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SPECIAL ISSUE REVIEW

DNA methylation remodeling in F1 hybrids

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SUMMARY

F1 hybrids derived from a cross between two inbred parental lines often display widespread changes in DNA methylation patterns relative to their parents. To which extent these changes drive non-additive gene expression levels and phenotypic heterosis in F1 individuals is not fully resolved. Current mechanistic models propose that DNA methylation remodeling in hybrids is the result of epigenetic interactions between parental alleles via small interfering RNA (sRNA). These models have strong empirical support but are limited to genomic regions where the two parental lines differ in DNA methylation status. However, most remodeling events occur in parental regions with similar methylation patterns, and seem to be strongly conditioned by distally acting factors, even in isogenic hybrid systems. The molecular basis of these distal interactions is currently unknown, and will likely emerge as an active area of research in the future. Despite these gaps in our molecular understanding, parental DNA methylation states are statistically associated with heterosis, independent of genetic information, and may serve as biomarkers in crop breeding.

Keywords: Heterosis, DNA methylation, sRNA, Hybrids, Paramutation, Epigenetics.

INTRODUCTION

Heterosis describes the superior phenotypes of F1 hybrids derived from a cross between two inbred parents (Birchler et al., 2010), most notably for traits such as biomass, seed yield, or developmental rate. First defined in the early work of Shull (1908), the molecular and phenotypic properties of this phenomenon have been intensively investigated, both in the academic and in the commercial sector. Many breeding programs target heterosis as a way to optimize crop production (Schnable & Springer, 2013). Yet, despite its importance, the underlying molecular mechanisms are still poorly understood (Schnable & Springer, 2013). A number of genetic explanations have been put forth, all of which center on classical models of dominance, overdominance, or epistasis (reviewed in Chen, 2010; Crow, 1948; Fujimoto et al., 2018; Jones, 1917; Schnable & Springer, 2013). These models predict that heterosis should scale positively with the genetic divergence between parental lines (Chen, 2010; East, 1936). There is certainly evidence to support this, as heterosis tends to be much more pronounced in interspecific crosses compared with intra-specific crosses. However, numerous exceptions to this prediction have emerged over the years, which undermine the generality of these models and open the door for alternative explanations. One intriguing model proposes that epigenetic, rather than genetic, differences serve as a trigger for heterosis (Groszmann et al., 2013; Lauss et al., 2018). This model is backed by observations that F1 hybrids undergo substantial DNA methylation remodeling relative to their parents (Greaves et al., 2012; Kakoulidou et al., 2022; Shen et al., 2012; Zhang et al., 2016) and that experimental perturbations of DNA methylation pathways alter the heterotic potential of hybrids (Kakoulidou et al., 2022; Kawanabe et al., 2016; Lauss et al., 2018; Rigal et al., 2016; Shen et al., 2012). The remodeling of DNA methylation patterns co-occurs with other chromatin modifications, which together reshape the regulatory landscape of hybrid genomes and could thus provide a molecular basis for heterosis. The fact that similar epigenetic changes have been observed in F1 offspring from isogenic parents that had been engineered to differ in their DNA methylation profiles (Dapp et al., 2015; Kakoulidou et al., 2022; Lauss et al., 2018) underlines that these events are not just consequences of genetic variation, and thus call for independent mechanistic explanations.

A number of studies have examined the DNA methylomes of F1 hybrids and their parents in detail (see Table 1).

Table 1 Examples of studies focusing on epigenetic remodeling mechanisms in diploid plants

Reference	Species	Cross	Epigenetic mechanism
Zhang et al. (2016)	Arabidopsis	C24 and Col	DNA methylation and sRNAs
Shen et al. (2012)	Arabidopsis	C24 and Ler	DNA methylation and sRNAs
Greaves et al. (2012)	Arabidopsis	C24 and Ler	DNA methylation and sRNAs
Chodavarapu et al. (2012)	Rice	Nipponbare and indica (93-11)	DNA methylation and sRNAs
Shen et al. (2017)	Oilseed rape	MB and ZY50	DNA methylation and sRNAs
Groszmann, Greaves, Albertyn, et al. (2011)	Arabidopsis	C24 and Ler	DNA methylation and sRNAs
Li et al. (2012)	Arabidopsis	Col and Ler	sRNAs
Sinha et al. (2020)	Pigeonpea	ICPA 2043 and ICPR 2671; ICPH 2740 and ICPR 2740	DNA methylation and sRNAs
Barber et al. (2012)	Maize	B73 and Mo17	sRNAs
Lauss et al. (2018)	Arabidopsis	Col and *ddm1-2*-derived epiRILs	DNA methylation
Kakoulidou et al. (2022)	Arabidopsis	msCol and *ddm1-2*-derived epiRILs	DNA methylation and sRNAs
Rigal et al. (2016)	Arabidopsis	*met1-3* (Col background) and Ler MET1 wild type	DNA methylation and sRNAs
Dapp et al. (2015)	Arabidopsis	Col and *met1-3*-derived epiRILs	Epigenetic regulation of transcription
Kawanabe et al. (2016)	Arabidopsis	Col, C24, Cvi, *ddm1-1* (Col), *ddm1-9* (C24), *met1-1* (Col), *nrpd1a-3* (Col), and *sde4-2* (C24)	DNA methylation and sRNAs
Greaves et al. (2016)	Arabidopsis	C24 and Ler	DNA methylation and sRNAs
Ma et al. (2021)	Rice	ZS97 and MH63	DNA methylation and sRNAs
Greaves et al. (2014)	Arabidopsis	C24 and Ler	DNA methylation and histone modifications
He et al. (2010)	Rice	*O. sativa* ssp. *japonica* cv Nipponbare and *O. sativa* ssp. *indica* cv 93-11	DNA methylation, histone modifications, and sRNAs

