EPIGENOME INTERACTIONS IN COMPLEX NEUROGENETIC DISORDERS

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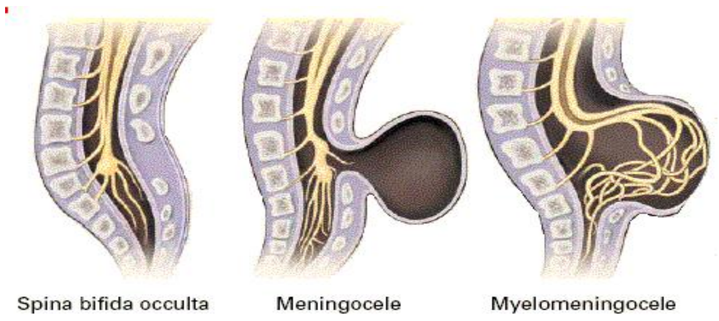
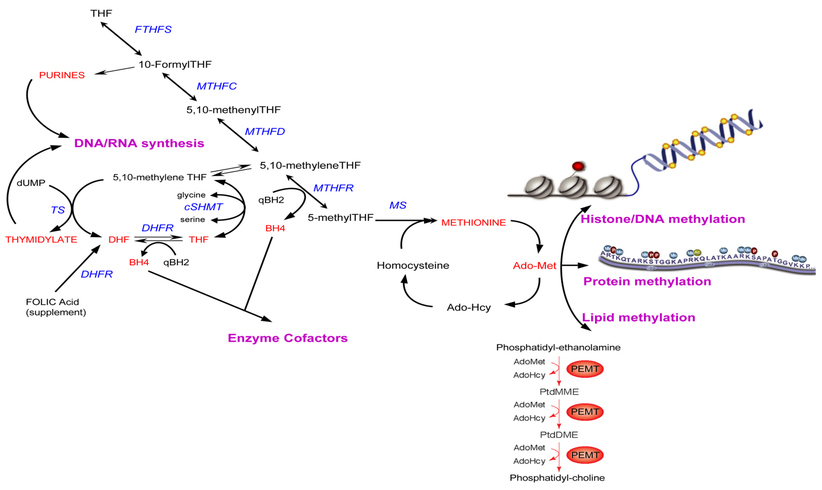
Introduction

Neural tube defects (NTD), such as spina bifida, affect 0.5-1 per 1,000 live births worldwide. In humans, folic acid has the ability to reduce the occurrence of NTDs by as much as 70%. However, there is evidence that excessive folate in certain genetic backgrounds can increase (rather then prevent) risk for NTDs.



Folate Metabolic Pathway is the Major source of methyl donor SAM



Both Genotype and Diet Alter Folate Metabolism & NTD risk

Mutations in the WNT signaling pathway lead to NTDs in mouse. Gain and loss of function mutations in the low density lipoprotein receptor-related protein 6 (Lrp6) respond differently to folic acid (FA) supplementation. Crooked tail (Cd) mice ($Lrp6^{Cd/+}$) bear a GOF mutation and are rescued from NTD by FA. NTDs in heterozygous Lrp6 nulls ($Lrp6^{+/-}$) are exacerbated by FA. Thus we intend to look for epigenetic-driven differences in NTD risk driven by diet and genotype, to obtain methylation patterns that may indicate loci with epigenomic dysregulation in human NTD patients. Our goal is to combine mouse with human data to probe the relationship between DNA methylation and expressivity of NTDs in genetically susceptible individuals. Eventually, a carrier-screening test for women of child bearing age may indicate their optimal NTD prevention regimen.

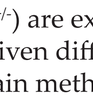
Genomic & Computational Methods

ERRBS
- Enhanced Reduced Representation Bisulfite Sequencing
- Assays DNA methylation at base-level resolution
- Targets regions enriched for CpG dinucleotides- CpG islands (CpGi's) and surrounding regions (shores)

methylKit and eDMR algorithms
- R packages designed by Mason lab for methylation analysis

RNA-seq
- Sequencing assay for quantification of expression over entire genome

limma-voom
- R package for RNA-seq analysis

Mouse Samples 
- Mothers fed on diet containing either 2ppm or 10ppm folic acid
- Lrp6 KO samples ($Lrp6^{+/-}$,$Lrp6^{+/+}$)
- Lrp6 CD samples ($Lrp6^{Cd/+}$,$Lrp6^{+/+}$)

Whole tissue from embryonic mice (E9.5)
- Four replicates for each biological condition (64 animals)
- Gender balanced

