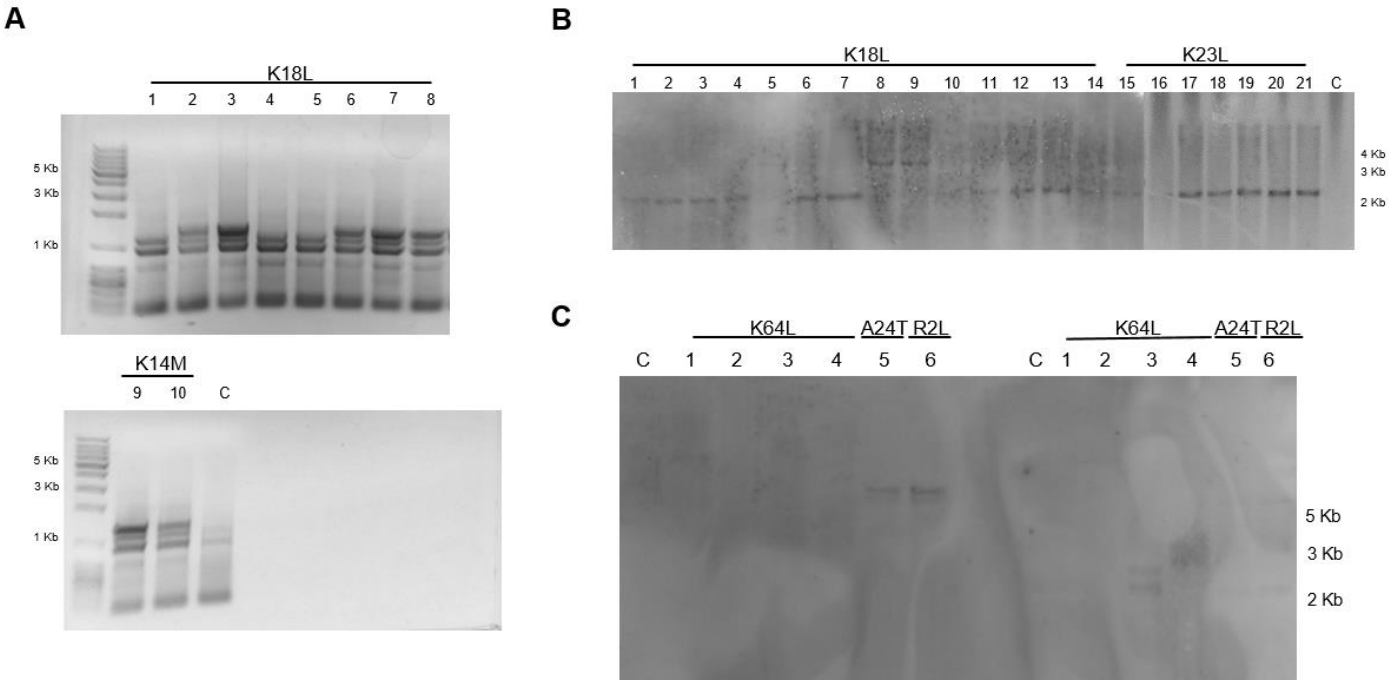

Figure 3 Localization of mutant hH3 alleles at the endogenous *hH3* locus in *Neurospora* and *Fusarium* was verified by Southern blots and PCR. (A) PCR amplification of the *hH3* 5' flank in *F. graminearum* mutants. Bands at 1.2 kb suggest proper targeting and integration of K18L and K14M histone alleles. C is the wildtype control, PH1. Numbered lanes are different transformant strains being verified. We observed numerous background bands that also appeared in the wildtype control. (B) Southern blot results for K18L and K23L histone mutants in *N. crassa* when probing with the *hph* gene. C is the wildtype control, NMF39. Numbered lanes indicate different transformants. (C) Southern blot results for K64L, A24T, and R2L histone mutants in *F. graminearum* when probing with *hph.* Samples were digested with *Hin*dIII (left) and *Sac*I (right). C is the wildtype control, PH1. Numbered lanes indicate different transformants.

To create double recombinants, targeted *F. graminearum* samples were crossed with FMF 651 and targeted *N. crassa* samples were crossed with NMF 426. Resulting progeny were screened by epifluorescence microscopy to confirm presence and localization of EED-GFP. EED is a unit of the PRC2 body and was chosen to represent the localization of the PRC2 body as a whole. The H3K14M mutant strain produced 4/5 *gfp*$^{+}$ progeny, all of which were localized in the same pattern as WT (See Figure 4).

