Structural Forms of the Human Amylase Locus and Their Relationships to SNPs, Haplotypes, and Obesity

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**Structural forms of the human amylase locus
and their relationships to SNPs, haplotypes, and obesity**

A dissertation presented

by

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to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

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Abstract

Hundreds of human genes reside in structurally complex loci that elude molecular analysis and assessment in genome-wide association studies (GWAS). One such locus contains the three different amylase genes (*AMY2B*, *AMY2A*, and *AMY1*) responsible for digesting starch into sugar. The copy number of *AMY1* is reported to be the genome's largest influence on obesity, yet has gone undetected in GWAS. Using droplet digital PCR (ddPCR), sequence analysis, and optical mapping, we characterized eight common structural forms of the amylase locus, their mutational histories, and their relationships to SNPs. We found that *AMY1* copy number has a unique distribution undetectable to earlier methods that can be understood from an underlying set of structural forms and their allele frequencies. Despite a history of recurrent structural mutations, *AMY1* copy number has maintained partial correlations to nearby SNPs; these SNPs do not associate with body mass index (BMI). To directly test for association, we measured amylase gene copy number using ddPCR in 1,000 Estonians selected for being either obese or lean and in two cohorts totaling ~3,500 individuals using sequence analysis. We had 99% power to detect even the lower bound of the reported effects on BMI and obesity, yet found no association. This study model of using multiple methods to analyze the copy number, structural haplotypes, and surrounding SNP haplotypes of multi-allelic variants will likely facilitate more robust disease association results in future studies.

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Introduction

Human genomes have thousands of deletion and duplication polymorphisms. These so-called copy number variants (CNVs) cause as many as ~0.78% of base pairs to differ in copy number between any two individuals' genomes¹. The first indication that these copy number alterations could influence human phenotypes came from sporadic diseases, now termed genomic disorders, caused by *de novo* structural alterations². The dominant mutations responsible tended to alter gene dosage and occur in regions of genomic instability caused by segmental duplications and low copy repeats³. It was not long until some inherited genomic disorders and mendelian diseases were linked to inherited CNVs in linkage studies⁴⁻⁶. However, despite the evidence of inheritance, early studies of CNV in normal individuals still referred to common CNVs as recurring, as if these CNVs had independent origins⁷ and were affecting as yet undefined phenotypes.

Though rare and *de novo* CNVs have well-known roles in disease, with many having strong odds ratios of 2–30⁸⁻¹⁰, we now know from microarray-based genome-wide CNV studies in phenotypically normal populations that most of the CNV in any individual's genome arises from a reservoir of polymorphisms that are common¹¹, stably

inherited¹¹, and mostly benign¹². Most deletions and simple duplications are now routinely assessed in genome-wide association studies, either directly with arrays¹³ or with imputation^{14,15}. However, there is a class of CNV that has been left behind by these genomic advances.

Multi-allelic CNVs

Out of the ~8500 CNVs found in Phase 1 of the 1000 Genomes Project¹⁶, 1,356 were more complex than simple deletions and duplications. These multi-allelic CNVs (mCNVs) varied widely in copy number, in patterns that imply the existence of three, four, five, or more segregating alleles. In that particular data set, 121 appeared to have four or more alleles, and 45 appeared to have five or more¹⁶.

When these multi-allelic CNVs are visualized by fiber FISH, they have often been found to involve tandem or inverted duplications of a genomic segment^{17,18}. Some of these duplications have been estimated (from sequencing data) to have up to about 50 copies, though the great majority appear to be present in copy numbers of 0–12^{16,19}. Many mCNVs are more complex than copy number can quantify, because they also involve additional duplications, deletions, and inversions, sometimes affecting multiple genes^{11,19-22}. An apt example is the 17q21.31 region, associated with fertility²³ and neurological diseases^{24,25}. It has nine alleles that affect five genes through various numbers of duplications, sequence changes, and a single large-scale inversion^{21,22}. It is one of the only mCNVs to be assembled into haplotypes on a sequence-level; however, investigations into other loci, such as the amylase locus, *FCGR3B/3A*, *CCL3LI*, and *C4*, indicate that mCNVs commonly have other variants within them^{17,26-29} (**Figure I-1**).

Figure I-1. Examples of known haplotypes at two mCNVs. The 17q21.31 haplotypes were assembled from copy number data²¹ and confirmed with fosmid sequencing²². The *CCL3L1* haplotypes were assembled using Fiber FISH on DNA from five individuals^{17,29}. In both mCNVs, the copy number of multiple genes is being affected and multiple DNA segments are being shuffled.

The large sequence modifications characteristic of mCNVs can disrupt the genes that overlap them, influencing disease and evolution in a variety of ways. All CNVs, simple and multi-allelic, may change the coding sequence of a gene^{30,31}, create paralogs that can diverge to create new or specialized functions³², and alter the overall gene expression level^{31,33,34}. mCNVs can have an even greater impact because they can have many duplications, and these effects are multiplied when several genes and non-coding elements are in the same mCNV locus, all being affected by the same, or different, disruptions. In fact, mCNVs account for 88% of the gene dosage variation in humans¹⁶.

In theory, an mCNV's impact on a disease should be easier to parse than that of a SNP. Many mCNVs have of a series of alleles with different numbers of gene duplications, creating an allelic series of growing phenotypic impact and testable hypotheses about how the mCNV is contributing to the phenotype. Their multi-allelic nature also ensures that they cannot be in high LD with any single SNP. Thus, their signal of association is easier to pinpoint in the genome, and their roles in disease are easier to decipher once an association has been found.

Yet only a handful of mCNV associations have been reported and most were ascertained through candidate gene studies, with only a few genes receiving most of the attention due to their known roles in immunity and disease progression: *FCGR3B* (binds the Fc region of gamma immunoglobulins), *CCL3L1* (ligand of the co-receptor for the HIV virus), beta-defensins (cluster of microbicidal and cytotoxic peptides), *HBA1/2* (α -chain of hemoglobin), and *C4* (part of the complement pathway)^{27,35-47}.

On top of the relatively few genes that are being investigated, many of these associations have been resistant to replication in independent cohorts with independent investigators⁴⁸, with some associations disappearing when different genotyping methods were used^{26,49,50} (**Table I-1**^{26,27,29,36-40,42,44-47,49-87}). A compelling example of this can be found in the Wellcome Trust Case Control study, which used an array-based genotyping technology to perform a genome-wide association study on eight common diseases. Despite good copy number measurements (that clustered at integers) and a large sample size (2000 cases), it did not replicate three previously published associations (*FCGR3B* on rheumatoid arthritis, *CCL3L1* on rheumatoid arthritis, B-defensins on Crohn's disease^{39,46,47}).

Table I-1. Reported associations with attempted replications. Nearly every reported disease association has failed to replicate in at least one follow up study.

Small sample sizes may be behind some of these non-replications, but not all. Entries are referenced by the first author and in the above text.

Gene	Disease	First study to find an effect by measuring copy number	Replication - independent cohort	Replication - precise genotyping method
<i>FCG3RB</i>	systemic lupus erythematosus	Fanciulli (536)	Molokhia (134), Mamtani (149), Willcocks (159), Willcocks (171), Aitman (187 sib pairs), Lv (202), Chen (846)	Neiderer (210), Neiderer (240), Morris (365 trios), Neiderer (880)
	Sjögren's syndrome	Mamtani (61)	Halderson (124), Lester (174)	
	anti-neutrophil cytoplasmic-antibody-associated vasculitis	Fanciulli (77)	Willcocks (556)	Niederer (567)
	Rheumatoid arthritis	McKinney (768)	Breunis (112), Mamtani (158), Graf (197), McKinney (250), Marques (518), McKinney (643), Chen (948)	Robinson (1188), Wellcome Trust Case Control Consortium (2000)*
<i>FCGR3A</i>	Rheumatoid arthritis	Chen (948)	Thabet (945)	Robinson (1188)
<i>CCL3L1</i>	HIV phenotypes	Gonzalez (1132)	Bhattacharya (46), Meddows-Taylor (46), Lee (48), Nakajima (95), Larson (153), Dolan (165), Shostakovich-Koretskaya (178), Shao (184), Rathore (196), Huik (208), Dolan (240), Kuhn (460), Gonzalez (470), Urban (1855)	Aklillu (1134)
	Kawasaki Disease	Burns (164)	Mamtani (133), Kim (459)	-
	Rheumatoid arthritis	McKinney (1136)	-	Carpenter (274), Nordang (900), Wellcome Trust Case Control Consortium (2000)
<i>B-defensins</i>	Crohn's disease	Fellermann (85)	Fellermann (165), Bentley (466)	Aldhous (1500), Wellcome Trust Case Control Consortium (2000)*
	Psoriasis	Hollox (179)	-	Hollox (319), Stuart (1396), Stuart (2616)
<i>C4</i>	systemic lupus erythematosus	Yang (233)	Lv (924)	Boteva (501), Boteva (527)
	Type 1 diabetes	Mason (50)	Mason (110), Pani (220 families)	-
	Cardiovascular health	Arason (233)	Blasko (142)	-
<i>A-globin</i>	Severe malaria	Allen (249)	-	Lell (100), Mockenhaupt(301), Williams (655), May (2591)

Red = no association found (p<0.05, unless otherwise stated)

Orange = association found in the opposite direction

Gray = studies that began the work of investigating surrounding LD

(#) = number of cases included in the study, provided to give an estimate of the power each cohort had. Controls normally equaled the number of cases

*Non-significant after an allowance for multiple testing

^Studies with shared cohorts were excluded from this group

Another example is *FCGR3B*'s impact on anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis, where three studies found three different results: association to a lower copy number, association to a higher copy number, and no association at all – despite the studies sharing the same cohort^{26,37,55} (in addition to other samples). These controversies likely have many causes, some global and some locus-specific. However, they most likely stem from the difficulties of measuring the copy numbers of mCNVs⁴⁸ (referred to as “genotyping”) and from not accounting for locus structure.

Imprecise genotypes

While the genotyping of simple CNVs and SNPs is mature – they are routinely analyzed by arrays and imputation^{15,88} – genotyping multi-allelic CNVs requires more sensitivity. An assay measuring the copy number of an mCNV must be able to discriminate within its range of copy numbers. The difference between a copy number of 4 and one of 5 is only 20%, a difference that can be difficult to detect using techniques like real-time PCR, a method that is used for the majority of mCNV association studies⁴⁸.

