Report

Current Biology

Robust DNA Methylation in the Clonal Raider Ant Brain

Highlights

- A large proportion of brain DNA methylation in the clonal raider ant is robust
- Genes with robust methylation show high and stable expression
- DNA methylation is not associated with different reproductive and behavioral states
- Evidence for caste-specific DNA methylation in social insects is weak

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In Brief

Libbrecht et al. show that in the clonal raider ant, brain DNA methylation is robust, particularly in genes with high and stable expression, and is not associated with different reproductive and behavioral states. They also report that currently there is little evidence of differential DNA methylation between the female castes of social insects.

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Robust DNA Methylation in the Clonal Raider Ant Brain

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SUMMARY

Social insects are promising model systems for epigenetics due to their immense morphological and behavioral plasticity. Reports that DNA methylation differs between the queen and worker castes in social insects [1–4] have implied a role for DNA methylation in regulating division of labor. To better understand the function of DNA methylation in social insects, we performed whole-genome bisulfite sequencing on brains of the clonal raider ant *Cerapachys biroi*, whose colonies alternate between reproductive (queen-like) and brood care (worker-like) phases [5]. Many cytosines were methylated in all replicates (on average 29.5% of the methylated cytosines in a given replicate), indicating that a large proportion of the *C. biroi* brain methylome is robust. Robust DNA methylation occurred preferentially in exonic CpGs of highly and stably expressed genes involved in core functions. Our analyses did not detect any differences in DNA methylation between the queen-like and worker-like phases, suggesting that DNA methylation is not associated with changes in reproduction and behavior in *C. biroi*. Finally, many cytosines were methylated in one sample only, due to either biological or experimental variation. By applying the statistical methods used in previous studies [1–4, 6] to our data, we show that such sample-specific DNA methylation may underlie the previous findings of queen- and worker-specific methylation. We argue that there is currently no evidence that genome-wide variation in DNA methylation is associated with the queen and worker castes in social insects, and we call for a more careful interpretation of the available data.

RESULTS AND DISCUSSION

The clonal raider ant *Cerapachys biroi* provides a good system to investigate insect DNA methylation, because age-matched individuals that are genetically identical can be collected easily [7]. *C. biroi* has no distinct queen and worker castes. Instead, all ants in a colony produce female offspring by parthenogenesis [8], and colonies undergo stereotypical cycles alternating between queen-like reproductive phases (ants lay eggs inside the nest) and worker-like brood care phases (ants do not lay eggs but nurse the brood and forage for food) [5]. To characterize the brain methylome of *C. biroi*, we sequenced eight samples of bisulfite-treated DNA extracted from pools of 20 brains dissected from age-matched ants collected in the reproductive phase (four samples) and in the brood care phase (four samples) from four source colonies belonging to two different clonal lineages (Experimental Procedures).

The average proportion of methylated cytosines across the eight samples was 2.1% ± 0.1% (mean ± SD), which is substantially higher than what has been reported for the honeybee (0.1%) [1] and other ant species (0.3% in *Camponotus floridanus* and 0.2% in *Harpegnathos saltator*) [2]. Methylation-sensitive AFLP on additional samples confirmed higher levels of methylation in *C. biroi* than in other social insects (Table S1; Supplemental Experimental Procedures). DNA methylation was found primarily in CpG dinucleotides (66.3% ± 1% of the methylated cytosines) and within genes (82.5% ± 0.6%), especially in exons (57% ± 0.9%). Such exonic CpG methylation has been reported in other insect species and in mammals, and it may affect gene function through histone modifications [9], nucleosome stability [10], and/or alternative splicing [1, 2, 11]. As previously shown in other ant species [2], levels of DNA methylation in *C. biroi* were associated with patterns of alternative splicing (Figure S1; Supplemental Experimental Procedures), and transposable elements were hypomethylated compared to the genome baseline (Wilcoxon rank-sum test, $W = 64$, $p = 0.0002$; Table S2; Supplemental Experimental Procedures).