The major questions that have emerged from these studies are: (i) What are the molecular mechanisms that trigger DNA methylation remodeling? (ii) How do these changes translate into regulatory and phenotypic alterations? (iii) And how can epigenetic information be used as a biomarker to predict heterosis? Here we provide a critical overview of these studies. We begin with a brief review of DNA methylation pathways, discuss different classes of DNA methylation remodeling events and their mechanistic underpinnings, and finally review the evidence for a link between DNA methylation remodeling and phenotypic changes in F1 hybrids. Our review highlights a promising role of DNA methylation as a biomarker and potential breeding target for heterosis, but also uncovers major gaps in our current understanding of the molecular mechanisms of DNA methylation remodeling in F1 hybrids.

DNA METHYLATION

DNA cytosine methylation is the addition of a methyl group (CH_3) to a cytosine nucleotide, which is one of the most widely conserved and extensively studied epigenetic modifications in eukaryotes. In plants, it occurs in sequence contexts CG, CHG, and CHH (where H = A, T, or C). Dense methylation is found mainly in pericentromeric heterochromatic regions of chromosomes, where it is associated with the repression of transposable elements (TEs) and repetitive sequences (Zhang et al., 2006). *De novo* methylation in all three sequence contexts is primarily catalyzed by the RNA-directed DNA methylation (RdDM) pathway, which involves 24-nucleotide (nt) small RNAs (sRNAs) acting as guide molecules for DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2). RdDM requires two RNA polymerases, Pol IV and Pol V, for the production of sRNAs and their targeting to specific loci (reviewed in Law & Jacobsen, 2010; Matzke et al., 2009). In addition to their role in heterochromatin, sRNAs also contribute to the regulation of gene expression via (post-) transcriptional silencing mechanisms, and are involved in plant development, reproduction, and phenotypic plasticity (Borges & Martienssen, 2015).

Once established, DNA methylation is maintained by a number of context-specific pathways. In a CG context, hemimethylated CG sites are recognized by the VARIANT IN METHYLATION (VIM1) family of proteins and recruit METHYLTRANSFERASE 1 (MET1) to catalyze CG methylation on the newly synthesized strand by way of template copying. Loss of MET1 results in complete genome-wide loss of CG methylation (Saze et al., 2003). CHG methylation is maintained mostly by the plant-specific methyltransferase CHROMOMETHYLASE 3 (CMT3), which acts in a self-reinforcing loop with histone H3 lysine 9 dimethylation (H3K9me2) and the histone methyltransferase SUPPRESSOR OF VARIEGATION 3–9 HOMOLOG 4 (SUVH4) (Johnson et al., 2007). At a subset of CHG and CHH sites, methylation is also maintained by the *de novo* activity of CMT2, which also requires the presence of

H3K9me2 (Law & Jacobsen, 2010). Studies of various DNA methylation mutants have uncovered substantial crosstalk between these different pathways (Stroud et al., 2014; To & Kakutani, 2022).

Beyond DNA methyltransferases, DNA methylation patterns are also indirectly controlled by the chromatin remodeler DECREASE IN DNA METHYLATION 1 (DDM1). DDM1 facilitates methylation by providing access for DNA methyltransferases to H1-containing histones, primarily within long TEs of heterochromatic regions (Zemach et al., 2013). Loss of DDM1 results in a significant reduction of DNA methylation in all three sequence contexts, as well as to a widespread overaccumulation of TE-related transcripts (Kakutani et al., 1995; Lippman et al., 2004; Vongs et al., 1993). DDM1 is also required to maintain histone H3 methylation patterns. Loss of DNA methylation is accompanied by replacement of methylation of lysine 9 with methylation of lysine 4 (Gendrel et al., 2002; Soppe et al., 2002), which is consistent with the transcriptional activation of otherwise repressed regions. Phenotypic studies of *ddm1* mutants show strong phenotypic effects, including altered flower morphology, late flowering, and low fertility, particularly after several rounds of inbreeding (Kakutani et al., 1996). Moreover, transient loss of DDM1 can induce heritable epialleles that segregate independently and contribute to phenotypic heritability (Colome-Tatche et al., 2012, p. 201; Cortijo et al., 2014; Johannes et al., 2009; Kooke et al., 2015, 2019; Roux et al., 2011).

OVERVIEW OF F1 EXPERIMENTAL SYSTEMS

A number of experimental studies have examined DNA methylation changes in F1 hybrids, including in *Arabidopsis thaliana* (Arabidopsis), *Oryza sativa* (rice), *Brassica napus* (oilseed rape), *Zea mays* (maize), and *Cajanus cajan* (pigeonpea) (Table 1). Several of these studies also included other data sources such as sRNA and RNA expression as well as phenotypic data as a way to delineate mechanistic causes and functional consequences. We focus here on intra-species crosses in diploids, although information is also available in polyploids (Chen, 2007; Ha et al., 2009; Ng et al., 2012; Ni et al., 2009). The majority of the studies utilized *A. thaliana* as a model. While the choice of this primarily selfing species may limit generalization to obligate outcrossers, its small genome size and extensive (epi)genetic resources make mechanistic insights feasible. Moreover, many basic epigenetic observations in *A. thaliana* hybrids seem to be generalizable to much more complex outcrossing species like maize (Barber et al., 2012), which suggests that this is a reasonable model.