The rough estimates of copy number that are produced with qPCR can result in a bell-shaped distribution of genotypes, characterized by no clustering at integers^{48-50,89,90}. This distribution can hide technical confounds caused by genotyping-batch effects, DNA-isolation batch effects, and other, unknown factors – some or all of which are suspected to have caused false associations at particular loci^{49,50,89,90} (**Figure I-2**). In addition, by adding noise to an assay, the rough estimates of copy number can further hide the signal of association, requiring larger patient cohorts and more genotyping replications to find the hidden signal. Imprecise genotypes can be avoided by using an appropriate

technology, such as the paralog ratio test (PRT), droplet digital PCR (ddPCR), or sequence analysis (with programs like Genome STRiP). However, these techniques still require knowledge of the genetic structure of the locus.

Figure I-2. Batch effects can lurk in imprecise copy numbers. To the left, a small technical artifact causing the cases' copy numbers to be shifted right can be identified (and accounted for), because the copy numbers of the cases are clustering at integers+0.2, which likely does not reflect real biology. In the imprecise copy numbers to the right, the small technical artifact can appear to be a real effect.

Locus structure

Many mCNVs are not only simple tandem duplications, but are complex, in that they also involve additional duplications, deletions, and inversions^{11,19-22}. The presence and number of these features vary by allele, and designing a copy number assay by only using the reference genome can result in inaccurate genotypes if an assay is in, or crosses a breakpoint of, one of these other variants. Other locus-specific features, such as

extensive homology in the resident genes, can interfere with the genotyping of a single target^{18,43}. In addition, some of these other variants may actually be the causal variant(s), simply transferring a disease association to the target multi-allelic gene through LD, a particularly serious concern for the HLA-located *C4* gene^{27,43,65,68,91,92}, a case where high LD to a plethora of SNPs and CNVs makes it difficult to pinpoint a single variant as causal.

On a positive note, SNPs in the vicinity of mCNVs may offer substantial information that is currently unexploited. SNP genotyping is a mature, reliable technology that has already been applied to millions of genomes^{93,94}. While an individual SNP cannot serve as a proxy for a multi-allelic variant, it is nonetheless likely that the individual structural alleles of an mCNV arose on specific SNP haplotypes. Depending on the mutation rate of the mCNV, as well as the age and number of alleles, the structural alleles may continue to bear relationships to those SNPs, and those SNPs may be used to inform on the copy number or allelic make-up of an mCNV in an individual.

The rationale of the dissertation

With the current controversies revolving around an inability to replicate mCNV associations, I wanted to investigate the unique challenges of associating mCNVs to phenotypes and to propose a study model that would yield replicable results. I hypothesized that this could be achieved by first obtaining precise copy numbers with multiple genotyping methods (to ensure the accuracy of the copy numbers), then characterizing the alleles of the mCNV and their relationship to SNPs and SNP haplotypes, allowing me to exploit that information when testing for associations. With these technical genotyping methods (measuring copy number) and theoretical models

(analyzing structural and SNP haplotypes) working towards the same goal of testing a locus for an association, the study would most likely be resistant to the confounds affecting a single method of experimentation and would produce replicable results.

The amylase locus

In order to investigate the challenges of associating mCNVs to phenotypes, I needed to select a model mCNV that had a range of copy numbers and a likely association to a phenotype. The amylase locus (**Figure I-3**) in humans contains the three related genes responsible for digesting starch into sugar and also contains inversions, deletions, and duplications⁹⁵. Each of the three amylase genes varies in copy number, with *AMY1* varying from 2–17 copies^{18,96}, *AMY2A* from 0–8⁹⁶, and *AMY2B* from 2–6 (as I report below).

Protein structure

The AMY1 protein has 496 amino-acid residues, 1 calcium ion, 1 chloride ion, and 170 water molecules (**Figure I-4**). It folds into a structure consisting of three

domains, A, B, and C. Domain A has a beta/alpha(8) barrel structure; it holds the chloride ion and contains the active site and catalytic residues. Domain B has no definite topology, but it holds the critical calcium ion. Domain C has a Greek-key barrel structure⁹⁷, most likely plays a role in secretion, and contains glycosylation sites⁹⁷⁻⁹⁹. During catalysis, a highly mobile glycine-rich loop near the chloride ion becomes ordered while binding the oligosaccharide and moves in toward the substrate¹⁰⁰. While the kinetic data suggest that the mobile loop may be involved in assisting catalysis during the transition state, crystallographic data suggest that the loop may play a role in the release of the product from the active site¹⁰⁰.

The role of the calcium ion is critical. It stabilizes the secondary and tertiary structure of the enzyme¹⁰¹. However, the role of the chloride ion in the function of amylase appears to be complicated. It is not critical like the calcium ion, but it allosterically activates the enzyme and modulates both maximal activity and pH optima¹⁰². The maximum hydrolysis rate without chloride is 10x lower than the rate with chloride, and that max rate is centered at 6 pH, as opposed to 7 for enzymes with chloride¹⁰³.

Figure I-4. The structure of *AMY1*. The white sphere is the Ca²⁺ ion, which is critical for proper folding, sits near the active site, and the green sphere is Cl⁻, which assists in normal function.

The pancreatic amylases, *AMY2A* and *AMY2B*, share a nearly identical structure and 97% homology with *AMY1*¹⁰⁴. The known differences lie in post-translational modifications. Whereas some salivary amylase proteins are glycosylated⁹⁸, very few of the amylase proteins from the pancreas are⁹⁹, though it is not known why or what the function of the glycosylation is. In addition, the N-terminal glutamine residue of pancreatic amylases undergoes a post-translational modification to form a stable pyrrolidone derivative, presumably to protect the enzyme from digestion¹⁰⁴.

Function

Amylases hydrolyze the 1,4-alpha-glucoside bonds in oligosaccharides and polysaccharides, thus catalyzing the first step in digestion of dietary starch and glycogen (**Figure I-5**). The human amylases tend to cut along the polysaccharides, producing maltose molecules¹⁰⁵, but may also leave longer oligosaccharides. They do not degrade the polysaccharide entirely to glucose molecules, but a single mutation can activate this functionality¹⁰⁶. Instead, they rely on other enzymes, such as maltase-glucoamylase, sucrase-isomaltase, and oligo-1,6-glucosidase, to completely degrade the dietary starch into glucose¹⁰⁷.

Figure I-5. The human amylases hydrolyze the 1,4-alpha-glucoside bond. Red line marks the cut site. They tend to degrade polysaccharides into maltose, though may leave longer oligosaccharides.

Booming amylase expression is found only in the salivary gland (*AMY1*) and pancreas (mostly *AMY2A* and *AMY2B*), though amylases can also be found in the serum, thyroid, liver, lung, and the female genital tract¹⁰⁸⁻¹¹². *AMY1* is expressed in the salivary acinar cells, where it gets packaged in secretory granules and released into the acinar lumen along with MUC5B, MUC7, aPRP, bPRP, DMBT, Cystatin, Histatin, Statherin, LYZ, LPO, LL-37, electrolytes, and water¹⁰⁷. Salivary amylase digests the polysaccharides in the oral cavity, both in solution and bound to the bacteria that constitute plaque¹¹³.

AMY1 is swallowed with the food and some of it is likely destroyed in the stomach, but with a sufficient amount of starch protecting it, up to 50% of the AMY1 proteins are predicted to survive through to the small intestine¹¹⁴. Even in the absence of AMY1, which some newborns naturally and briefly lack¹¹⁵, the pancreatic amylases can digest the dietary starch in the small intestines. In a pathway similar to AMY1, AMY2A and AMY2B are produced in pancreatic acinar cells and packaged into zymogen granules, which are later released into the small intestine¹⁰⁷.

Phenotypic Effects

In the past, *AMY1* was mostly used as a marker for stress. Increased AMY1 amount and activity correlates with cortisol and has been used to measure stress in burn victims, new parents, students, and psychiatric patients¹¹⁶⁻¹²². Though it is usually associated with stress, it would perhaps be more correct to state that it associates with arousal, as good emotions can trigger *AMY1* expression as much as bad emotions¹²³, and exercise can also increase expression¹²⁴.

In addition to functioning as a marker for arousal, amylase has also been investigated for being a marker for cancer. Chromophobe renal cell carcinoma nearly always has deletions in the 1p21.1 region encompassing the amylase genes¹²⁵. In addition, *AMY2A* was identified as a possible tumor-suppressor gene in gastric carcinoma¹²⁶, and autoantibodies against AMY2A are present in cases of pancreatitis and fulminant type 1 diabetes¹²⁷.

Amylase specifically binds with high affinity to several oral commensal streptococcal species, including *Streptococcus gordonii*, *Streptococcus mitis*, *Streptococcus parasanguinis*, *Streptococcus cristatus*, and *Streptococcus salivarius*¹²⁸⁻¹³¹. Amylase-binding bacteria constitute a substantial proportion of the total cultivable flora on human teeth and only colonize the mouths of animals with salivary amylase activity¹²⁸. Amylase binding facilitates biofilm growth *in vitro*, though the addition of saliva inhibits this growth¹¹³. If amylase interacts with microbes in the mouth, it may also interact with microbes in other areas that express it such as the intestines or female genital tract. Indeed, in the mucosa of the genital tract, amylase breaks down glycogen, allowing colonization by *Lactobacillus*¹³².

AMY1 copy number associates with ancestral diet; populations that have eaten starch-based diets having more copies of *AMY1*¹⁸. This implies that *AMY1* copy number may affect phenotypes related to ingesting starch. Swallowing food without chewing, thus giving AMY1 less time and surface area to work, reduces the glycemic response food¹³³. Conversely, people who are classified as having more amylase activity than average in saliva have lower blood glucose concentrations after eating, which the study authors interpreted as meaning those individuals are better adapted for ingesting starch¹³⁴.

Variation in blood glucose levels could affect the propensity for diabetes, and it has been reported that T2D-associated end-stage renal disease (ESRD) in African Americans is significantly associated with a CNV encompassing *AMY2A* and *AMY2B*¹³⁵. It is also possible that *AMY1* could influence diabetes or obesity through its action in the mouth. It has already been shown to alter the oral perception of starch viscosity¹³⁶ and may contribute to how much or little a person eats.