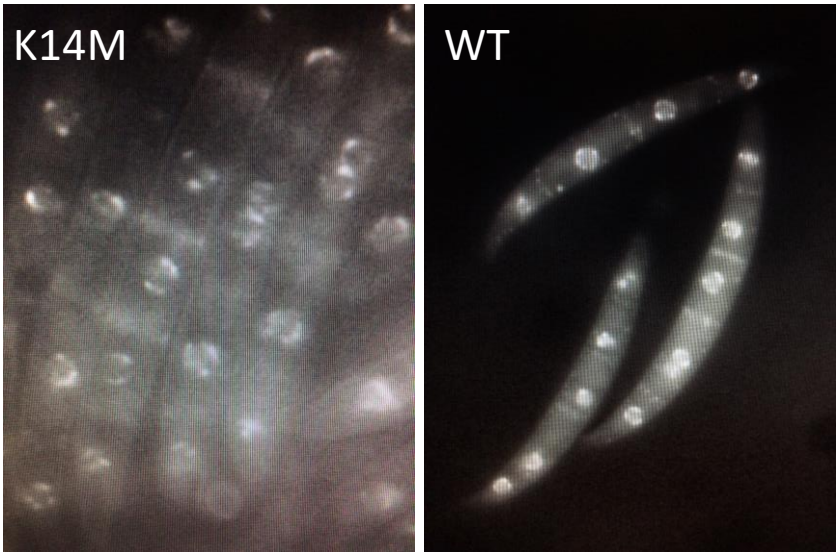

Figure 4 H3K14M mutant expresses *eed-gfp*$^{+}$, and localizes EED-GFP similar to wildtype. (Left) conidia of H3K14M *F. graminearum* mutant showing wildtype (right) localization of the PRC2 component EED-GFP.

Histone alleles can be created by Quick Change PCR

Not all Quick Change PCR reactions yielded accurate products. Of 95 planned mutations, 43 point mutant *hH3* genes were successfully created by Quick Change PCR, as confirmed by Sanger sequencing (See Tables S2 and S3). The creation of 52 other point mutant *hH3* genes by Quick Change PCR was attempted, but returned Sanger sequencing identical to WT *hH3* (See Tables S2 and S3). Two attempts were made to produce histone H3 alleles T3A, K4R, K4Q, K23M, K37Q, K42A, K56Q, and K79R**.** Both attempts failed to produce proper plasmids with the histone allele of interest (See Tables S2 and S3). Attempts at creating point mutant plasmids for transformation through standard cloning yielded no appropriate plasmid product for *F. graminearum* transformation.

hH3 Mutants Show Developmental Defects

Two *F. graminearum* lysine mutants in H3 were generated during this study. The H3K14M mutant exhibited a “sick” phenotype. The H3K18L mutant exhibited wildtype phenotypes after initial transformation, but expressed a “sick” phenotype after crossing with FMF651 (*eed*+*gfp*). Self-crosses revealed that all generated histone mutants were self-sterile. Linear growth was measured in a progeny containing the H3K14M allele and is shown in Figure 5**.** The H3K14M mutant exhibited slower growth when compared to FMF 278 (Δ*kmt6)*, FMF651, and WT. The H3K14M mutant resembled FMF 278 in color and morphology.