Robust DNA Methylation Is Associated with Highly Expressed Genes Involved in Core Functions

On average, 29.5% ± 1.7% of the methylated cytosines in a given sample showed robust methylation, as they were methylated in all eight samples, despite behavioral, reproductive, and genotypic differences among samples. Additionally, the percentage of sequencing reads indicating methylation was higher for the cytosines that were methylated in all samples (58.2% ± 0.4%) than for those that were methylated in only a subset of samples (17.4% ± 1.9%). Strikingly, 99.3% ± 0.1% of the cytosines with more than 60% reads indicating methylation were methylated in all samples (Figure S2). This suggests that DNA methylation is not only robust across samples but also within samples, hence across individual brains. However, to more definitively assess variation in DNA methylation across

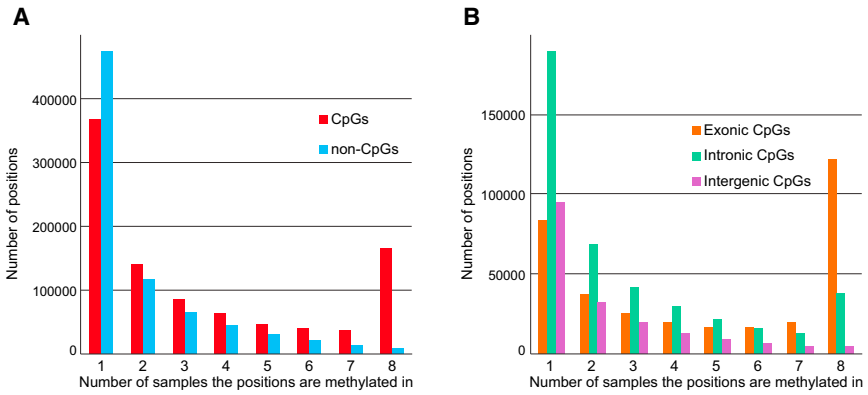

Figure 1. Robust Methylation Is Context and Location Dependent

The graphs show the number of methylated cytosines that are methylated in different numbers of samples (from one to eight) for CpG and non-CpG contexts (A) and for exonic, intronic, and intergenic CpGs (B). Most methylated cytosines are methylated in one sample only (random or sample-specific methylation) or in all eight samples (robust methylation). See also Tables S1 and S3 and Figure S2.

(A) Levels of robust methylation differ between CpG and non-CpG contexts, as illustrated by the sharp increase observed between seven and eight samples for CpGs, but not for non-CpGs.

(B) Levels of robust methylation differ across genomic locations: DNA methylation is more robust in exons compared to introns (sharper increase between seven and eight samples for exonic CpGs than intronic CpGs) and in introns compared to intergenic regions (increase between seven and eight samples for intronic CpGs, but not intergenic CpGs).

individuals would require very deep sequencing coverage from single brains.

The degree of robust DNA methylation differed between CpG and non-CpG contexts and across genomic locations. While 164,258 CpG positions (41.3% ± 2.2% of the methylated CpGs) were methylated in all eight samples, only 9,047 non-CpG positions (4.8% ± 0.4% of the methylated non-CpGs) were methylated in all samples, revealing that CpG methylation is more robust than non-CpG methylation (Figure 1A). Similarly, while 121,858 exonic CpGs (60.9% ± 3.8% of the methylated exonic CpGs) were methylated in all eight samples, only 38,036 intronic CpGs (26.2% ± 1.5% of the methylated intronic CpGs) and 4,364 intergenic CpGs (8.3% ± 0.5% of the methylated intergenic CpGs) were methylated in all samples, revealing that DNA methylation is more robust in exons compared to introns and in genic (exons and introns) compared to intergenic regions (Figure 1B).