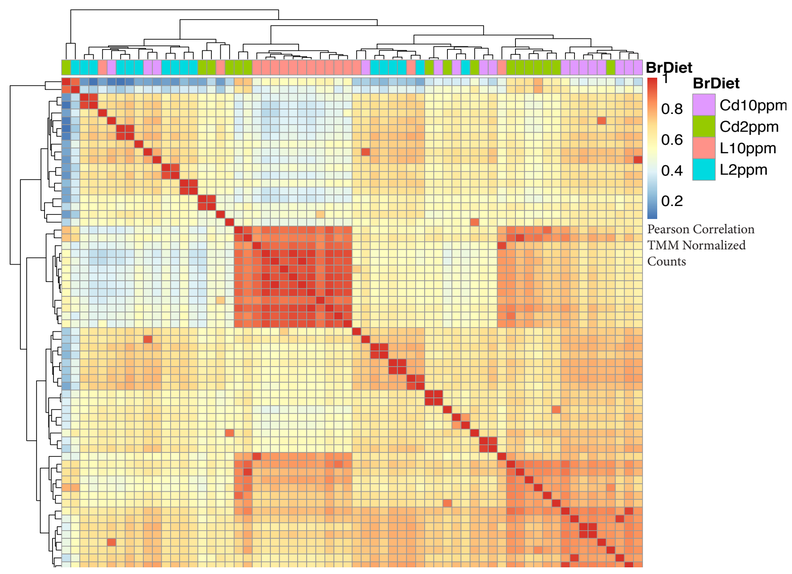
Blood from postnatal mouse pups, day 2 (P2)
- 4 replicates for each KO condition
- 6 replicates for each Cd Condition
- Not gender balanced

Human 
- Blood samples from US NTD collections
- WGS Illumina Sequencing
- 129 cases, 164 related controls
- 108 unrelated Qatari Controls

Results

Genetic Background and FA have Large Effect on Gene Expression in E9.5 Mice

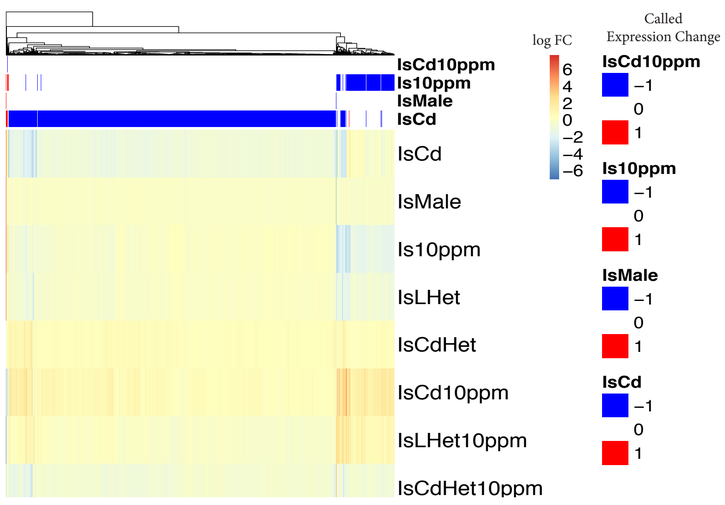
# Genes (q<0.05)	IsCd	IsMale	Is10ppm	IsLrp6$^{Cd/+}$	IsLrp6$^{+/-}$	IsCd 10ppm	IsLrp6$^{Cd/+}$ 10ppm	IsLrp6$^{+/-}$ 10ppm
Under-expressed	2653	2	408	0	0	1	0	0
Over-expressed	22	4	11	0	0	2	0	0



Top 10 FA DEGs	logFC	q-value
Ftl1	**-5.373418841**	**1.74E-05**
Lars2	**2.979358198**	**0.003068425**
Cd4	-5.970177381	0.001155491
Sox3	-1.83060133	0.013355498
Gm1943	1.891594492	0.021958133
Hipk4	-3.931997359	0.012745624
Vill	-1.827438277	0.02217474
Rmrp	-2.840377421	0.02217474
Mdfi	-2.531792626	0.02217474
Scoc	1.052890556	0.022485895

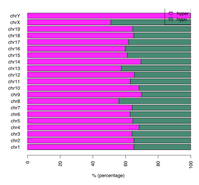
Genes with Cd/FA Interaction	logFC	q-value
Ftl1	**6.167921521**	**0.000131615**
Lars2	**-3.915483725**	**0.017925452**
Rps3a1	3.406238565	0.029101235

limma-voom,
Empirical Bayes, F-test
Benjamini-Hochberg correction, q-value<0.05



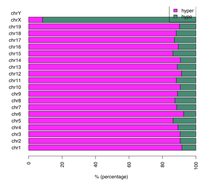
Lrp6$^{+/-}$ and Lrp6$^{Cd/+}$ alleles Induce Hypermethylation in P2 mice