The majority of the studies above were conducted by measuring amylase activity or protein amount. Amylase activity, though most likely more biologically relevant than pure copy number, can fluctuate based on arousal, circadian rhythms, diet, and the time of the most recent meal¹³⁷. Despite the environmental influences, *AMY1* copy number correlates to *AMY1* amount with an r^2 of 0.35–0.5^{18,28,136}, and across time and conditions, *AMY1* amount is relatively robust and stable¹¹⁷.

If amylase copy number does affect a phenotype, it would be a tempting therapeutic target because it is present in an easily accessible part of the body (digestive tract) and has known inhibitors¹³⁸. Amylase inhibitors appear to have some efficacy in reducing the glycemic response to food¹³⁹ and assisting with weight loss¹⁴⁰. Some of these inhibitors are easy to purchase and can mix easily with food¹⁴⁰, which can create popular, yet dangerous, diet crazes – the most well known of which is the white kidney bean incident in Japan, where a weight loss TV program advertised eating food with toasted white kidney beans on it (known to contain an amylase inhibitor). This resulted in 100 hospitalizations for lectin poisoning¹⁴¹.

The poisoning was unrelated to inhibiting amylase. Indeed, there is no known phenotype associated with missing amylase activity. A family with a total deletion in

AMY2A has been found, but it was not associated with any phenotype¹⁴². On a population-scale, some newborns do not express *AMY1* until one month of age and most do not express it at near adult levels until three months of age, with the analyzed newborns displaying no difference in growth, weight, or tolerance of starchy foods¹¹⁵. This indicates that there is likely enough redundancy in the function of the amylase genes to create a robust network that can withstand the deletion of one type. On the other hand, with no reported instances of a complete and life-long depletion in *AMY1* or *AMY2B*, it is possible that these could be embryonic lethal or under extreme negative selection.

Evolution

Though most eukaryotes contain amylase genes¹⁴³, the origin and organization of humans' *AMY2B*, *AMY2A*, and *AMY1* gene set is found only in the apes¹⁴⁴. Though it was the last to be discovered in humans, *AMY2B* is actually the ancestral amylase gene that is shared among mammals, and perhaps, even shared with fungi¹⁰⁴. *AMY2B*-only is found in New World and Old World Monkeys, though Old World Monkey express *AMY2B* in saliva, due to an unknown mechanism¹⁴⁴ (**Figure I-6**).

In the common ancestor of the apes, *AMY2B* duplicated to create a progenitor *AMY1*, and soon after, an endogenous retroviral element (ERV) inserted into the promoter region, conferring salivary expression and creating the modern *AMY1* gene. Then, *AMY1* duplicated into a progenitor *AMY2A*, which soon deleted most of the ERV in its promoter by recombining the ERV's LTR regions – thus forming the modern *AMY2A*¹⁴⁴. About a million years ago, after the divergence from apes, *AMY1* began to duplicate, creating an array of copy numbers in modern humans¹⁴⁴(**Figure I-7**). As evidenced by my research, *AMY2B* and *AMY2A* also duplicated, but to a lesser extent.

Non-allelic homologous recombination (NAHR) is commonly assumed to be the mutational mechanism behind these duplications¹⁴⁵, and models based on NAHR exist that could explain how the ancestral *AMY2B-AMY2A-AMY1* cassette mutated into the human reference sequence's *AMY2B-AMY2A-AMY1-AMY1-AMY2A pseudo-AMY1* cassette¹⁴⁵ (**Figure I-8**). However, multiple NAHR steps would have to occur over several generations without leaving any apparent intermediate haplotypes in the modern populations.

Another likely mutational mechanism could be the set of replication error mechanisms, FoSTes/MMBIR (fork stalling and template switching/microhomology-mediated break-induced replication). In these mechanisms, the DNA replication fork either stalls or collapses and an extending strand invades another replication bubble to continue synthesis for a brief time¹⁴⁶. Unlike NAHR, these mechanisms can result in inversions and only rely on microhomology. However, with only one of the *AMY1* genes inverted, it is difficult to imagine how a mechanism such as this could produce the human reference cassette with only one mutational event, though one is suggested in **Figure I-9**.

Figure 1-8. Model for how NAHR could have generated the human amylase locus. NAHR between the homologous amylase genes could have duplicated them, though it does not explain the inversion and deletion.

Figure I-9. Possible replication fork-linked mutational mechanism.

If the replication fork (marked by solid lines in the shape of a V) stalled or collapsed, the leading strand (dotted line) may invade a nearby replication bubble, copy the *AMY2A* and *AMY1*, switch to the other side of the bubble to copy an inverted *AMY1* before the original fork restarts to finish copying part of the *AMY2A* and all the *AMY1*.

Humans are not the only species to have had duplication events at the amylase locus. Dogs also have duplicated *AMY2B*, resulting in up to 30 copies in a single genome¹⁴⁷. In addition, other proteins in the dog starch metabolism pathway, a maltase and a glucose transporter, are under positive selection. Dogs do not express amylase in their saliva, so these other adaptations may have been necessary to cope with a starch-rich human-influenced diet¹⁴⁷. Mice have also duplicated their *AMY2B* into two versions: one expressed in the pancreas and the other in the salivary glands¹⁴⁸. Coincidentally, the 4 or 5 pancreatic amylase genes are named *Amy2a1–5*, the salivary amylase is *Amy1*, and a pseudogene is named *Amy2b*^{149,150} (**Figure I-10**). There are indications that the copy

numbers of these genes may vary across strains^{151,152}. In addition, there is direct and indirect evidence that salivary amylase is duplicated in other species such as bonobos, gorillas, and orangutans^{18,153}.

As such, I selected the amylase locus for its variation in copy numbers and possible phenotypic impact.

Chapter 1. Measuring the amylase locus

In order to investigate the challenges of associating mCNVs to phenotypes, I needed to select a model mCNV that had a range of copy numbers and a likely association to a phenotype. The amylase locus contains the three related genes responsible for digesting starch into sugar and also contains inversions, deletions, and duplications⁹⁵. Each of the three amylase genes varies in copy number, with *AMY1* varying from 2–17 copies^{18,96}, *AMY2A* from 0–8⁹⁶, and *AMY2B* from 2–6 (as I report here).

Given their role in starch metabolism and *AMY1*'s greater average copy number in three populations with high starch diets¹⁸, it was long hypothesized that *AMY1* copy number plays a role in dietary and metabolic disorders, such as obesity and diabetes, but at the start of this study, nothing convincing had yet been reported (see **Chapter 4** for the recently reported association).

After selecting the amylase locus as a testing ground, I wanted to eventually perform an association. Yet, first I needed to be able to accurately measure copy number, preferably in such a way that I could definitively call an integer copy number for most

individuals. Southern blot and qPCR have both been used previously at this locus, but Southern blot is not high-throughput, and qPCR gives imprecise *AMY1* copy numbers that form a bell-shaped distribution^{18,95}. I decided to use droplet digital PCR (ddPCR)^{154,155} and Genome STRiP^{16,156}, which have been used to precisely measure copy number at other loci in large sample sizes^{16,21}.

In short, ddPCR can be thought of as a qPCR that has been emulsified into droplets. After amplification, the droplets that contain the target sequences are fluorescent, due to the action of the TaqMan assays, and the number of fluorescent droplets is counted. In rough terms, one fluorescent droplet represents one target sequence that was present in the 20- μ l reaction. Thus, ddPCR gives a near-exact estimate of the number of target and control sequences, which can be calculated back to a CNV copy number. The advantage of ddPCR over qPCR lies in the fact that ddPCR counts target and control sequences (as opposed to comparing PCR curves) and does so after amplification (which reduces the effect of differential PCR efficiencies between the control and target regions).

Despite the advantages of ddPCR, it still requires access to ~20 ng of quality DNA. Genome STRiP, a computational method that can determine copy number from sequencing data, can increase the number of known-copy number individuals by utilizing public sequencing databases. This algorithm counts how many sequencing reads align to a target region, compared to those aligning elsewhere. Regions containing more reads than the genome-wide average are called as duplications, whereas those that have less are deletions. This change is quantitative and can be translated into a copy number measurement at the specified locus. This method has the advantage of genotyping any

particular locus relatively quickly (after the sequencing has already been done) and allows for genotyping refinement, in that certain parameters of the algorithms can be adjusted and optimized until the copy numbers cluster at integers.

While Robert Handsaker, the creator of Genome STRiP, used his program to measure amylase gene copy number in 569 individuals sequenced in Phase 1 of the 1000 Genomes Project¹⁵⁷, I used ddPCR to genotype an overlapping set of individuals: 114 parent-offspring trios from HapMap¹⁵⁸. This parallel effort allowed us to separately investigate copy number and to draw our own conclusions – if our conclusions matched, then there was a higher likelihood of our findings being correct.

Optimizing the measurement of *AMY1*

Though ddPCR has worked well on other loci, those loci tended to have fewer than 6 copies^{16,21} (and unpublished findings). *AMY1* has an average of 6 copies and can have as many as 17, which requires an increased level of sensitivity and precision. Using ddPCR worked well for genotyping *AMY2A* and *AMY2B*, but *AMY1* initially gave poor genotypes and, thus, required an extensive amount of optimization, as described below.

(1) The *AMY1* assay: Normally, qPCR assays function the same or better in a ddPCR reaction (unpublished data). However, using a previously published qPCR assay¹⁸ failed to give tight, separate clusters of target-positive, control-positive, and double-positive droplets (**Figure 1-1**). Additionally, the assay failed to give copy number measurements that clustered at integers in both ddPCR and qPCR¹⁸. I ordered and screened four custom assays that were predicted to only bind within the *AMY1* gene (as opposed to within *AMY2B* or *AMY2A*, based on Clustal analysis¹⁵⁹), to amplify efficiently (based on Primer 3 analysis¹⁶⁰), and to avoid SNPs (UCSC Genome Browser¹⁴⁹). This group yielded two assays, *AMY1_assay1* and *AMY1_assay2* (**Appendix B**), that produced tight droplet clusters (**Figure 1-2**) and copy numbers that were closer to integers, though more optimization was needed.