The comparison between genes with and without robust methylation revealed that genes with robust methylation (i.e., with at least one cytosine methylated in all eight samples) were significantly enriched for gene ontology (GO) terms related to core processes, such as DNA repair; RNA binding and processing; and protein translation, folding, transport, and binding (Table S3). Genes with robust methylation also were more expressed than genes without robust methylation (Wilcoxon rank-sum test, $W = 5{,}216{,}694$, $p < 0.0001$). More generally, there was a positive relationship between the level of expression and the level of methylation (Spearman rank-correlation test, $rho = 0.59$, $p < 0.0001$; Figure 2A). DNA methylation may preferentially target highly expressed genes and/or DNA methylation may enhance gene expression.

DNA Methylation Is Not Associated with Reproduction and Behavior

To determine whether parts of the *C. biroi* methylome are associated with reproduction and behavior, we performed two analyses to investigate whether DNA methylation differs between brains of age-matched ants in the reproductive phase and in the brood care phase. First, we compared the proportion of methylated reads between the two phases for each CpG. There was no CpG for which the proportion of methylated reads significantly differed between phases after correcting for multiple testing (all p values > 0.22). Second, we used the methylation status of each CpG (methylated or not methylated) to calculate the number of CpGs that were methylated in all four samples from one phase and not methylated in all four samples from the other phase. Then we determined whether such a number of differentially methylated CpGs could be expected by chance by repeating the analysis for all possible sample randomizations. We found 1,560 differentially methylated CpGs between the reproductive phase and the brood care phase, while random comparisons returned an average of 1,727 ± 222 differentially methylated CpGs (median = 1,705; ranging from 1,418 to 2,115; Figure S3). This suggests that the 1,560 apparently differentially methylated CpGs were false positives. Therefore, our analyses did not detect any significant differences in DNA methylation between brains of ants in the reproductive phase and brains of ants in the brood care phase.

In line with the finding that DNA methylation is not associated with reproduction and behavior in the context of colony cycles in *C. biroi*, there was a strong negative relationship between the level of DNA methylation and the level of differential gene expression. Genes that were differentially expressed between the reproductive phase and the brood care phase had fewer methylated sites, while genes with a stable expression between phases tended to be more methylated (Spearman rank-correlation test, $rho = -0.32$, $p < 0.0001$; Figure 2B). Because our analyses did not detect differentially methylated CpGs and DNA methylation is less likely to be found in genes that are differentially expressed between phases, it is unlikely that DNA methylation is involved in the regulation of the clonal raider ant colony cycles.

Re-evaluating the Evidence for Caste-Specific DNA Methylation

Our finding that DNA methylation is robust and not associated with changes in reproduction and behavior in *C. biroi* seems to contradict previous studies that reported DNA methylation differences between the queen and worker castes in four social insect species. Although the findings of caste-specific DNA methylation have been reviewed extensively in the

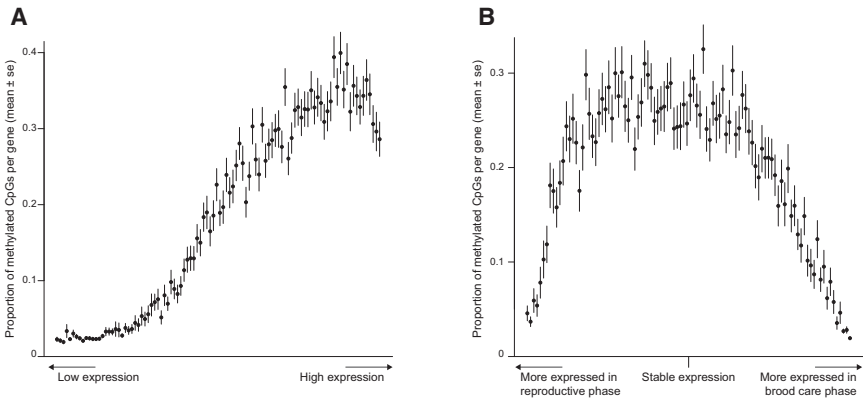

Figure 2. Relationship between DNA Methylation and Gene Expression and between DNA Methylation and Proportional Change in Gene Expression between the Phases of the Colony Cycle

(A) There is a positive relationship between the proportion of methylated CpGs per gene and gene expression. Genes were ranked according to their mean expression across the eight samples before being divided into 100 bins. For each bin, we plotted the mean ± SE proportion of methylated CpGs per gene.