The initial use of *A. thaliana* was also motivated by early observations that genetically very similar ecotypes (e.g., C24 × Ler) can produce extensive vegetative heterosis (rosette diameter and biomass) (Groszmann, Greaves, Albert et al., 2011; Groszmann, Greaves, Albertyn, et al., 2011). This has led to the hypothesis that mostly epigenetic, rather than genetic, differences between the parents trigger these heterotic phenotypes. More concerted attempts to delineate the role of parental epigenetic differences in facilitating DNA methylation remodeling and heterosis in F1 hybrids have been made by Dapp et al. (2015), Rigal et al. (2016), Lauss et al. (2018), and Kakoulidou et al. (2022). These latter studies examined F1 hybrids from crosses between isogenic parents that had been engineered to differ only in their epigenetic profiles. These parental lines were chosen from existing panels of *ddm1*- or *met1*-derived epigenetic recombinant inbred lines (epiRILs; Johannes et al., 2009; Reinders et al., 2009).

CLASSIFICATION OF DNA METHYLATION REMODELING EVENTS

DNA methylome analyses of the different F1 systems have revealed that the majority of the parental methylation states combine additively in hybrids (Kakoulidou et al., 2022; Lauss et al., 2018; Ma et al., 2021; Zhang et al., 2016). Additivity here means that the methylation states of the two parental alleles are stably and independently maintained (Figure 1). This observation is consistent with the faithful transgenerational inheritance of DNA methylation, particularly in highly methylated repressed regions of chromosomes. However, a considerable proportion of hybrid genomes do appear to undergo some type of methylation remodeling event. These events can be in the form of methylation gains or losses in genomic regions where the two parents are differentially methylated (DMRs); we refer to these events as DMR gains and DMR losses, respectively (Figure 1). In the case of DMR gains, hybrids experience a monoallelic increase of methylation on the previously unmethylated parental allele (Figure 1). Conversely, DMR loss events involve a monoallelic decrease of methylation on the previously methylated parental allele (Figure 1). In addition to these DMR-centered remodeling events, a large proportion of changes also occur in regions where the two parents are similarly methylated (SMRs). One can distinguish SMR gains and SMR losses, which involve (mostly) biallelic methylation increases or decreases in regions where the parents are either both unmethylated or methylated, respectively (Figure 1).

Methodologically, the classification of these different remodeling events is not trivial. Delineating monoallelic from biallelic methylation changes in the hybrids requires allele-specific DNA methylation data. This information can be obtained for genomic regions where the two parents are genetically polymorphic as the parental origin of the sequencing reads can be ascertained. In isogenic F1 hybrid systems (*ddm1*-derived or *met1*-derived epiHybrids), or in non-polymorphic regions of genetically divergent parental lines, such information is not available. In these latter cases, classification must rely on studying changes of DNA

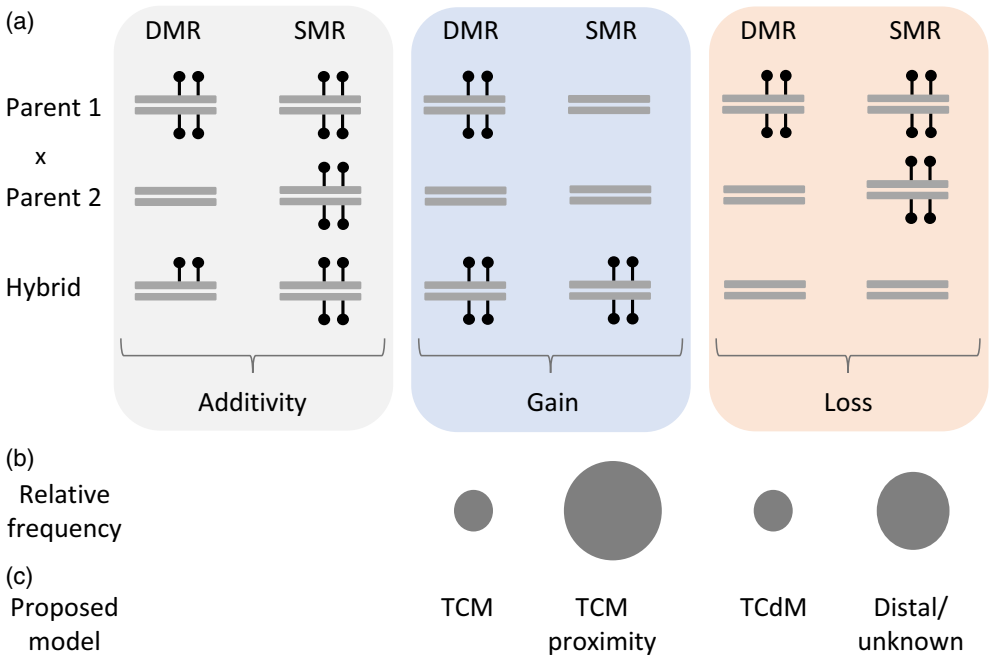

Figure 1. Classification of DNA methylation remodeling events observed in F1 hybrids.
(a) Schematic representation of the different remodeling events. Additivity: Stable inheritance of parental DNA methylation states and thus no remodeling; DMR gain and DMR loss: Hybrids gain or lose DNA methylation in regions where the parents are differentially methylated; SMR gain and SMR loss: Hybrids gain or lose DNA methylation in regions where both parents are unmethylated or methylated. Gray bars indicate DNA, while black lollipops indicate DNA methylation.
(b) Relative frequency of remodeling events across the genome approximated from Kakoulidou et al. (2022), Lauss et al. (2018), and Zhang et al. (2016). Larger circles denote higher frequency.
(c) Proposed mechanistic models for remodeling events. DMR, differentially methylated region; SMR, similarly methylated region.

methylation levels in the hybrids relative to the parents. Although an approximation, this approach seems to work well, particularly for parental regions with extreme methylation levels (i.e., clearly methylated or unmethylated). Indeed, classification of remodeling events based on changes in methylation levels produces similar frequencies of the different remodeling events as allele-specific analyses.