Figure 1-2. Replicability of *AMY1* copy number between technical replicates and different assays. For the most part, *AMY1* copy number is concordant across replicates, more so if the same assay is used, reflecting that there are assay-specific factors at play.

Figure 1-3. Effect of control assay on copy number call. Amylase is in a late-replicating region, whereas *RPP30* is not. This leads to calls that are ~10% below the true value, as estimated by a replication-matched control.

(2) The control assay: A standard commonly used in ddPCR is an assay to *RNaseP Protein P30 (RPP30)*^{154,155}, which is a housekeeping gene located in a genomic region that replicates early in the cell cycle¹⁶¹. However, since the amylase locus is in a late-replicating region, DNA isolated from replicating cells will naturally have less of it than *RPP30* (**Figure 1-3**). This artificially deflates the copy number calls by an

amount corresponding to the percent of cells that had already replicated *RPP30*, but not *AMY1*, at the time the DNA was extracted. Because the raw copy number calls using the *RPP30* probe are about 10% less than those with a replication-matched control, I can calculate the percent of cells caught between early and late replication at ~20% of cells, though the exact number most likely varies by cell line. To counteract the replication effect, I used a control assay targeted to just outside the amylase region, referred to as Near_AMY (**Appendix B**).

- (3) Batch effects: A plate-wide correction factor was applied to each plate to correct for batch effects. The factor was generally between -5 and 5%, and was calculated by averaging the distance to the nearest integer for the best genotypes (the ones with a confidence interval range less than 0.75 and having more than 10% negative droplets, indicating the input DNA was at an optimal concentration). Because the same DNA samples needed different correction factors for different genotyping runs, the factor(s) causing the discrepancy are most likely technical and/or assay-specific.
- (4) DNA concentration: The sometimes high copy numbers of *AMY1* can oversaturate the ddPCR reaction, resulting in an inaccurate genotype call. The likelihood of this happening correlates with the amount of input DNA and the *AMY1* copy number; generally, many samples oversaturated at DNA concentrations greater than 5 ng/μl in the ddPCR reaction. Combined with the fact that samples in the same plate often vary in DNA concentration, one plate-wide ddPCR reaction nearly always resulted in some samples being oversaturated (<10% double-negative droplets) and others under-saturated. Precise copy number calls could only be obtained by performing two reactions – one at a high sample concentration designed to ensure each sample has the

minimum DNA input (1 ng/μl in the reaction) and one at a low sample concentration, to genotype those that oversaturated their first reaction (using a concentration that was determined by evaluating the first run).

- (5) After our initial investigations into 1000 Genomes, we found that *AMY1* copy number was only even when *AMY2A* copy number was even, and odd when *AMY2A* was odd (discussed below). This observation led to a genotyping pipeline where the *AMY2B* and *AMY2A* assays were done first to determine the concentrations of the samples (enabling the high-low *AMY1* genotyping scheme discussed above). Then, if the two *AMY1* runs disagreed on the genotype of a particular sample, the *AMY2A* call was used to decide which *AMY1* call was correct (**Figure 1-4**).

In parallel, Robert Handsaker was optimizing Genome STRiP to work on the low-coverage sequencing data of 1000 Genomes. He optimized the genotyping of amylase by specifying various ranges of base pairs as the “official” *AMY1* duplicated region; certain ranges yielded better copy numbers than others, and the best range was used for genotyping. In this way, he defined to near-base pair resolution which segments of the amylase locus were commonly duplicated. **Figure 1-5** displays these breakpoints for the GRCh37/hg19 human reference sequence. **Figure 1-6** gives the unrounded copy number measurements for both ddPCR and Genome STRiP for the three amylase genes. Though there was still noise present in the measurements, the copy number calls clustered near integers and were concordant between both methods.

Figure 1-4. Copy number distribution during an *AMY1* genotyping workflow.

The top two histograms are for the concentrated and diluted sample DNA concentration runs, respectively. The bottom two are the average of the runs and the *AMY2A*-adjusted copy number, respectively. The bars are colored gray and white for easier viewing. Copy numbers are closer to integers when adjusting for *AMY2A*, indicating that it screens out imprecise *AMY1* copy number calls, whereas simple averaging allows those imprecise calls to distort the good calls.

The distribution of amylase gene copy number

After optimization, we had copy number measurements for 569 individuals of the 1000 Genomes Project¹⁵⁷ (covering three populations: European American, Chinese/Japanese, and West African) and an overlapping set of 114 European American

and West African parent-offspring trios from HapMap¹⁵⁸. These data, which were concordant across overlapping samples, revealed two novel relationships (**Figure 1-7**):

- 1) Individuals are four times more likely to have an even copy number (2, 4, 6, etc.) than an odd copy number (1, 3, 5, etc.) of *AMY1*.
- 2) *AMY1* and *AMY2A* share parity – the copy numbers of *AMY1* and *AMY2A* are almost always both odd, or both even.

These features – which we observed by two independent methods (ddPCR and Genome STRiP) – had not been reported in studies that used lower-precision methods, such as real-time qPCR or array CGH, to measure amylase gene copy number^{18,28,162}.

These copy numbers must have arisen from an underlying set of structural haplotypes of the amylase locus. However, the known alleles could not account for these patterns, particularly the variation in *AMY2B* and *AMY2A* copy number^{18,95,145}. I needed to characterize the novel structural haplotypes and confirm the known ones before I could investigate their relationships with each other and with the surrounding SNPs. Though I could technically perform a phenotype association study using only the copy numbers, I wanted to first investigate the haplotypes and alleles, because they could have held clues on how to better perform such an association study.

The structural alleles of the amylase locus

To determine the structural alleles of the amylase locus, I extended an approach used for the 17q21.31 locus, one of the first structurally complex loci to be resolved into a set of common structural haplotypes^{21,22}. I followed the segregation of each amylase gene's copy number in the 114 father-mother-offspring trios and inferred the copy numbers of the amylase genes on the transmitted and untransmitted chromosomes (a process known as phasing) by comparing the copy numbers between parent and child. Assigning only the haplotypes that had been previously described^{18,95,145} to the chromosomes resulted in successful phasing for only 7% of the trios and 15% of individuals.

In the remaining unphased trios, there were patterns that could only be explained by novel haplotypes – in particular, patterns concerning duplications in *AMY2B* and partial deletions/duplications of *AMY2A* (**Figure 1-8**). When I added the three novel haplotypes that best explained the data to the phasing program, 98% of the 480 European-ancestry individuals in the 1000 Genomes Project had copy numbers that could

be explained. The West Africans had additional, rare novel haplotypes that could not be fully identified with certainty, and when two of these haplotypes were added into the phasing program (totaling 10 haplotypes), 27% of trios and 39% of individuals, in both populations, could be successfully phased, with the vast majority of the remainder having copy numbers that were consistent with multiple combinations of haplotypes.

In summary, I inferred 8 common haplotypes consistent with 5 of the 6 haplotypes previously identified using cosmids^{95,145} and fiber FISH¹⁸, along with 3 novel haplotypes in the European American trios and evidence for additional, rarer haplotypes in the West African trios (**Figure 1-9**). To complete the list of haplotypes, I would need to know the allele frequencies. These frequencies could also provide clues about the evolution of the amylase locus.

Figure 1-9. Evidence for novel haplotypes in other populations. The copy numbers of 1000 Genomes and the GPC cohort (768 European-ancestry individuals) are plotted. Evidence for each novel haplotype is circled in the corresponding color. The haplotypes also in **Figure 1-8** were also found in family-segregation data.

I could not calculate the allele frequencies of the haplotypes from the individuals I could phase, since this would artificially enrich for haplotypes that create unique, phaseable genotypes. Instead, I utilized the individuals carrying haplotype AH2, who can be identified because of their lower *AMY2A* copy number. I selected individuals carrying AH2 from the GPC cohort (768 European-ancestry individuals) and the European Americans of 1000 Genomes (142 individuals total) and identified their other haplotype by subtracting copy numbers. I calculated the frequency of each haplotype in this pool of other haplotypes. Unfortunately, the frequency of AH4 could not be determined this way, since it causes an increase in *AMY2A* that balances AH2's decrease. Instead, its frequency was determined by identifying individuals that carry AH4 (marked by an increase in *AMY2A*) in GPC and 1000 Genomes, and dividing it by the total.

One reason for characterizing the common haplotypes and their frequencies was to discover what was driving the patterns of *AMY1* variation. The enrichment in diploid even copy numbers was caused by 89% of chromosomes carrying an odd number of *AMY1* copies (referred to as *AMY1*-odd haplotypes). The parity between *AMY1* and *AMY2A* was caused by the *AMY1*-odd haplotypes each having one copy of *AMY2A*, while the *AMY1*-even haplotypes have either zero or two copies of *AMY2A*.

Yet, I could not be sure of the order of genes on the haplotypes, which could help determine the evolutionary history of the locus and explain the presence or absence of SNP correlations to copy number – a feature potentially usable in a SNP-based association study. To arrive at the haplotypes presented in **Figure 1-8**, I conducted a literature review to assemble known haplotypes and assembled the novel haplotypes using optical mapping.

Chapter 2. Assembling the alleles of the amylase locus

Published assemblies

To assemble the amylase haplotypes I took two broad approaches: re-analyzing previous literature in light of our discoveries and *de novo* assembly. For the published assemblies, I started with Gumucio et al., who assembled a partial amylase haplotype through restriction mapping¹⁶³. Groot et al. expanded on the work by assembling the first full amylase haplotype with probes¹⁴⁵. Perry et al. followed, with fiber FISH assemblies of four haplotypes¹⁸, and Teague et al. used genome-wide optical mapping on three individuals, with the amylase locus uninvestigated¹⁶⁴. How these haplotypes relate to the ones I found is a story best told through pictures; thus **Figures 2-1–4** step through each study's findings and methods^{9,63-65} and how they relate to the findings of this study. Please note that the intergenic region presented in **Figure 1-8** is divided into two regions in the graphics below, because it was later found to consist of two segments.