(B) Genes with stable expression between phases tend to be more methylated than genes with differential expression. Genes were ranked depending on how differential their expression was before being divided into 100 bins: in the center are genes with stable expression, on the left those that are more expressed in the reproductive phase compared to the brood care phase, and on the right those that are more expressed in the brood care phase compared to the reproductive phase. For each bin, we plotted the mean ± SE proportion of methylated CpGs per gene. See also Table S2 and Figure S1.

literature [12–27], there are only four empirical studies that used whole-genome bisulfite sequencing to report such differences in ants and bees [1–4]. All those studies investigated differential methylation using the same statistical method, which does not require biological replicates but is prone to producing false positives stemming from sample-specific DNA methylation.

We used the *C. biroi* methylome to assess the validity of the statistical method used in previous studies. First, we investigated whether sample-specific DNA methylation occurred in *C. biroi* by comparing DNA methylation across the eight samples. We found that, on average, 105,321 ± 18,935 cytosines (17.8% ± 2.7% of the methylated cytosines) and 46,027 ± 6,453 CpGs (11.5% ± 1.3% of the methylated CpGs) showed sample-specific DNA methylation. Second, we applied the statistical method used in previous studies to our own data (Supplemental Experimental Procedures). Instead of performing one analysis with four replicates, we performed four separate analyses, each comparing the reproductive phase and the brood care phase of one source colony. We found several hundred differentially methylated exons between the phases for all four source colonies (Figure 3), which is in striking contrast to our combined analysis of the four replicates. However, overlapping the results from the four separate comparisons revealed no exon that was consistently significantly differentially methylated between the two phases in all four analyses (Figure 3). This shows that the lists of differentially methylated exons generated by the statistical method used in previous studies are random or colony specific, and they likely stem from sample-specific DNA methylation.

To our knowledge there are only two empirical genome-wide studies of DNA methylation in social insects that used a replicated experimental design to test whether methylation differs between queens and workers in honeybees [28], *Dinoponera* ants, and *Polistes* wasps [29]. Neither of the two studies detected significant differences in DNA methylation between queen and worker brains (Supplemental Experimental Procedures), which is consistent with our finding that brain DNA methylation does not differ between the reproductive and brood care phases in the clonal raider ant.

Conclusions

The use of biological replicates allowed us to conduct a proper study of the brain methylome of the clonal raider ant *C. biroi*. Our analysis reveals that a large proportion of methylation is robust both across and within samples, especially in exonic CpGs of highly expressed genes involved in general processes. We also report that DNA methylation is unlikely to be involved in regulating the reproductive and behavioral dynamics of the *C. biroi* colony cycle. Finally, evaluating the statistical method used in previous studies with our data indicates that there currently is no empirical evidence for genome-wide variation in DNA methylation associated with the queen and worker castes in other social insect species. Such a lack of well-supported evidence does not necessarily imply that caste-specific methylation does not exist, but rather calls for more controlled and carefully replicated studies of DNA methylation in insect societies.

EXPERIMENTAL PROCEDURES

Sample Preparation

In *C. biroi*, the presence or absence of larvae triggers the switch between the phases of the colony cycle [30]. We used this effect of the larvae to prepare the samples for our study. We first collected 500 callow (recently eclosed) workers, which are light-colored age-matched ants, from a source colony in the brood care phase. We split those callows into two subcolonies, from one of which we removed all the larvae. The subcolony with the larvae remained in the brood care phase, while the other entered a new reproductive phase. We then waited a complete cycle (circa 34 days) until the two subcolonies were again at opposite ends of the cycle. The subcolony in the brood care phase was flash frozen 6 days after the ants started foraging, while the subcolony in the reproductive phase was flash frozen when the first eggs were laid. Thus, the ants collected in the brood care phase and in the reproductive phase were the same age, and they were morphologically and genetically identical (all came from the same source colony, i.e., the same clonal genotype).