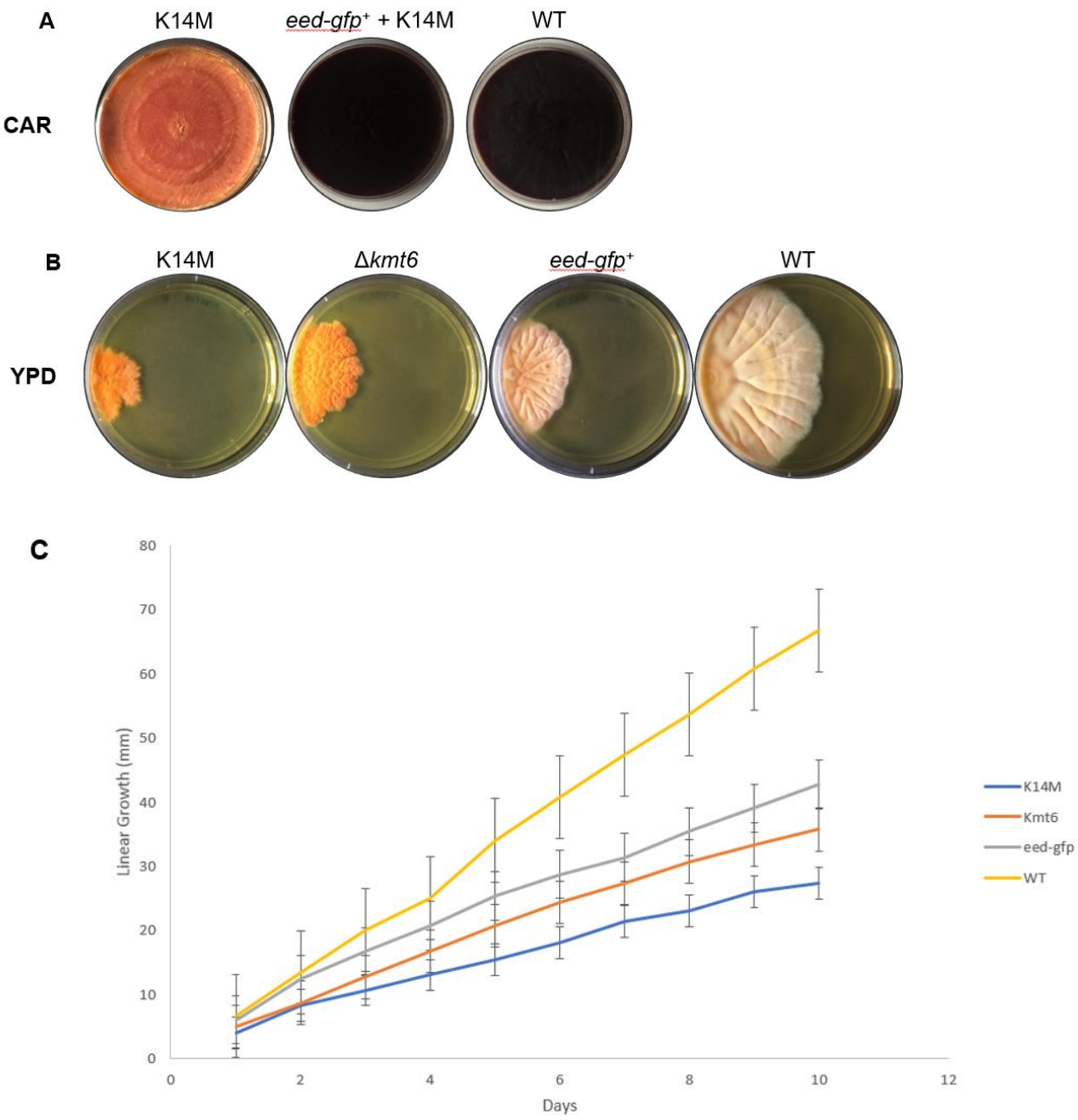

Figure 5. Growth phenotype and linear growth assay of H3K14M shows similarities to the *Δkmt6* strain. (A) Self-crosses of K14M (left) showed that the strain is self-sterile. H3K14M crossed with a *F. graminearum* strain containing *eed-gfp*$^{+}$(middle) showed normal production of fruiting bodies and spores when compared to wildtype (right). (B) Linear growth assay of H3K14M showed that the mutant had a similar phenotype to the *Δkmt6* strain. (C) Plot of linear growth assay showed that H3K14M mutant was slower growing than *Δkmt6* strain. However, the difference is not statistically significant (p>0.05). The H3K14M mutant was significantly slower growing than WT (p=0.009).

DISCUSSION

Overall, 57% of all plasmids produced by Quick Change PCR were found to contain the desired *hH3* allele. Almost half (43%) of the Quick Change plasmids were found to contain WT *hH3*, and 8% of these plasmids were found to contain WT *hH3* even after multiple, separate attempts to produce the allele of interest. This is likely due to error in primer sequences, execution of the experiments, sequencing, low concentration of plasmid DNA for sequencing, or poor condition of chemically competent *E. coli* used for transformation.

Transformants 5, 8 and 9 shown in the Southern blots in Figure 3 had 4 kb bands, varying from the expected bands of 2.2 kb for a successfully integrated histone allele. This is likely due to either incomplete restriction enzyme digestion, poor quality of genomic DNA used, or modification of a restriction enzyme cut site. Southern blots using different gene specific probes will return results of higher resolution concerning the integration of the histone alleles.