AMY2B
 AMY2A
 AMY1
 Intergenic A
 Intergenic B
 AMY2A pseudogene
 ← Inverted

36

Key:

Figure 2-3. Haplotypes AH4 and AH3 in Perry et al. Fiber FISH is a modification of the FISH technique. DNA fibers are stretched on a glass slide and allowed to hybridize with markers. The resulting fluorescence gives the order of markers on the strand. In the original picture (left), red corresponds to *AMY1* and green to *AMY1*'s promoter¹⁸. Because this only informs on the location and orientation of *AMY1*, the haplotypes to the right have only the inferred locations of *AMY2B* and *AMY2A*. Of note, we did not find evidence for the upper haplotype in our samples, and the structure we found for the second-most upper haplotype (AH4) did not agree with this assembly. These two haplotypes could potentially be fiber FISH artifacts (personal correspondence with John Armour).

Key:

Novel assemblies

After analyzing previously assembled haplotypes, I wanted to assemble the haplotypes that had not yet been captured. To do this, I used optical mapping, the technology of Teague et al., but on the BioNano Genomics platform. The techniques are similar, in that strands of whole DNA are stretched and subjected to a restriction enzyme. However, with the BioNano platform, this enzyme leaves fluorescence at the cut sites (as opposed to the Teague et al. platform, where only the intact DNA is fluorescent). This creates patterns that can be used to assemble a whole genome, or pieces thereof, in a manner similar to restriction mapping. Each amylase gene has its own restriction pattern, and because optical mapping uses whole strands of DNA, I can determine the order and orientation of the genes from these patterns.

I selected two individuals who together had two unreported haplotypes (AH2 and AH2B2), one partially assembled haplotype (AH4), and one known haplotype to serve as a positive control (AH3). The BioNano team did the work of extracting and analyzing the DNA, while I worked with Alex Hastie to align the data (**Figure 2-5**). The haplotypes that had already been assembled (AH3 and AH2) were largely consistent. On the other hand, the AH4 structure contradicts the Perry et al. structure (**Figure 2-3**) with one fewer inverted *AMY1* copies, though Perry et al. could have characterized another variant that was not present in our copy number data. In addition, anonymous samples that BioNano had access to, as well as a European American family, contained AH1, AH3, and AH5, which assembled into structures consistent with the known haplotypes. Of note, in several haplotypes, the *AMY2A* pseudogene is inverted. This feature appears to be stably inherited, but has not been confirmed using a second technology.

Figure 2-5. Optical mapping assembly of structural haplotypes. For optical mapping, strands of whole, labeled DNA (nick labeled and backbone stained with YoYo1) DNA are electrophoresed through a NanoChannel array. The array straightens the DNA and the labeling enzyme leaves fluorescence at the nick sites, creating patterns that can be used to assemble a whole genome, or pieces thereof, in a manner similar to restriction mapping. Each amylase gene has its own restriction pattern, so we can determine the order and orientation of the genes from these patterns.

Figure 2-5 (Continued).

As a set, the structural haplotypes suggest the mutational history of the amylase locus. The more common *AMY1*-odd haplotypes (haplotypes containing an odd number of *AMY1*) differ in the number of copies of a cassette containing two head-to-head *AMY1* genes separated by the last 6 exons of *AMY2A* (a pseudogene)(**Figure 2-6**). Frequent non-allelic homologous recombination¹⁶⁵ (NAHR) involving this tandem array could have generated these many structural forms, but that hypothesis is complicated by the fact that the intergenic region varies, something we would not expect in NAHR¹⁶⁵. This variation could be coincidental or it could be the scars left behind by other, unknown mutational mechanisms. Either way, with each *AMY1*-odd haplotype having at least two different structural forms when accounting for the intergenic region, it suggests these haplotypes each have multiple origination events (**Figure 2-7 and -8**).

As opposed to the *AMY1*-odd haplotypes, the *AMY1*-even haplotypes do not appear to have a mutational history in common. The lack of commonalities in this group suggests these haplotypes most likely have separate origination events, one each. A possible exception is AH4B2, which has variation in its intergenic region, though it is unclear whether that indicates two origins or a mutation after the origin.

Given that the more common *AMY1*-odd haplotypes originated multiple times, it was unlikely there were any SNPs that would correlate well with haplotype or with copy number. Polymorphisms within the actual, varying part of the amylase locus proved hard to genotype, and with the multiple structural forms of the amylase locus, surrounding SNPs likely had low correlations with copy number. However, even low correlations can be useful given a large enough sample size. Because of this, I went on to investigate and quantify the relationships the surrounding SNPs have to the amylase locus.

Chapter 3. SNP correlations to the amylase locus

I first visualized the relationships SNPs had with the amylase locus by making a spiderplot using the individuals I could phase ($n = 158$)(**Figure 3-1**). In this plot, the SNP haplotypes on either side of the locus are clustered as a phylogenetic tree, with the amylase structural haplotypes on them plotted at the distal ends.

AH2 clustered on one branch, as did AH2B2 and AH4B2, in both the GPC cohort and 1000 Genomes, suggesting that the structural data are correct: each of these haplotypes has one origin, with the possible exception of AH4B2. Haplotype AH4 did not cluster consistently between cohorts, though it is a low frequency haplotype with less than three instances per cohort.

Unlike the *AMY1*-even haplotypes, the more common *AMY1*-odd haplotypes are intermixed on nearly every branch, indicating that most have arisen at least 7 times (one for each SNP haplotype branch). It is possible that each branch has its own set of haplotype variants, as marked by the intergenic region; however, I did not have a large enough cohort to investigate these sub-groups of haplotypes. Such an endeavor would likely require hundreds of parent-child trios to obtain enough phase-able individuals and a method that can genotype which intergenic regions have which structure.

SNPs capturing structural haplotypes

Table 3-1. The analysis displayed was conducted in the GPC cohort, due to better SNP coverage and genotyping.

Haplotype (AH#)	Best Associated SNP	Minor Allele Freq.	r^2 of SNP	p- value	Replicated in 1000 Genomes?	r^2 of Imputation	p-value	Replicated in 1000 Genomes?
1	rs113922683	0.19	0.22	$<10^{-6}$	yes	0.23	$<10^{-6}$	yes
3	rs34848656	0.26	0.08	$<10^{-6}$	no	0.23	$<10^{-6}$	yes
5	rs1566154	0.24	0.07	$<10^{-6}$	no	0.03	$<10^{-3}$	no
7	s75133138	0.16	0.19	$<10^{-6}$	yes	0.09	$<10^{-5}$	yes
2	rs72694406	0.43	0.32	$<10^{-6}$	yes	0.43	$<10^{-6}$	yes
4	rs12740780	0.12	0.26	$<10^{-6}$	no	0.37	$<10^{-6}$	no
2B2	rs12076610	0.18	0.11	$<10^{-6}$	yes	0.44	$<10^{-6}$	no
4B2	rs79043596	0.17	0.27	$<10^{-6}$	yes	0.24	$<10^{-6}$	no

Table 3-2. The “BMI association P value” refers to data from the GIANT consortium, discussed in **Chapter 4**.

		<i>AMY1</i> copy number association							
Gene	SNP	Minor Allele Freq.	Change in copy number /minor allele		r^2		P value		BMI association P value
			GPC	GoT2D	GPC	GoT2D	GPC	GoT2D	
<i>AMY1</i>	rs4244372	0.33	-1.23	-1.25	0.111	0.118	$<10^{-6}$	$<10^{-6}$	0.09
	rs11577390	0.07	2.08	1.88	0.104	0.089	$<10^{-6}$	$<10^{-6}$	0.13
	rs1566154	0.19	0.90	0.88	0.044	0.038	$<10^{-6}$	$<10^{-6}$	0.11
	rs1930212	0.18	-0.89	-1.05	0.041	0.053	$<10^{-6}$	$<10^{-6}$	0.74
	rs10881197	0.35	-0.66	-0.73	0.037	0.042	$<10^{-6}$	$<10^{-6}$	0.75
	rs2132957	0.03	-1.95	-1.29	0.036	0.022	$<10^{-6}$	$<10^{-6}$	0.73
	rs11185098	0.26	0.70	0.79	0.032	0.035	$<10^{-6}$	$<10^{-6}$	0.80
	rs1999478	0.18	-0.76	-0.92	0.030	0.042	$<10^{-5}$	$<10^{-6}$	0.53
	rs1330403	0.14	0.82	0.75	0.029	0.020	$<10^{-6}$	$<10^{-6}$	0.42
	rs6696797	0.35	-0.60	-0.72	0.028	0.041	$<10^{-5}$	$<10^{-6}$	0.63
<i>AMY2B</i>	rs12076610	0.11	0.80	0.61	0.582	0.479	$<10^{-6}$	$<10^{-6}$	ND
<i>AMY2B</i>	rs11185098	0.26	0.35	0.24	0.207	0.166	$<10^{-6}$	$<10^{-6}$	0.80
<i>AMY2A</i>	rs28558115	0.11	0.90	0.72	0.398	0.270	$<10^{-6}$	$<10^{-6}$	ND
<i>AMY2A</i>	rs11185098	0.26	0.42	0.32	0.154	0.112	$<10^{-6}$	$<10^{-6}$	0.80

Confirming the patterns of clustering in the spiderplot, I did not find a SNP that tagged any structural haplotype with an r^2 greater than 0.32, and imputation fared only slightly better (imputation is a process that involves correlating whole SNP haplotypes with the structural haplotypes) (**Table 3-1**). In both methods, AH2 was the best correlated, with an r^2 of 0.32 for a tag SNP (rs72694406) and 0.43 for imputation. With

its spiderplot clustering, one would expect the correlations for AH2 to be higher, but there were other amylase haplotypes on its branch and AH2 appeared on other branches - at a low frequency, possibly indicating errors in genotyping or phasing.

SNPs capturing copy number

Even if SNPs do not have good correlations ($r^2 > 0.8$) to amylase structural haplotypes, they can still have a correlation with gene copy number, a measurement that is used in association studies. I correlated the *AMY1* copy number of the European-ancestry individuals of the 1000 Genomes Project¹⁵⁷ to their SNP genotypes and found SNPs that had an average difference of 0.6 to 2.0 *AMY1* copies per SNP minor allele. Because of the non-normal distribution of amylase copy numbers, the *P* value of the correlations had to be determined by permutation

tests. The amylase copy numbers were shuffled and a new set of correlations were calculated one million times; the *P* value was the fraction of permutations that produced a better result than the observed result. These tests established that the correlations were statistically significant. These effect sizes and r^2 values replicated in two independent cohorts: 768 and 2,807 European-ancestry individuals in the GPC and Got2D cohorts, respectively (**Figure 3-2 and Table 3-2**). This shows that, despite poor correlations, the SNPs do contain information on the amylase locus.