For each subcolony, we dissected the brains of 30 individuals with two ovarioles [8], pooled 20 brains to extract DNA for whole-genome bisulfite sequencing, and pooled ten brains to extract RNA for RNA sequencing (see the Supplemental Experimental Procedures for DNA and RNA extraction protocols). We repeated this entire process four times using four different source colonies spanning two clonal lineages: source colonies A1 and A2 (C1 and C16 from clonal lineage A or MLL1 in [31]), and B1 and B2 (STC1 and STC6 from clonal lineage B or MLL4 in [31]). This resulted in eight DNA samples and eight

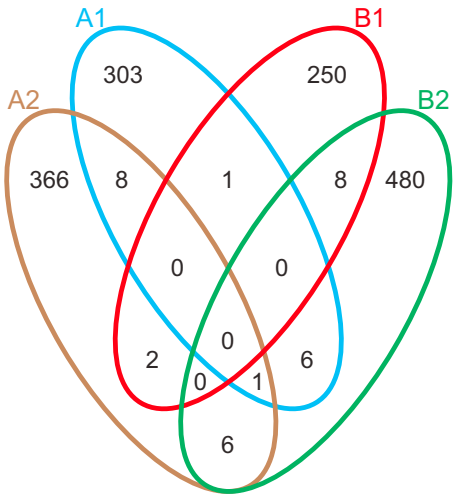

Figure 3. The Lists of Differentially Methylated Exons Returned by the Statistical Method Used in Previous Studies without Biological Replicates Are Random or Colony-Specific Lists of Exons

This graph shows the number of differentially methylated exons between the reproductive phase and the brood care phase for each source colony: 319 in colony A1, 383 in colony A2, 261 in colony B1, and 501 in colony B2 (see details in the Supplemental Experimental Procedures). There was no exon that was consistently differentially methylated between phases in all four source colonies. This shows that the statistical method used in previous studies, especially when used without biological replicates [1–4, 6], is prone to return random or colony-specific lists of exons. See also Figure S3.

RNA samples (four in the reproductive phase and four in the brood care phase for both DNA and RNA).

Library Preparation and Sequencing

Library preparation for whole-genome bisulfite sequencing and RNA sequencing, sequencing, and post-processing of the raw data were performed at the Epigenomics Core at Weill Cornell Medical College (see the Supplemental Experimental Procedures for details). Each phase and each clonal lineage was equally represented in each of the two batches of library preparation and sequencing.

Methylated Cytosines

For each position with coverage $\geq$ 10 in each sample (on average 63.6% ± 4.6% of the cytosines had a coverage $\geq$ 10), the methylation status (methylated or not methylated) was determined by comparing the proportion of sequencing reads indicating methylation (methylated reads) to a binomial distribution, where the number of trials is the number of reads (coverage), the number of successes is the number of methylated reads, and the probability of success is the conversion rate of the bisulfite sequencing treatments. If the proportion of methylated reads could not be explained by chance ($p < 0.05$ after correcting for multiple testing [32]), the position was considered methylated. If it could, the position was considered unmethylated.

Differentially Methylated CpGs

Quantitative Method

For each CpG with coverage $\geq$ 10 in all samples, we performed a paired t test to compare the proportion of methylated reads between the reproductive phase (four replicates) and the brood care phase (four replicates), and then we corrected the p values for multiple testing [32].

Permutation Method

We counted the number of CpGs with coverage $\geq$ 10 in all samples that were methylated in the four samples of one phase but unmethylated in the four samples of the other phase. We then compared this number to the numbers for all possible combinations of four and four samples to assess the number of differentially methylated CpGs that could be expected by chance.

ACCESSION NUMBERS

The accession number for the sequencing data reported in this paper is NCBI BioProject: PRJNA304722.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.12.040.

AUTHOR CONTRIBUTIONS

R.L., P.R.O., L.K., and D.J.C.K. designed the experiments. R.L. conducted the experiments. R.L. and P.R.O. analyzed the data. R.L., P.R.O., L.K., and D.J.C.K. wrote the manuscript.

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