Fifteen transformations produced no transformants. It is possible that these transformations failed because the integration of the specific histone allele produced a lethal phenotype. However, it is more likely that the transformations failed because of low DNA concentrations used in the transformation process. This study will continue after the submission of this thesis and these transformations will be attempted again with higher concentrations of plasmid DNA.

Transformants found to contain the histone allele of interest were used to examine phenotypic difference. Most strains resembled the Δ*kmt6* strain, FMF 278, in

color and form. The H3K14M mutant was slower growing than a Δ*kmt6* strain (Figure 5), although not significantly (p>0.05). H3K14M was, however, significantly slower growing (p=0.009) when compare to the wildtype control. This indicates that these mutants could be deficient in silencing in similar ways to FMF 278. Alternatively, histone proteins serve a multitude of different functions within an organism, hence the consequences of mutating even a single residue can lead to pleiotropic consequences. Further experiments will be conducted to assess changes in PTMs across the genome, and understand if the observed phenotype of these histone mutants is related to a lack of silencing or other pleiotropic effects.

All *F. graminearum* transformants were self-sterile and did not produce sexual structures, perithecia, and failed to form spores (Figure 5.). When crossed with FMF 651, however, the transformants produced viable spores. This is likely due to the pleiotropic nature of histone H3. It is possible that the mutation of *hH3* led to dysfunction in the complicated function of self-fertilization commonly observed in wildtype *F. graminearum* strains. FMF 651, which has no mutations of critical replication or recombination machinery, was able to compensate for the deficiencies in the transformants and successfully produce spores.

Conidia obtained from *gfp*+ progeny showed fluorescent localization patterns similar to that of WT in the H3K14M mutant (Figure 4). This indicates that, although integration of the mutant histone alleles resulted in a sick phenotype, the localization of the PRC2 body was not affected. It is possible that the mutation of K14 and K18 had little effect on the localization of PRC2 because of their location outside of the PRC2 targeted area, but produced a sick phenotype due to pleiotropic effects. The creation

and screening of additional mutants will reveal if localization is unaffected in all of the mutations or varies with the integrated histone allele and its corresponding point mutation.

The continuation of this study will lead to more information on the importance of each residue along the H3 protein in both *F. graminearum* and *N. crassa.* In this portion of the study two histone H3 mutants were successfully created in *F. graminearum.* However, 132 mutants remain to be created, 69 in *F. graminearum* and 63 in *N. crassa.* Mutants produced in the future of this project will not only be screened through microscopy, but will also be subject to western blotting and ChIP-sequencing. These screens may show if localization of H3K27me3 and H3K9me2/3 is affected by their own substitution or the substitution of the residues around them. Differences in PTM of genetic mimic may also be exposed. Based on previous studies it is expected that the mutations created in the future of this project will produce a depth of data showing the many functions and consequences of mutating histone H3.

Ultimately, the creation and screening of these mutants may reveal new functions of the histone H3 protein, the importance of the (TARK^{9}ST) and (AARK^{27}SA) targeting locations, how genetic mimics affect histone activity, and further confirm the differences between H3.3 and H3.1.

SUPPLEMENTAL MATERIAL

Table S1. List of primers used in this study (OMF, oligos M. Freitag laboratory).