The above classification captures the main types of remodeling events that have been observed in F1 hybrids. In reality, classification is much fuzzier, and should be understood as existing on a continuum of remodeling possibilities (e.g., DMR gain can occur on both alleles). Nonetheless, this classification has been useful for guiding the development of mechanistic models.

MECHANISMS OF DNA METHYLATION REMODELING

The vast majority of DNA methylation remodeling events in F1 hybrids seem to occur in parental SMRs rather than in DMRs (Greaves et al., 2012; Kakoulidou et al., 2022; Lauss et al., 2018; Ma et al., 2021; Zhang et al., 2016). However, considering that only a small fraction of parental genomes are typically differentially methylated, the sheer number of DMR-based remodeling events presents a significant enrichment. That is, parental DMRs – when they exist – are more likely to be subject to methylation changes in F1 hybrids than SMRs. Therefore, and perhaps also because they lend themselves more readily to mechanistic explanations involving known epigenetic processes, DMR-based remodeling events have become the major focus in the literature.

A trans-chromosomal methylation model for DMR gain events

A common molecular model for DMR gain events posits that the two parental alleles interact in the hybrids via 24-nt sRNAs. In this model, the sRNAs are initially produced from the methylated parental allele and subsequently targeted to the unmethylated allele for *de novo* methylation (Figure 2). Once established, the methylation status of the recipient allele is then maintained independently. This process has also been termed 'trans-chromosomal methylation (TCM)' (Greaves et al., 2012). The term 'trans', in this case, is supposed to emphasize that the allelic interactions are between the two homologous chromosomes (i.e., at the same genomic locus). It should not be confused with the more common use of the word '*trans*' in genetics, which denotes interactions or associations between two distal loci in the genome.

Terminological issues aside, a number of observations support the TCM model. First, sRNA sequencing analysis

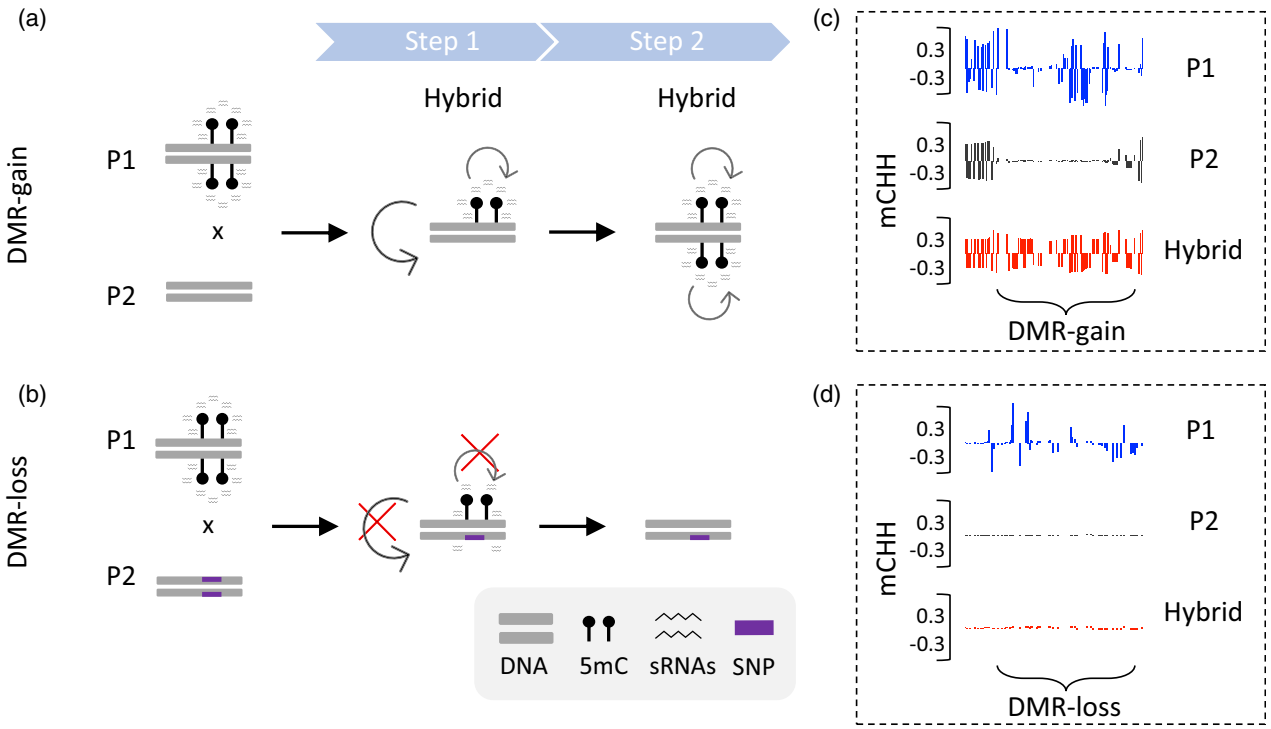

Figure 2. Trans-chromosomal (de)methylation models for DMR events.
(a) A TCM model for DMR gain events: In the hybrids, 24-nt sRNAs from the methylated parental allele target the unmethylated parental allele for *de novo* methylation. This process is similar to paramutation.
(b) A TCdM model for DMR loss events: In the hybrids, 24-nt sRNAs from the methylated parental allele fail to effectively target the unmethylated parental allele because of insufficient sequence homology, leading to a dilution of sRNA copies and eventual loss of methylation reinforcement on the methylated allele. (c, d) Example browser shots for the CHH methylation context taken from the data of Kakoulidou et al. (2022) for (c) DMR gain and (d) DMR loss events. DMR, DMR, differentially methylated region; TCM, trans-chromosomal methylation.