Lrp6$^{+/-}$ vs. WT
2ppm Folic Acid Diet



4,496 Differential CpGs
2,869 hypermethylated
1,627 hypomethylated

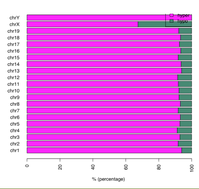
Lrp6$^{Cd/+}$ vs. WT
2ppm Folic Acid Diet



8,723 Differential CpGs
7,572 hypermethylated
1,151 hypomethylated

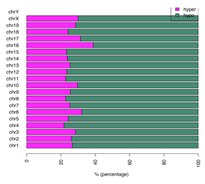
Elevated FA diet Increases Hypermethylative Effect of Lrp6$^{+/-}$, Reverses Effect in Lrp6$^{Cd/+}$

Lrp6$^{+/-}$ vs. WT
10ppm Folic Acid Diet



51,113 Differential CpGs
46,949 hypermethylated
4,164 hypomethylated

Lrp6$^{Cd/+}$ vs. WT
10ppm Folic Acid Diet



13,442 Differential CpGs
3,467 hypermethylated
9,955 hypomethylated

Comparisons
Methylation Change >25%
methylKit Wald Test (Logistic Regression)
FDR correction, q-value <0.01

Results (Cont'd)

Re-analysis of P2 Methylation Data Shows Genes with Consistent Epigenetic Interaction with FA

#DMCs (q<0.01)	IsCd	IsMale	Is10ppm	IsLrp6$^{Cd/+}$	IsLrp6$^{+/-}$	IsCd 10ppm	IsLrp6$^{Cd/+}$ 10ppm	IsLrp6$^{+/-}$ 10ppm
Hypo-methylated	2324	139	1378	0	0	5	0	0
Hyper-methylated	2226	4	5542	0	0	178	0	0

Genes with DMCs in Promoter & DE as a result of FA
1810019J16Rik
ADAM11
ARSI
cyp26c1
DKKL1
Espn
Hmg20b
nudt22
relA
SLC26A10
Sstr4

Genes with Expression affected by FA in Embryonic Mice are Enriched for Variants in Human NTD Cases

SNPs and Indels in the human samples were filtered for common variants (MAF<0.05) in public repositories. The cases were additionally filtered for variants found in either control cohort. Homologs of the 419 genes whose expression was affected by FA in the E9.5 mice were then tested to look for enrichment in the human cases. Taken as a set, they were enriched (q-value= 2.481E-40, logistic regression). There were additionally 13 genes which showed enrichment individually (q-value<0.05, logistic regression):

Gene	q-value (logistic regression)
Triobp	0.001582398
Tcf3	1.12341E-07
NRXN1	0.018974215
MRAP2	3.49179E-39
KDM4B	1.25739E-05
Lars2	0.005721352
LMTK3	2.58217E-05
INTS1	0.010748032
DHX34	0.001699661
Crocc	0.005179627
Caskin2	0.019563813
CLEC2L	2.28314E-08
AHDC1	0.000787798

Discussion

The effect of genetic background was much larger than anticipated. In both mice cohorts, which were assayed for different epigentic marks at different points in development, it was one of the dominant effects observed. The lack of direct effects of either Lrp6 allele that were detected, as well as the lack of interactions with FA, in both the RNA-seq and integrated methylation analysis was extremely surprising, and may require alternate modeling approaches to capture. In both cohorts, initial analysis failed to detect the effect of FA. Only after correcting for the possible interaction between genetic background and FA that the primary effect was observed. However only three genes were significanly affected by such interactions, none with a known strong tie to Lrp6 or FA metabolism. This suggests that the interactions may be mildly affecting many genes. Ftl1 and Lars2, in addition to showing potential interactions, were also strongly affected by FA over all conditions, and may be candidates for further study. The methylation data suggests that hypermethylation, and hence potentially gene under-expression, are epigenetic lesions associated with the Lrp6 alleles, and hence NTD state. This concords with the RNA-seq, where almost all the observed effects were inhibitory. Similarly the effect of FA was to increase methylation, as expected and in line with previous work. The concensus genes between the mouse and human analysis may point towards FA disregulation, as opposed to deficiency, asa potential driver of NTDs in humans. The collection of further data from the mice will allow us to do analysis within each time point, and more confidenely identify biomarkers that are persistent over development. We are also collecting further human samples, which can be used to validate both our current and future findings.

Conclusions

- Genetic background and dietary FA can have large effects on epigentic landscape, and may have significant interactions with each other
- Evidence for persistent epigentic effects of elevated FA over development in mice
- Strong hypermethylation correlates with NTD in mice
- Potential evidence that disregulation of FA metabolism may increase NTD risk in humans

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Acknowledgments

I would like to thank my mentor Dr. Chris Mason, as well as the vital staff of Pbtech at the ICB. Support was provided by the Tri-Institutional Training Program in Computational Biology and Medicine (via NIH training grant 1T32GM083937).