OMF#	Name	Sequence (5' to 3')
4691	pSF6_FghH3XbaF	CGCGGTGGCGGCCGCTCTAGAATACTGAGCATGTACTACC
4692	pSF6_FghH3XhoR	GGTACCGGGCCCCCCCTCGAGGGAGGTTTACGGTGTCGAGG
4693	NcH3_SeqR	CTGGGTAGCGACGCGTTAGC
4694	NcH3_SeqF	GCTAACGCGTCGCTACCCAG
4695	R2L_F	GCCCTCACTAAGCAGACCGCC
4696	R2L_R	TAGTGAGGGCCATTGTTGATGATTCT
4697	T3S_F	CCCGCTCTAAGCAGACCGCCC
4698	T3S_R	GCTTAGAGCGGGCCATTGTTGATGATTC
4699	T3A_F	CCCGCGCTAAGCAGACCGCCC
4700	T3A_R	GCTTAGCGCGGGCCATTGTTGATGATTC
4701	K4L_F	GCACTCTCCAGACCGCCCGCAA
4702	K4L_R	TCTGGAGAGTGCGGGCCATTGTTG
4703	K4M_F	GCACTATGCAGACCGCCCGCAA
4704	K4M_R	TCTGCATAGTGCGGGCCATTGTTG
4705	K4R_F	CACTCGCCAGACCGCCCGCAA
4706	K4R_R	TCTGGCGAGTGCGGGCCATTGTTGAT
4707	K4Q_F	GCACTCAGCAGACCGCCCGCAA
4708	K4Q_R	TCTGCTGAGTGCGGGCCATTGTTGA
4709	T6A_F	GCAGGCCGCCCGCAAGTCCACC
4710	T6A_R	GCGGCCTGCTTAGTGCGGGCC
4711	A7M_F	AGACCATGCGCAAGTCCACCGGTGGC
4712	A7M_R	TGCGCATGGTCTGCTTAGTGCGGGC
4713	R8A_F	CGCCGCCAAGTCCACCGGTGGCAAG
4714	R8A_R	ACTTGGCGGCGGTCTGCTTAGTGCGG
4715	K9L_F	CCGCCTCTCCACCGGTGGCAAGGCCC
4716	K9L_R	GTGGAGAGGCGGGCGGTCTGCTTAGTG
4717	K9M_F	CCGCATGTCCACCGGTGGCAAGGCCC
4718	K9M_R	GTGGACATGCGGGCGGTCTGCTTAGTG
4719	K9R_F	CGCCGCTCCACCGGTGGCAAGGCCC
4720	K9R_R	GTGGAGCGGCGGGCGGTCTGCTTAGTG
4721	K9Q_F	CCGCCAGTCCACCGGTGGCAAGGCCC

4722	K9Q_R	GTGGACTGGCGGGCGGTCTGCTTAGTG
4723	S10A_F	GCAAGGCCACCGGTGGCAAGGCCCCC
4724	S10A_R	GGTGGCCTTGCGGGCGGTCTGCTTAG
4725	S10T_F	GCAAGACCACCGGTGGCAAGGCCCCC
4726	S10T_R	GGTGGTCTTGCGGGCGGTCTGCTTAG
4727	T11A_F	AGTCCGCCGGTGGCAAGGCCCCC
4728	T11A_R	ACCGGCGGACTTGCGGGCGGTCTGC
4729	G12P_F	CACCCCTGGCAAGGCCCCCGTAAGC
4730	G12P_R	GCCAGGGGTGGACTTGCGGGCGG
4731	K14L_F	TGGCCTCGCCCCCCGTAAGCAGCTC
4732	K14L_R	GGCGAGGCCACCGGTGGACTTGCG
4733	K14M_F	TGGCATGGCCCCCCGTAAGCAGCTC
4734	K14M_R	GGCCATGCCACCGGTGGACTTGCG
4735	K18M_F	CCGTATGCAGCTCGCTTCCAAGG
4736	K18M_R	GCTGCATACGGGGGGCCTTGCCA
4737	K18L_F	CCGTCTCCAGCTCGCTTCCAAGG
4738	K18L_R	GCTGGAGACGGGGGGCCTTGCCA
4739	K23M_F	CTTCCATGGCTGCCCGCAAGTCCG
4740	K23M_R	CAGCCATGGAAGCGAGCTGCTTACG
4741	K23L_F	CTTCCCTCGCTGCCCGCAAGTCCG
4742	K23L_R	CAGCGAGGGAAGCGAGCTGCTTACG
4743	A24T_F	CCAAGACTGCCCGCAAGTCCGCC
4744	A24T_R	GGCAGTCTTGGAAGCGAGCTGC
4745	A25M_F	AGGCTATGCGCAAGTCCGCCCCCT
4746	A25M_R	GCGCATAGCCTTGGAAGCGAGCTG
4747	R26A_F	CTGCCGCCAAGTCCGCCCCCTCC
4748	R26A_R	ACTTGGCGGCAGCCTTGGAAGCGAGC
4749	S28T_F	CAAGACCGCCCCCTCCACCGGCG
4750	S28T_R	GGGCGGTCTTGCGGGCAGCCTTGG
4751	A29M_F	GTCCATGCCCTCCACCGGCGGTG
4752	A29M_R	GGGCATGGACTTGCGGGCAGCCTTG
4753	A29T_F	GTCCACCCCCTCCACCGGCGGTG
4754	A29T_R	GGGGGTGGACTTGCGGGCAGCCTTG
4755	K36R_F	TGTCCGCAAGCCCCACCGTTACAAG
4756	K36R_R	GCTTGCGGACACCGCCGGTGGAGG