revealed that TCM loci are genuine targets of 24-nt sRNAs, with parental DMRs often being accompanied by differential sRNA abundance (Greaves et al., 2016). Second, the monoallelic methylation gains observed in hybrids tend to co-occur with a gain of 24-nt sRNAs on the same allele (Zhang et al., 2016). Finally, and importantly, TCM events are largely abolished in RdDM mutants (Greaves et al., 2016; Zhang et al., 2016), indicating that 24-nt sRNAs are required for TCM.

Interestingly, the establishment of TCM is a gradual process that emerges only slowly during plant development (Greaves et al., 2014). This could indicate that the reinforcement of methylation on the recipient allele is partly replication-dependent. However, even within the same system, there are differences in the timing of the TCM regions, most likely due to the strength of the TCM signal involving sRNAs (Greaves et al., 2014).

Given the tight relationship between DNA methylation and various histone modifications, TCM events are often accompanied by alterations in other epigenetic marks. For instance, Greaves et al. (2014) showed that specific TCM-induced methylation gains correlate with a decrease of H3K9ac (Groszmann et al., 2013). This would seem to indicate that TCM serves as a trigger for higher-level chromatin state changes in hybrids. However, other situations exist where the reverse is true. An example is the *FLOWERING WAGENINGEN A* (*FWA*) gene, a well-known flowering time regulator in plants. *FWA* is transcriptionally silenced by methylation of repeat elements in the promoter and the 5′ untranslated region (Kinoshita et al., 2007, p. 200), which is mediated by sRNA. The sRNA sites can be targeted for TCM, but not when *FWA* on the recipient allele is expressed and marked by active chromatin (Chan et al., 2004). This suggests that active chromatin hinders sRNA-directed TCM (reviewed in Groszmann, Greaves, Albert, et al., 2011).

The above-described TCM model leans heavily on previous work on paramutation in maize (Chandler, 2007). In fact, the initial step of paramutation follows a TCM process as the inducing methylated allele serves as a template for Pol IV and the production of sRNAs, in order to impose their epigenetic state onto the sensitive (unmethylated) allele. sRNAs produced at the inducing allele reinforce its silenced state in *cis* and target the other allele to subsequently trigger *de novo* methylation (reviewed in Hövel et al., 2015).

Similar to paramutation, many TCM-induced methylation changes can also be transmitted beyond the F1 generation, and thus represent a source of heritable epialleles (Greaves et al., 2014; Zhang et al., 2016). In Arabidopsis,

using crosses of C24 and either Ler or Col, it was found that many TCM-induced gains can still persist among F2 individuals (Greaves et al., 2014; Zhang et al., 2016). However, this pattern (of inheritance) can be disrupted by perturbations of the RdDM pathways (Zhang et al., 2016) and it is not always stable (Greaves et al., 2014). The most stable paramutable alleles seemed to be those that, in addition to RdDM, are also redundantly targeted by other methylation pathways, like MET1 and DDM1 (Greaves et al., 2016). These alleles are enriched for CG dinucleotides, which may confer the necessary transgenerational stability.

By contrast, the lack of stability of many TCM-induced methylation states is of interest in its own right: It may provide a molecular basis for the breakdown of hybrid vigor that often occurs in the F2 or F3 generation (Greaves et al., 2014). This hypothesis is plausible, but requires a demonstration of a direct link between TCM and phenotypic heterosis and a way to rule out alternative explanations such as the loss of epistatic genotype combinations due to segregation or recombination, both of which are experimentally difficult to test.

A trans-chromosomal demethylation model for DMR loss events

The reverse of a TCM process has been invoked to also explain DMR loss events (Greaves et al., 2012; Zhang et al., 2016). By analogy, this model has been termed trans-chromosomal demethylation (TCdM; Greaves et al., 2012). The model is supported by the observation that monoallelic methylation losses are accompanied by a reduction of matching 24-nt sRNA on the same allele (Zhang et al., 2016) (Figure 2). However, this is only correlative evidence, and it remains unclear how the unmethylated allele can actually trigger the cessation of sRNA production on the methylated allele; that is, the molecular basis of the 'trans-chromosomal' signal is not well understood. Moreover, TCM and TCdM events at DMRs are not likely to be orthogonal processes as TCdM events are more prevalent in regions that are enriched in CHH methylation, which would point to a different mechanism than the one that leads to establishment.

One attempt at a mechanistic explanation was provided by Zhang et al. (2016). The authors found that DMR loss events occurred more frequently in regions of high genetic variation among the parents. They proposed that sRNA from the methylated alleles cannot efficiently target the unmethylated alleles because of a lack of sequence homology. These unsuccessful targeting attempts lead to a dilution of sRNA copies from the donor alleles, which in turn weakens its own sRNA-mediated methylation reinforcement (Zhang et al., 2016). This proposed mechanism is interesting and may hold for highly polymorphic sites. However, it is unlikely to provide a general framework for thinking about DMR loss events, as a similar frequency of such events is observed in isogenic F1 hybrid systems (epiHybrids) (Kakoulidou et al., 2022), where a polymorphism-based explanation is obviously not valid.