Figure 3-2. Replication of SNP correlations. SNP correlations to *AMY1* copy number were compared across cohorts, revealing that the effects generally replicate.

Chapter 4. Associating the amylase locus to obesity

After I (with the help of Bob Handsaker and Alex Hastie) finished characterizing the locus, it was time to test it for associations to phenotypes. As chance would have it, during our investigation into the amylase locus, Falchi et al. reported that each copy of *AMY1* decreased the risk of obesity 1.2 fold²⁸, a profound effect considering *AMY1* copy number varies so widely. This effect would explain 11% of the genetic contribution to obesity, far greater than the effect of fat mass and obesity-associated protein (*FTO*) – the largest effect detected thus far – yet amylase went undetected in other association studies, particularly the GIANT Consortium’s GWAS of 339,224 people for body mass index (BMI)^{28,94,166}. This finding provided a good opportunity to accomplish the goal of my dissertation; I could demonstrate how precise genotypes, knowledge of the locus, and utilizing the surrounding SNPs could lead to a well-supported, and potentially replicable, result that would confirm or contradict the previous study²⁸.

I first used the surrounding SNPs to interrogate the locus. Although the SNPs capture only a small fraction of *AMY1* copy number variation, statistical power is the product of linkage disequilibrium (r^2) and sample size. Given the GIANT Consortium’s large cohort of 339,224 individuals^{94,166} and the reported *AMY1* copy number effect on

BMI²⁸, the SNPs most correlated with amylase would be 99% likely to be nominally associated ($P < 0.05$, calculated by Tõnu Esko) in the GIANT GWAS. However, none of the 17 SNPs covered by GIANT reached even nominal significance, and the SNPs as a group showed no trend toward low association statistics (**Table 3-1** and **Figure 4-1**).

Since this lack of evidence for *AMY1*'s association to BMI is indirect, we conducted our own association analyses by directly measuring the copy numbers of the amylase genes using our high-resolution methods in three different cohorts of Europeans. Tõnu Esko did the majority of the statistics, Robert Handsaker ran Genome STRiP, Jennifer Moon did the SNP genotyping, and I led the project.

We began by analyzing 1,000 Estonians selected from a broader Estonian Biobank cohort¹⁶⁷ (51,535 individuals) for

Figure 4-1. SNPs' correlation to *AMY1* copy number and BMI. BMI Data from GIANT Consortium. *AMY1* data from this study. Black points have a significant correlation to *AMY1* ($P < 0.05$, after Bonferonni correction). With an effect as big as what was reported, there was a 99% likelihood of a nominal association. There are some points approaching nominal significance in the BMI study (in the same direction of effect as Falchi et al.). If those effects are real, it suggests a much lower effect size for *AMY1* in BMI.

being in the tails of the BMI distribution – 500 individuals with BMI<22 and 500 individuals with BMI>33, making a lean/obese case-control cohort. The individuals were randomized onto 96-well plates, and we were blinded to their case/control status. We observed an association to the SNPs that were associated with BMI in earlier studies⁹⁴, including SNPs at the fat mass and obesity-associated protein (*FTO*, $P = 3.5 \times 10^{-7}$), SEC16 homolog B (*SEC16B*, $P = 5.3 \times 10^{-4}$), and mitochondrial carrier homolog 2 (*MTCH2*, $P = 9.6 \times 10^{-3}$) loci. For added power to detect a positive-control association, we also tested a group of 11 known obesity/BMI SNPs, referred to collectively as the polygenic score, and found the score was associated ($P = 3.7 \times 10^{-12}$) (**Table 4-1, Appendix B: Table B-2**).

With these positive controls validating the study design and demonstrating power to detect the effect of *AMY1*, I used ddPCR to obtain crisp, integer genotypes of all three amylase genes, again observing the abundance of even *AMY1* copy numbers. With our study design, we had >99% power to detect (at nominal significance) effects as strong as those reported²⁸. However, we did not observe any association between obesity and the copy number of any amylase gene ($P = 0.70$ for *AMY1*) (**Figure 4-2**).

We then analyzed two other cohorts of Europeans - one consisting of 2,807 individuals (1,437 type 2 diabetes cases and 1,370 controls) sequenced to >5x average coverage (GoT2D cohort), and the other of 657 Europeans sequenced to 7x median coverage (InCHIANTI cohort¹⁶⁸, completed with the help of Michael Weedon and Marcus Tuke). Analysis of amylase gene copy number using Genome STRiP again showed the enrichment of even copy numbers, replicating our earlier results and validating the precision of the analysis within the new cohorts. The GoT2D cases and

controls each had 95% power, while InCHIANTI had 77% power, to detect the reported effect²⁸ of *AMY1* at nominal significance. Yet *AMY1* copy number did not associate with BMI in any group ($P = 0.31$ for GoT2D controls, $P = 0.24$ for GoT2D cases, $P = 0.53$ for InCHIANTI), or in a meta-analysis of all 3,464 replication samples ($P = 0.38$). By contrast, SNPs at *FTO* and other loci showed the associations expected given sample size and statistical power.

Table 4-1. The association results of the three cohorts.

Cohort	Sample size	Gene	Variant genotyped	Power	Odds ratio, obesity	<i>P</i> value
Estonian	1000	<i>FTO</i>	rs1558902	0.96	1.61 (1.34-1.93)	3.5 x 10 ⁻⁷
		Polygenic	11 SNPs	>0.99	1.61 (1.41-1.84)	3.7 x 10 ⁻¹²
		<i>AMY1</i>	Copy number	>0.99	1.01 (0.96-1.06)	0.7
					Beta, BMI	
InCHIANTI	657	<i>FTO</i>	rs1558902	0.32	0.41 (-0.04-0.86)	0.07
		Polygenic	11 SNPs	0.64	0.50 (0.20-0.81)	0.001
		<i>AMY1</i>	Copy number	0.77	0.04 (-0.08-0.15)	0.53
GoT2D controls	1370	<i>FTO</i>	rs1558902	0.58	0.03 (-0.02-0.07)	0.22
		Polygenic	11 SNPs	0.91	0.08 (0.04-0.12)	4.2 x 10 ⁻⁴
		<i>AMY1</i>	Copy number	0.95	0.01 (-0.01-0.03)	0.31
GoT2D cases	1437	<i>FTO</i>	rs1558902	0.60	0.03 (-0.01-0.07)	0.21
		Polygenic	11 SNPs	0.93	0.06 (0.02-0.10)	6.3 x 10 ⁻³
		<i>AMY1</i>	Copy number	0.95	0.01 (-0.01-0.03)	0.24
GoT2D meta-analysis	2807	<i>FTO</i>	rs1558902	0.87	0.05 (0.02-0.08)	4.2 x 10 ⁻³
		Polygenic	11 SNPs	>0.99	0.10 (0.06-0.13)	3.9 x 10 ⁻⁸
		<i>AMY1</i>	Copy number	>0.99	0.01 (-0.01-0.02)	0.44

These results contrast with those of Falchi et al., who found that *AMY1* copy number influenced obesity with an odds ratio of 1.19 per copy and changed BMI by -0.15 per copy, both with a P value on the order of 10^{-10} . Though I have not found a definitive explanation for this contradiction (discussed in the **Discussion**), I believe it likely stems from my use of higher-resolution genotyping approaches. Though low-resolution calls cannot cause a spurious association, they may conceal confounds that can.

Though we cannot specify a reason why our results contradict, I did accomplish what I set out to do: propose and utilize a study design that is resistant to the known confounds and can replicate across cohorts and genotyping methods.

Discussion

Discussion of the previous report

My results contrast with those of Falchi et al., who found that *AMY1* copy number influenced obesity with an odds ratio of 1.19 per copy and changed BMI by -0.15 per copy, both with a *P* value on the order of 10^{-10} . Though I have not found a definitive explanation for this contradiction, I believe it likely stems from my use of higher-resolution genotyping approaches.

The previous study failed to see an enrichment in even *AMY1* copy numbers in any of their reported genotyping runs (6868 samples with qPCR). The qPCR assay was purchased from Life Technologies, mapping with undisclosed coordinates to somewhere in exon 1, which is 98% identical to the exon 1 of *AMY2A* and *B*. The copy numbers obtained from this assay do not appear to cluster near integers, based on the raw data in their Figure 3a,b (presented as $\Delta\Delta C_t$, copy number = $6/(2^{\Delta\Delta C_t})$). Despite their assay potentially binding the other amylase genes, their *AMY1* copy number distribution appeared to be shifted left from my distribution. To make my distribution match theirs, I had to multiply my calls by a random number between 0.5 and 1.1, suggesting that their assay was noisier than mine and had a tendency to underestimate copy number.

The copy numbers from their replication runs with ddPCR and read-depth (the algorithm mrCaNaVar) actually clustered near integers and had an enrichment in particular copy numbers; however, only a few samples were analyzed with those alternative methods (32 for read depth and 96 for ddPCR) and no association analyses were conducted using those calls. Two other methods were used for association analysis: an RNA expression microarray using adipose tissue and expression assays using serum. I was unable to find the probe data or the genomic coordinates of the microarray probes (proprietary property of Affymetrix), so I cannot comment on them. However, whether *AMY1* is expressed in adipose appears to be controversial, with some studies finding it^{169,170} and others not¹¹⁰. The expression assay using serum was based on enzymatic activity and utilized a product that had, afterwards, been recalled and discontinued by Roche for producing falsely low results in particular samples.