4757	K36M_F	TGTCATGAAGCCCCACCGTTACAAG
4758	K36M_R	GCTTCATGACACCGCCGGTGGAGG
4759	K36Q_F	TGTCCAGAAGCCCCACCGTTACAAG
4760	K36Q_R	GCTTCTGGACACCGCCGGTGGAGG
4761	K37L_F	TCAAGCTCCCCCACCGTTACAAGCC
4762	K37L_R	GGGGAGCTTGACACCGCCGGTGG
4763	K37M_F	TCAAGATGCCCCACCGTTACAAGCC
4764	K37M_R	GGGCATCTTGACACCGCCGGTGG
4765	K37R_F	TCAAGCGCCCCCACCGTTACAAGCC
4766	K37R_R	GGGGCGCTTGACACCGCCGGTGG
4767	K37Q_F	TCAAGCAGCCCCACCGTTACAAGCC
4768	K37Q_R	GGGCTGCTTGACACCGCCGGTGG
4769	R40A_F	CCACGCTTACAAGCCCGGTACCGTCG
4770	R40A_R	CTTGTAAGCGTGGGGCTTCTTGACACC
4771	Y41A_F	CCGTGCCAAGCCCGGTACCGTCGC
4772	Y41A_R	CTTGGCACGGTGGGGCTTCTTGACAC
4773	K42A_F	CGTTACGCGCCCGGTACCGTCGCT
4774	K42A_R	GCGCGTAACGGTGGGGCTTCTTGAC
4775	K56Q_F	CCAGCAGTCCACTGAGCTTCTGATC
4776	K56Q_R	TGGACTGCTGGTAGCGACGAATCTC
4777	K56R_F	CAGCGCTCCACTGAGCTTCTGATC
4778	K56R_R	TGGAGCGCTGGTAGCGACGAATCTC
4779	K56M_F	CCAGATGTCCACTGAGCTTCTGATC
4780	K56M_R	TGGACATCTGGTAGCGACGAATCTC
4781	K56L_F	CCAGCTCTCCACTGAGCTTCTGATC
4782	K56L_R	TGGAGAGCTGGTAGCGACGAATCTC
4783	K64L_F	CCGCCTCCTCCCCTTCCAGCG
4784	K64L_R	GAGGAGGCGGATCAGAAGCTCAGTGG
4785	Q68A_F	CTCCCCTTCGCCCGTCTCGTAAGTTT
4786	Q68A_R	GCGAAGGGGAGCTTGCGGATCAGAAGC
4787	Q68S_F	CTCCCCTTCTCCCGTCTCGTAAGTTT
4788	Q68S_R	GAGAAGGGGAGCTTGCGGATCAGAAGC
4789	K79Q_F	GACTTCCAGTCCGACCTCCGCTTCC
4790	K79Q_R	GGACTGGAAGTCCTGGGCAATCTCACGG
4791	K79R_F	GACTTCCGCTCCGACCTCCGCTTCC

4792	K79R_R	GGAGCGGAAGTCCTGGGCAATCTCACGG
4793	K79M_F	GACTTCATGTCCGACCTCCGCTTCC
4794	K79M_R	GGACATGAAGTCCTGGGCAATCTCACGG
4795	K79L_F	GACTTCCTCTCCGACCTCCGCTTCC
4796	K79L_R	GGAGAGGAAGTCCTGGGCAATCTCACGG
4797	K122L-F	GAGCCTCGACATCCAGCTCGCCC
4798	K122L-R	TGTCGAGGCTCTGGATGGTGACACG
4799	E133A_F	GCGGTGCGCGCAACTAAGCGACTC
4800	E133A_R	GCGCACCGCGGAGGCGGCGGG
4801	R134A_F	GTGAGGGCAACTAAGCGACTCTTCG
4802	R134A_R	TTGCCCTCACCGCGGAGGCGGC
4663	hH3K9M_F	CCCGCATGTCCACCGGTGGCAAGG
4664	hH3K9M_R	CACCGGTGGACATGCGGGCGGTCT
4665	hH3TK27T_F	CTCGCTTCCAAGACTGCCCGCAAGTCCACCCCCTCCACCG
4666	hH3TK27T_R	CGGTGGAGGGGGTGGACTTGCGGGCAGTCTTGGAAGCGAG
4667	hH3AK9A_F	CTAAGCAGGCCGCCCGCAAGTCCGCCGGTGGCAAGGCCCC
4668	hH3AK9A_R	CACCGGCGGACTTGCGGGCGGCCTGCTTAGTGCGGGCCAT
4324	hH3K27M_F	AGGCTGCCCGCATGTCCGCC
4662	hH3K27M_R	GGCGGACATGCGGGCAGCCT