The fact that TCdM events have no clear 'trans-chromosomal' signal in '*cis*' (i.e., at a given genomic position) raises the possibility that the trigger originates from remodeling events in distal regions. This is an area of research that is largely unexplored. As we will see, the need for models that incorporate interactions between 'distal' loci becomes particularly relevant for explaining SMR-based methylation remodeling in F1 hybrids.

A putative TCM proximity model for SMR gain events

Mechanistic models of DMR-based remodeling events rely on molecular interactions between parental alleles. It should be obvious that such models cannot be readily extended to SMRs, because the parental alleles have the same methylation state. The trigger to initiate remodeling events in SMRs must therefore come from 'distal' regions outside of homologous alleles. Kakoulidou et al. (2022) recently provided evidence that many SMR gain events could possibly be fitted into what may be called a 'TCM proximity model' (Figure 3). They found that many such events occur nearby TCM sites (about 350 bp), suggesting that they are simply a byproduct of a TCM process in the genomic 'neighborhood'. Although many details of this model remain untested, one could hypothesize that the sRNA-mediated targeting of the unmethylated allele at a DMR (i.e., DMR gain) is imprecise and occasionally initiates DNA methylation spreading into flanking regions on the targeted allele, a process that has been well documented in the context of TE biology (Hollister & Gaut, 2009, and reviewed in Diez et al., 2014). That this spreading is monoallelic could not be directly verified in the isogenic hybrid system used by the authors. However, in many cases, SMR gain events flanking TCM loci display clear intermediate methylation levels (Kakoulidou et al., 2022), which is at least consistent with this idea. Further support for this comes from the fact that many SMR gain events show a correlative gain in sRNA abundance in the flanking regions relative to the parents. It would be interesting to explore if the spreading also leads to an occasional re-targeting of the original donor allele for a second round of TCM, but now in the opposite direction (Figure 3). This latter possibility could explain why many SMR gain events appear to be biallelic. Exploring the TCM proximity model in detail would require allele-specific analyses (genetically diverse crosses) along with time-resolved data (i.e., different developmental stages).

In search of a molecular model for SMR loss events

One can argue that the above proximity model could also be used to account for SMR loss events, but in this case as

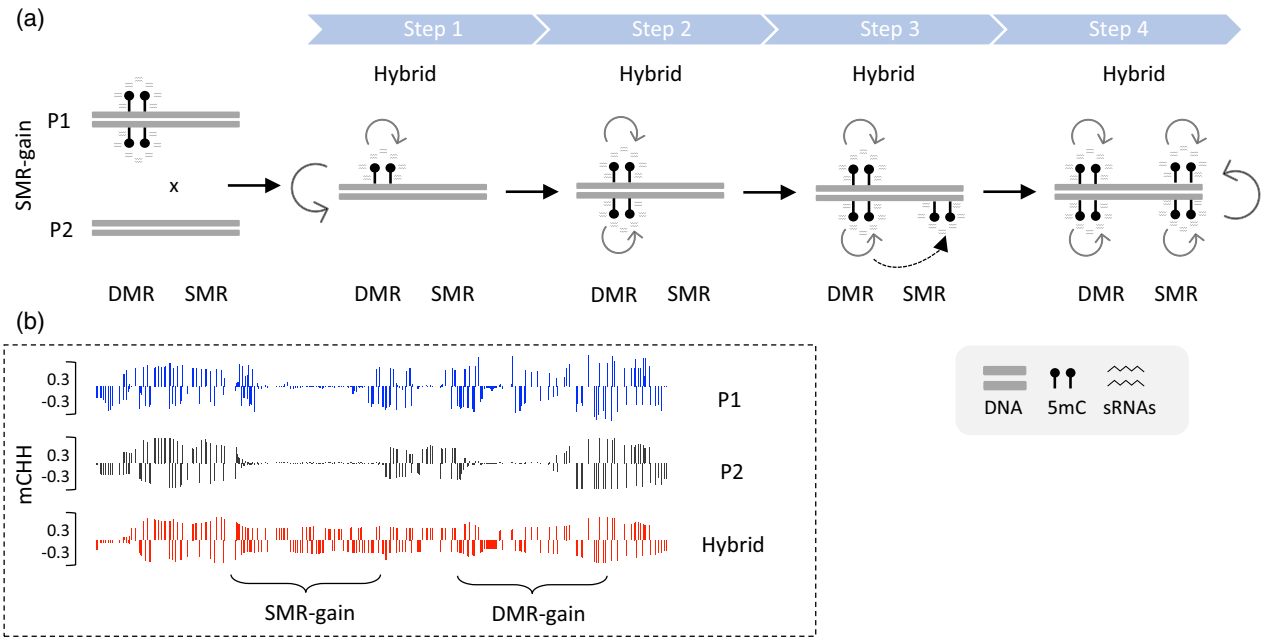

Figure 3. A TCM proximity model for SMR gain events.
(a) SMR gain events are the result of DNA methylation spreading from proximal DMRs through TCM (Steps 1–3). Spreading is a monoallelic process (Step 3). It is plausible that spreading leads to a second round of TCM in the opposite direction, thus producing a biallelic gain of methylation at the SMR (Step 4).
(b) Example browser shots for the CHH methylation context taken from the data of Kakoulidou et al. (2022). DMR, differentially methylated region; SMR, similarly methylated region; TCM, trans-chromosomal methylation.