Though the low-resolution qPCR calls cannot cause a spurious association, they may conceal confounds that can. However, it is difficult for me to pinpoint a single confound that could cause an association in all the cohorts of Falchi et al., because they had multiple cohorts drawn from populations and case/control studies. If it is indeed a spurious association, it may be caused by a constellation of small, hard-to-identify artifacts, perhaps some cohort-specific. I sought to determine how the cohorts were collected, how the DNA was collected, and whether there were different ancestries in the cohort (Falchi et al. stated they were all Europeans, with the exception of the Singapore cohort, and so did not appear to correct for cryptic ancestry differences). Unfortunately, there was not much information available on some of these cohorts. What I found is assembled below:

- TwinsUK. Used by Falchi et al. as a population cohort and as case/control cohort (using the tails of the BMI distribution). It is from the St. Thomas' UK adult twin registry, part of the Wellcome Trust¹⁷¹. This cohort has both DNA from blood and lymphoblastoid cell lines, and I cannot determine which DNA was used. This cohort also appears to contain non-European ancestry individuals, though presumably Falchi et al. screened those out.
- DESIR. Used by Falchi et al. as a population cohort and as a case/control cohort (using the tails of the distribution). It is from The DESIR Study Group¹⁷²: French individuals in a longitudinal study on Diabetes. Falchi et al. used the controls and freshly extracted the DNA, but didn't state whether it was from blood or tissue and I cannot determine which source DESIR normally collects. This group is composed of French citizens, so may contain non-European individuals.
- AOB. Used as a case/control cohort. This cohort is from the Pasteur Institute. Falchi et al. refers to them as French, but the original study states they had collected different subsets of European¹⁷³. The original study did not state what they did to avoid batch effects in DNA extraction, and their cases were defined as either having morbid obesity or early onset obesity (before 6 years of age). Falchi et al. used what appears to be an undefined subset of 563 samples (out of 2796).
- SP2. Used as a case/control cohort. It is a Singaporean-based cohort with a population mix of Chinese, Malays, and Asian-Indians¹⁷⁴. Though it appears to be used in many studies, I cannot find information on the methods of collection. It appears to have originally been a population sample, so presumably Falchi et al used the tails of the BMI distribution to assemble a case/control cohort.

- ABOS. Used in the serum expression assays. This cohort is from Lille University Hospital, France. It does not specify a population on their website (<https://clinicaltrials.gov/ct2/show/NCT01129297>), but Falchi et al. refers to them as French samples.
- Swedish Families. Used as a case/control cohort in the RNA expression assay. I believe the official name is the SOS Sib Pair study¹⁷⁵, in which siblings discordant for obesity were collected along with their families. The original study analyzed gene expression in adipose tissue, and Falchi et al. appears to have re-used that data. I cannot find any information on what the cohort collectors did to prevent batch effects.

We also considered the possibility that our study could have failed to see a real effect. We used an Estonian cohort, which is a population with a slightly different diet than that of other European countries¹⁷⁶, though it appears to be similarly rich in starch¹⁷⁷. In addition, our two European cohorts had an elevated average BMI, and though it is possible that amylase acts in ways that are specific to lean individuals, we saw no evidence for this in our BMI cohorts (**Appendix B, Table B-3**) and note that other BMI-associated variants have tended to associate in ways that are consistent across the BMI spectrum¹⁷⁸.

Discussion of my proposed study model

Fully understanding human genetic variation and its relationship to phenotypes will require characterizing hundreds of complex loci that mutate at high frequencies, causing large-scale changes to gene dosage and confounding older methods of

genotyping. The amylase locus offers several insights to guide future studies of these loci:

- 1) The copy numbers found at any particular locus most likely arise combinatorially from a modest number of structural alleles, and understanding these alleles may assist association studies. In the case of amylase, the knowledge that *AMY1* and *AMY2A* share parity, confirmed by the structural haplotypes, led to better genotypes (**Chapter 1**).
- 2) Structurally complex loci may have both common and rare variation within them, reflecting both ancient and recent mutations, and may be best understood through combinations of analysis methods developed for each, including tagging, imputation, and direct measurement. In the case of amylase, direct measurement was ideal, but if the duplication of *AMY2B* was of scientific interest, the use of tagging or imputation may have been possible due to its evolutionary history.
- 3) Although GWAS may miss or greatly under-estimate the relationships of structurally complex loci to phenotypes, accurately-typed SNP markers are useful for understanding and constraining expectations about the strength of an mCNV's potential effect on phenotypes.

Each of these points relies on having accurate measurements of copy number, which can be difficult when using the older genotyping methods^{48-50,89,90}. Several emerging technologies have promise for acquiring precise copy number measurements that cluster at integers. The paralog ratio test (PRT, a technique that compares the amount of amplification product between a control and target locus that both use the same primers) has been used effectively in mCNV associations, often giving copy number

measurements with enough resolution to detect batch effects^{50,89}, and ddPCR, as shown here, may also be used. However, PRT and ddPCR can only target one locus at a time. As large disease studies based on whole genome sequencing are just beginning, it is likely that the field of mCNV associations will move towards a genome-wide model, much like SNP associations did 10 years ago with the advent of arrays and GWAS methodology. Though there are notable genome-wide CNV studies done with arrays^{57,179}, read-depth algorithms like Genome STRiP will most likely become the genotyping method of choice for mCNVs, because they allow for genotyping refinement, in that certain parameters can be adjusted and optimized until the copy numbers cluster at integers.

Yet, a gestalt approach that accounts for the structural haplotypes and SNP-correlations of the mCNVs will likely still be necessary. mCNVs can contain elements that frustrate both computational and PCR-based approaches, such as stretches of extensive homology and varying breakpoints, elements that can be found and accounted for by the analysis of alleles. The SNPs in the vicinity of mCNVs also offer substantial information that is generally unexploited. While it is true that an individual SNP cannot serve as a proxy for a multi-allelic variant, it is also likely that the SNPs maintain some correlation between the copy number and/or haplotypes of an mCNV. As a result, a SNP's ability to capture an mCNV should not be thought of as a binary 'true' or 'false', but as a continuum. With some phenotype cohorts having as many as 120,000 individuals⁹⁴, even a SNP with a low r^2 can provide information on an mCNV's association.

Querying surrounding SNPs and SNP haplotypes can be used as a preliminary check for association in candidate gene studies, and upon completion of a genome-wide

catalog of SNP to mCNV associations, for assaying mCNVs genome-wide in a hypothesis-blind manner. This analysis method would allow the field to make full use of current patient cohorts and avoid the time-consuming process of measuring the copy number of single candidate genes with PCR-based technologies. These computational results would likely have to be followed by direct measurement of the copy number of the mCNV, but they can serve as a preliminary check to ensure that the disease field's resources are being focused on the most promising candidates.

This study was done with a focus on mCNV disease associations, but these methods could also be applicable to evolutionary biology. Being able to accurately measure the copy number of an mCNV across species, or even across time, may help unravel the evolutionary history of a species or genera. It may be that some mCNVs duplicated in response to the same selection pressure in separate species, resulting in convergent evolution – as in the case of amylase duplications in domestic dogs and humans^{18,147}. Using optical mapping or other methods to assess mCNV haplotype structure may also be used to glean insights into how the mCNV, itself, evolved into its many forms. This could, perhaps, lead to a better understanding of mutational mechanisms, such as in the case of amylase in humans, where the duplications appear to have not have originated from the known mutation mechanisms.

Future Directions

What locus next?

Someone seeking to enter the field of mCNV associations may ask which locus I recommend studying next. I could answer by pointing out loci that are expected to have a phenotypic effect because they contain genes that function in immunity, metabolism, development, the nervous system, or outward appearance. I could also point out genes that have a wide range of copy numbers, which could imply recent selection. However, following these suggestions means using a candidate-gene approach, in which there is a declared hypothesis that a particular gene influences a particular phenotype (or set of phenotypes). Though candidate gene studies have done much in the past, the field of human genetics has been moving towards a hypothesis-blind, genome-wide approach.

To this end, I would recommend a hypothesis-blind approach for finding the next mCNV to study. It is now possible to globally determine which SNPs most correlate to the copy numbers of mCNVs¹⁶. I would cross-reference the most correlated SNPs with GWAS databases to determine their P values for phenotypic associations. After cross-referencing, I would search the SNPs (binned by the mCNV they correspond to) for an enrichment in lower P values for any given phenotype, suggesting that the mCNV may be driving the association. From this, I would assemble a list of potentially phenotypically-influential mCNVs that could be investigated further.

Technologies

As hinted above, locus-specific techniques like qPCR and ddPCR will likely be supplanted by genome-wide approaches such as genome sequencing. With each year, more cohorts of genomes are becoming available, though many are only exome-sequenced. It is easy to assume that exome-sequencing is not suitable for algorithms like GenomeStrip, because the selection process would distort the ratios of reads originating from a CNV versus the surrounding genome. However, it is possible that, though precise measurements may be impossible, there could still be a correlation between read depth and copy number. Across large numbers of samples, this correlation may provide information on the relative copy number status of the individuals.

Genome sequencing and other genome-wide techniques will still need secondary technologies for validating results and addressing technical limitations. Fiber FISH and optical mapping will likely be needed to assemble structural haplotypes, and ddPCR can target smaller regions that are difficult to assess with read depth. Even in situations where a single technology may suffice, a second technology should still be used to validate the initial results. An apt example would be Falchi et al.'s qPCR results. Though they had replicated their genotype calls with other technologies, they had used so few samples in the replications (<96) that it was not apparent how imprecise their qPCR really was.

Quality standards

Having a second technology evaluate and replicate the results of the first technology should be a strived-for quality check, but it is not the only quality standard

that should be used in future studies. To increase confidence in the association results, the copy number genotypes should cluster at integers (and confirm a lack of confounding factors) and SNPs should be utilized in the association as a positive control (if surrounding SNPs are correlated with the mCNV, then they should be modestly associated with whichever phenotype that mCNV is).

However, quality standards should extend beyond study design and into study reporting. At a minimum, the genotyped copy numbers should be clearly displayed within a figure of the paper, in a way that makes assessing their precision simple (no rounding the calls, masking the copy numbers with a box plot, or using a cryptic axis name or log scale). Ideally, all of the copy number calls would be published in the supplemental methods, though ethics and confidentiality agreements may make that impossible. In addition, the genotyping protocol should also be available, not only stated in paragraph form within the Methods; this ensures that no changes to the manufacturer's instructions are left accidentally unstated and that the reader does not miss an important point that was buried in a paragraph.