Table S2. Results of Quick-Change PCR into pRG9 and transformation of *F. graminearum.*

Residue	Substitution	*Sanger Sequencing results*	Number of Transformations	Fraction with targeted gene insertion	Phenotype	Localization of PRC2 body
R2	R2L	R2L	1			
T3	T3S					
	T3A	WT				
K4	K4L	K4L	0			
	K4M					
	K4R	WT				
	K4Q	WT				
T6	T6A	T6A				
A7	A7M	A7M	0			
R8	R8A	R8A				
K9	K9L	K9L				
	K9M	K9M	0			
	K9R	K9R				
	K9Q					
S10	S10A	WT				
	S10T					
T11	T11A					
G12	G12P	WT				
G13	G13M					
K14	K14L	K14L	0			
	K14M	K14M	4	2/4	Sick	Wild Type
	K14R	WT				
	K14Q	WT				
A15	A15M	WT				
	A15P					
P16	P16A					
R17	R17L					
K18	K18L	K18L	10	5/10	Sick	
	K18M	K18M	1	0/1		
	K18R	K18R				
	K18Q					
K23	K23L	K23L	0			
	K23M	WT				

	K23R		
	K23Q		
A24	A24T	A24T	1
A25	A25M	A25M	0
A26	R26A	R26A	
K27	K27L	WT	
	K27M	WT	
	K27R		
	K27Q		
S28	S28T	WT	
	S28A		
A29	A29M	A29M	
	A29T	A29T	
K36	K36L	WT	
	K36M	K36M	
	K36R	K36R	0
	K36Q	K36Q	
K37	K37L	K37L	0
	K37M	K37M	0
	K37R		
	K37Q	WT	
R40	R40A	R40A	0
Y41	Y41A	Y41A	0
K42	K42A	WT	
K56	K56L	K56L	
	K56M	K56M	0
	K56R	K56R	0
	K56Q	WT	
K64	K64L	K64L	4
Q68	Q68A	Q68A	
	Q68S	WT	
K79	K79L	K79L	
	K79M		
	K79R	WT	
	K79Q		
K122	K122L	K122L	0

E133	E133A	E133A
R134	R134A	WT

Table S3. Results of Quick-Change PCR into pSF6 and transformation of *N. crassa*

Residue	*Substitution*	*Sanger Sequencing results*	*Number of Transformations*	*Fraction with targeted gene insertion*	*Phenotype*	*Localization of PRC2 body*
T3	T3S					
	T3A	WT				
K4	K4M					
	K4R	WT				
	K4Q	WT				
K9	K9L	WT				
	K9M	WT				
	K9Q					
S10	S10A	S10A				
	S10T					
K14	K14L					
	K14M	K14M	0			
	K14R	K14R				
	K14Q	K14Q				
A15	A15M	A15M				
	A15P					
P16	P16A					
R17	R17L					
K18	K18L	K18L	14	11/14		
	K18M	WT				
	K18R	WT				
	K18Q					
K23	K23L	K23L	7	7/7		
	K23M	WT				
	K23R					
	K23Q					
A24	A24T	WT				
A25	A25M	A25M				
A26	R26A					
K27	K27L	K27L				
	K27M	K27M				

	K27R	
	K27Q	
S28	S28T	S28T
	S28A	
A29	A29M	WT
	A29T	A29T
K36	K36L	K36L
	K36M	WT
	K36R	WT
	K36Q	
K37	K37L	K37L
	K37M	K37M
	K37R	
	K37Q	WT
R40	R40A	WT
Y41	Y41A	WT
K42	K42A	WT
K56	K56L	WT
	K56M	K56M
	K56R	K56R
	K56Q	WT
K64	K64L	K64L
Q68	Q68A	Q68A
	Q68S	Q68S
K79	K79L	
	K79M	
	K79R	WT
	K79Q	
K122	K122L	WT
E133	E133A	E133A
R134	R134A	E134A

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