a byproduct of TCdM at DMR loss sites. But since no clear molecular mechanism is currently known to actually trigger DMR loss, such a model would not provide a satisfactory molecular explanation. Moreover, compared to SMR gain, SMR loss events appear to be much further away from DMR loss events on average (approximately 1.6 kb, on average) (Kakoulidou et al., 2022), thus rendering the proximity argument void. An alternative hypothesis is that SMR loss events are a consequence of remodeling of distal regions. This hypothesis predicts that SMR loss at a given genomic locus is somehow correlated with methylation remodeling elsewhere in the genome. Kakoulidou et al. (2022) showed that there is strong support for this hypothesis. The authors analyzed the DNA methylomes of hundreds of *A. thaliana* epiHybrid families and uncovered strong positive and negative correlations among remodeling events across the genome (e.g., DNA methylation loss in one region was associated with methylation loss in another region in the hybrids, or gain in one region with loss in another, etc.). Pericentromeric regions of chromosomes emerged as major hubs, displaying high correlation not only locally, but also between pericentromeric regions of different chromosomes and with euchromatic loci, including genes (Kakoulidou et al., 2022; Zhang et al., 2021).

These latter results highlight a major contribution of distally acting factors to DNA methylation remodeling and provide a framework to begin to think about possible molecular mechanisms. One possibility is that correlated remodeling events are the result of both regions being targeted by *trans*-acting sRNA (here '*trans*' refers to 'distal'). Initial support for this comes from the fact that the correlation structure at the DNA methylation level in the hybrids is also visible at the sRNA level (Kakoulidou et al., 2022). That is, correlated loss of DNA methylation in two regions is accompanied by correlated loss of sRNA abundance. Of course, this mechanisms only applies to regions that have sufficient sequence homology so that they can be co-targeted by the same sRNA. However, this mechanism would be restricted to positive correlations only, and would not be able to explain situations where DNA methylation gain in one region correlates with loss in another region. This limitation suggests the presence of an alternative or complimentary mechanism.

One possible process that could account for distally correlated remodeling events is that hybrid DNA methylomes are subject to homeostatic adjustments as a result of the new parental epigenome combination being brought together. In this case, DNA methylomes must be understood as being the outcome of a balancing act between methylases and demethylases trying to adjust global or locus-specific methylation levels toward specific target values (review in Williams & Gehring, 2020). One example of how this might work is the autoregulation of the demethylase REPRESSOR OF SILENCING 1 (ROS1). The ROS1 promoter acts as a methylation sensor.

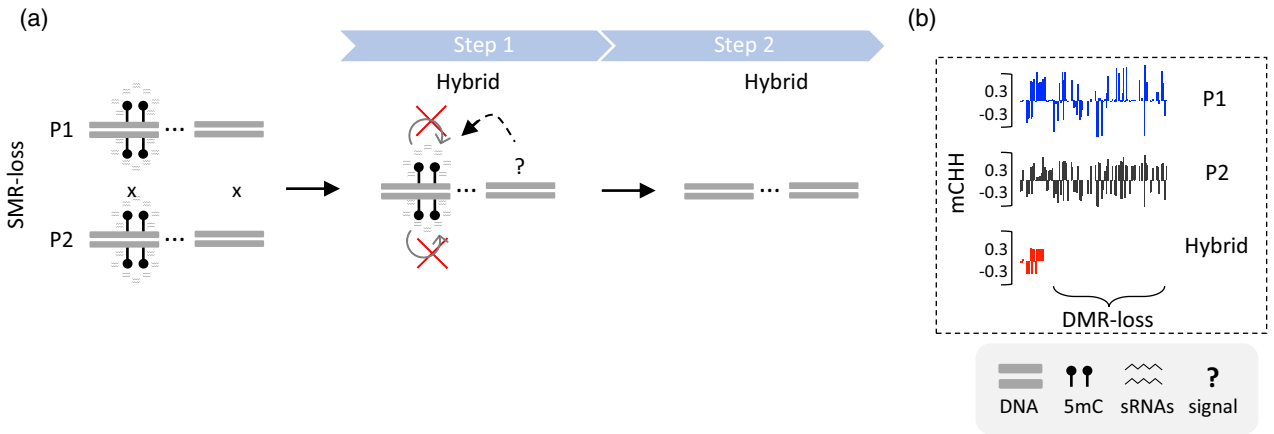

Figure 4. A 'distal' model for SMR loss events.
(a) SMR loss events cannot be explained by TCdM of neighboring DMRs as they do not tend to occur in close proximity. Instead, current data indicate that SMR loss events are triggered by a distal signal, possibly originating from a DMR remodeling event elsewhere in the genome. The molecular basis of the distal signal is currently unknown.
(b) Example browser shots for the CHH methylation context taken from the data of Kakoulidou et al. (2022) for SMR loss events. DMR, differentially methylated region; SMR, similarly methylated region; TCdM, trans-chromosomal demethylation.

Hypermethylation of the promoter sequence, via the RdDM pathway, results in upregulation of ROS1, which initiates demethylation in a self-regulatory negative feedback loop, ultimately resulting in decreased expression of ROS1 (reviewed in Williams & Gehring, 2020). It is plausible that similar homeostatic mechanisms underlie the correlated DNA methylome remodeling patterns found in F1 hybrids. The challenge is to quantify these processes, as neither the system's target values nor all relevant 'sensor' regions are fully known.

LINKS TO NON-ADDITIVE GENE EXPRESSION AND HETEROSIS

Regardless of the molecular mechanisms underlying DNA methylation remodeling in F1 hybrids, numerous studies report that such events are associated with non-additive gene expression changes (Chen et al., 2022; Greaves et al., 2012; Groszmann, Greaves, Albertyn, et al., 2011; He et al., 2010; Sinha et al., 2020) This is particularly true when remodeling occurs in promoter regions of genes. Gene-proximal gains or losses of methylation typically correlate with the upregulation or downregulation of genes, respectively. These associations seem to be dependent on specific developmental stages and tissue types (Fujimoto et al., 2018). Genes whose expression levels are altered through DNA methylation remodeling tend to be enriched in pathways regulating the circadian rhythm (Ni et al., 2009), hormones (Chen et al., 2022; Shen et al., 2012; Sinha et al., 2020) and metabolism (reviewed in Chen, 2013). This makes sense in light of the changes in photosynthetic activity, growth, and flowering time that often occur in F1 hybrids. Other candidates include genes that encode components of the epigenetic machinery, such as SU(VAR)3–9 RELATED 5 (SUVR5) and CLSY4 (a chromatin-remodeling protein of the CLASSY family) (Kakoulidou et al., 2022). While these latter transcriptional changes may be expected in view of the epigenomic remodeling events discussed here, they also point at an intriguing autoregulation of those genes by the very same pathways in which they are active.

Methodologically, it remains challenging to establish in F1 hybrids whether DNA methylation drives gene expression or the other way around. Causal inference approaches based on statistical models suggest that DNA methylation alterations precede expression changes in the majority of the cases (Kakoulidou et al., 2022). Similar methodological limitations apply to attempts to link DNA methylation remodeling events to phenotypic heterosis. Work with DNA methylation mutants shows that experimental manipulation of DNA methylation pathways can affect the heterotic potential of F1 hybrids for key traits such as vegetative biomass, pathogen resistance, and flowering time (Johannes et al., 2009; Kawanabe et al., 2016; Reinders et al., 2009). Loss of DDM1, for instance, results in a significant reduction in heterosis for rosette diameter (Kawanabe et al., 2016). However, the picture is less clear for RdDM, which is surprising given its central role in TCM. Studies by Barber et al. (2012), Zhang et al. (2016), and Kawanabe et al. (2016) report that loss of Pol IV, Pol V, or MODIFIER OF PARAMUTATION 1 (MOP1) does not impact F1 heterosis significantly. By contrast, Shen et al. (2012) found significant effects when abolishing sRNA production by knocking down the Arabidopsis RNA methyltransferase HUA ENHANCER1 (HEN1). Technical differences aside, this discrepancy may point to the possibility that phenotypic heterosis is at least partially independent of RdDM.

More recent studies have tried to link phenotypic heterosis with specific features of parental DNA methylomes. In a proof of principle demonstration, Lauss et al. (2018) and Kakoulidou et al. (2022) employed an epigenetic quantitative trait locus mapping approach in a large panel of isogenic *ddm1*-derived epiHybrid families. Parental pericentromeric DMRs emerged as major determinants of leaf area heterosis. Interestingly, these same DMRs were also associated with thousands of DNA methylation remodeling events across the genome, both locally and in distal regions. How these parental DMRs manage to reorganize hybrid methylomes is unclear, but this likely involves a combination of the mechanistic models outlined above (Figures 2–4). The statistical associations between parental DMRs and heterosis illustrate that epigenetic data collected on the parental generation could be developed further into biomarkers, independently of genetic determinants. These insights are consistent with statistical approaches that have successfully used parental differences in sRNA abundance as a predictor of yield heterosis in maize (Seifert et al., 2018).

CONCLUSION

Hybrids display widespread changes in DNA methylation and sRNA patterns relative to their parental lines. These changes appear to be consistent across diverse species, such as Arabidopsis (Greaves et al., 2012; Kakoulidou et al., 2022; Lauss et al., 2018; Rigal et al., 2016; Shen et al., 2012; Zhang et al., 2016), rice (Chodavarapu et al., 2012; He et al., 2010; Ma et al., 2021), pigeonpea (Sinha et al., 2020), maize (Barber et al., 2012), and oilseed rape (Shen et al., 2017). Within the same species, DNA methylome remodeling might vary across growing conditions, developmental stages, and tissue types. However, in F1 hybrids grown under the same conditions, a high proportion of remodeling events are recurrent across families, indicating that they are a reproducible rather than random feature of hybrid genomes (Kakoulidou et al., 2022). The question of how these DNA methylation changes have a causal role in driving non-additive gene expression levels and phenotypic heterosis in F1 hybrids is not fully resolved. Current mechanistic models propose that DNA methylation remodeling in hybrids is the result of epigenetic interactions between parental alleles by way of sRNA. We have shown that – while they have strong empirical support – these models are restricted to genomic regions where the two parental lines differ in DNA methylation status. This is a noteworthy limitation because most remodeling events occur in parental regions with similar methylation patterns. A solid molecular model for these latter events is currently missing. However, recent data suggest a major involvement of distally acting factors (particularly for SMR loss events). The molecular basis of these distal interactions is unknown, and will likely emerge as an active area of research in the future. Despite these gaps in our mechanistic understanding, statistical evidence has firmly linked parental DNA methylation states and sRNA abundance with heterosis (Kakoulidou et al., 2022; Lauss et al., 2018; Seifert et al., 2018), which may therefore emerge as biomarkers in crop breeding (Kakoulidou et al., 2021).

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CONFLICT OF INTEREST

The authors have not declared a conflict of interest.

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