In the end, the final step of quality control lies with the reviewers and the readers. As a field, we need to be educated and vocal about the need for quality mCNV studies. We need to hold mCNV studies to the same standards we now use for SNPs and simple CNVs. We should also be open to the fact that, though there are controversies, there is a correct answer that we need to find. We cannot assume the original authors are correct, and we cannot assume that all controversial results are wrong. Together, we can investigate the mysteries of the human genome and potentially find therapeutic targets for devastating diseases.

Appendix A. Dissecting the timing & origin of gene expression variation

CONTRIBUTIONS

Tom Mullen: Provided the initial idea for the project and did the majority of the wet-lab work.

Jim Nemesh: Did all of the groundwork computational analysis, such as sequencing read alignments and *P* value adjustments.

Christina Usher: Did most of the analyses for the results, such as allelic imbalance comparisons across genes and across the genome.

ABSTRACT

Human gene expression variation contributes to the etiology of common diseases, yet it is unknown when and how these allelic differences in RNA abundance emerge. To address this, we developed a genome-wide approach (Het-Seq) to measure gene expression within lymphoblastoid cells using seven fractions representing different stages of the RNA lifecycle: nascent, early, nuclear, cytoplasmic, polyA, and ribosomal-bound RNA. We used a custom capture strategy and an informatics pipeline to quantify the RNA abundance from each allele of each gene, allowing us to measure the timing and spatial origin of expression differences. We found that ~75% of variation is already present in nascent RNA, indicating a large role for transcription and a small, prominent role for post-transcriptional mechanisms. Overall, the allele expression differences were modest (less than 1.6 fold), but influenced 20–24% of the genes and correlated with local

genetic variation (r^2 of 0.82). These results indicate an extensive opportunity for new mutations and inherited polymorphisms to modify gene expression.

INTRODUCTION

Genome-wide association studies (GWAS) have mapped hundreds of traits to thousands of single nucleotide polymorphisms (SNPs) and the haplotypes those variants form¹⁸⁰. While more than 80% of phenotype-associated haplotypes overlap with protein-coding genes, fewer than 15% contain a protein-altering allele¹⁸¹. This implies that intronic, synonymous, intergenic, and other untranslated variants are affecting phenotypes, most likely through gene expression. Supporting this idea, several studies have shown that, within a disease-relevant tissue, inter-individual variation in mRNA abundance tends to associate with the same SNPs as the disease^{28,33,182-185}.

Allele-differential expression (ADE) studies, which compare the expression of alleles within an individual, have estimated that 4–30% of genes have unequal expression^{94,186-189}, and expression quantitative trait loci studies (eQTL studies) have found many *cis* variants that could be causing this ADE^{33,182,190-195}. But eQTL studies are not well-powered to detect *trans* effects on mRNA levels and cannot measure the impact of epigenetic variation that is not genetically controlled such as imprinting and random monoallelic expression¹⁹⁶⁻¹⁹⁹.

In addition, it is unknown when in the RNA lifecycle these variants alter expression. Transcription seems to be a key step, with genetic variants affecting transcription factor binding and epigenetic variants influencing the openness of chromatin^{183,184,200-203}, yet SNPs influencing splicing, polyadenylation, decay, and

miRNA have been shown to affect expression levels, whereas other mechanisms, like nuclear export and translational regulation, have been understudied²⁰⁴⁻²⁰⁶. There is most likely a temporal distribution of effect, with some variants affecting transcription and others affecting post-transcriptional processes. This distribution could be determined using an ADE or eQTL study, but previous studies have used total mRNA – thus, mixing the effects of transcription, splicing, polyadenylation, and decay into a single measurement of RNA abundance.

We determined when variants exerted their influence over the lifecycle of RNA and whether those variants were solely genetic. We used two human lymphoblastoid cell lines (LCLs) from the 1000 Genomes Project – GM12878, a female with European ancestry, and GM19240, a female with Yoruban ancestry²⁰⁷. We isolated specific fractions of their transcriptome - nascent RNA (maximum of 5 minutes old), early RNA (60 minutes old), nuclear RNA, cytoplasmic RNA, polyribosome-associated RNA, total mRNA, and total RNA. We took the ratio of the two alleles present for each gene and charted the emergence of allele-differential expression spatially within the cell (using the nuclear and cytoplasmic fractions) and temporally over the lifecycle of RNA. Though this experiment cannot map the variants affecting a gene, it can identify when those variants act, hint at the regulatory mechanisms they modify, and determine whether they tend to be local and genetic.

RESULTS

Experimental dissection of the maturing transcriptome

RNA derived from whole cells reflects the influences of many regulatory mechanisms, including those that act upon transcription, splicing, nuclear export, translation, and mRNA stability. To parse the influences of different regulatory mechanisms, we first dissected the transcriptome into biologically meaningful fractions (**Figure A-1A**), combining many earlier innovations in RNA analysis²⁰⁸⁻²¹⁰. We purified *nascent RNA* by metabolically-labeling new transcripts with a pulse of 4-thiouridine (4SU) for 5 minutes in live cells before RNA extraction. The 4SU-labeled RNAs were harvested by biotinylating the 4SU tag and then capturing on streptavidin-coated beads. We similarly purified *early RNA* by 4SU-labeling for 60 minutes before extraction. We separated *nuclear RNA* from *cytoplasmic RNA* by hypotonically swelling living cells and dounce-homogenizing the resulting suspension, then centrifuging at a force sufficient to pellet nuclei. We further fractionated the cytoplasmic RNA to identify actively translating, *polyribosome-associated RNA* by ultracentrifugation through sucrose gradients, eluent collection, and identification of the polyribosome-containing fractions. Within each fraction, we sequenced total RNA without poly(A) selection, after depleting fractions of ribosomal RNA (rRNA). For comparison to more conventional RNA sequencing techniques, we also sequenced *total mRNA* and *total RNA*.

To evaluate the integrity of each cellular RNA fraction, we analyzed the RNA sequences using both genome-scale and locus-specific analyses (**Figure A-1B**). At the genome scale, the maturity of RNA can be monitored from the portion of RNA arising from intergenic, intronic, and exonic segments of the genome as currently annotated (albeit imperfectly) in the human reference sequence. In genomic DNA, 55% of sequence reads arose from annotated intergenic regions, and only 1–2% from annotated exons,

consistent with the size and number of these features in the reference genome sequence²¹¹. In nascent RNA, only 8% of sequence reads arose from intergenic regions, while 70% arose from annotated introns. This was followed, as the transcriptome matured, by a gradual increase in the contribution of annotated exonic sequences, and a concomitant decrease in the contribution of annotated intronic sequences, reflecting processes of RNA maturation, splicing, and intron degradation. Total mRNA corresponded to the most mature of these fractions, while total cellular RNA appeared to be a mixture of all of them.

Figure 1. Efficient isolation of important fractions of the transcriptome. (A) The method of extracting important fractions of the transcriptome - nascent, early, nuclear, cytoplasmic, and polyribosome-associated RNA transcripts. (B) The proportion of sequence reads arising from exons, introns, and intergenic regions for each transcriptome fraction. Maturation of the transcriptome is apparent with the loss of intronic reads. (C) Maturation of RNAs from example genes. Normalized read depth is plotted in red and binned in 25-bp segments over the gene structures in black. Our results agree with the current knowledge that *MYC* encodes a typical mRNA transcript, *HIST1H2AE* RNA is not polyadenylated, and *XIST* encodes a nuclear-retained RNA.

We further evaluated these transcriptome fractions utilizing genomic loci whose transcriptional biology is well understood (**Figure A-1C**). Most multi-exonic, protein-coding genes produced sequence reads across their exonic and intronic regions in nascent RNA, then matured to exon-only profiles in mature RNA fractions. Transcripts with eccentric, and well characterized, life cycles also showed the expected behaviors in our transcriptome fractions. For example, *XIST* RNA, which is retained in the nucleus²¹², was

abundant in nascent, early, and nuclear RNA fractions, but absent from cytoplasmic and polyribosome-associated fractions. Histone-protein-encoding RNAs, which are not polyadenylated and are transcribed at low levels but accumulate to high abundance due to low turnover in dividing cells²¹³, showed low relative expression in nascent RNA, greater abundance in more-mature fractions, and absence from the total mRNA fraction. As expected, polyA-selected mRNAs showed a 3' bias, but our compartmentalized RNA fractions (which were sequenced in total, without poly(A) selection) did not.

Thus, this series of RNA fractions form a five-scene movie of the maturing transcriptome. The next step was to identify the scenes in which allele-specific effects appeared. We sought to understand to what extent regulatory sequence variation shapes each fraction, and what distribution of large and small effects such variants encode.

Het-Seq: Measuring allelic effects with precision and accuracy

To monitor allele-specific regulatory effects in a precise and well-controlled way, we utilized the heterozygosity that exists within each diploid genome to separately follow the expression of homologous copies of each gene (one derived from each parent) (**Figure A-2A**). Alleles of the same gene in the same individual are potentially powerful controls for each other, as both are subject to the same trans-acting genetic and environmental influences²¹⁴. Cis-acting genetic variation can cause alleles to diverge from the expected 1:1 ratio, displaying “allelic skew” or “allelic imbalance”.

Figure 2. Equal capture of alleles allows precise measurements of AR. (A) The Het-Seq capture strategy allows the equal capture of cDNA from both alleles by using RNA baits that bind the non-variable flanking sequence around each SNP. (B) This capture strategy is effective for increasing coverage on sites of interest. (C) SNPs in genomic DNA exhibit a nearly uniform distribution of p-values when captured with Het-Seq probes and analyzed with Het-Seq informatics. (D) Biological replicates showed a good concordance for both modest and large allelic skew events. (E) Confirmation of a subset of Het-Seq measurements was done on the ddPCR platform.

Allelic ratios (AR) in RNA can be obtained by RNA sequencing, which has previously been used to detect the extreme forms of allelic skew found at imprinted loci¹⁴⁷ and to validate eQTLs^{192,193}. Standard RNA sequencing, however, is not sensitive enough to monitor modest allelic skew in most genes. Because the expression levels of different genes vary across many orders of magnitude, a few highly expressed genes consume much of the bandwidth of a sequencing experiment (**Figure A-2B**). Thus, while a few genes may achieve average read depth of 100 reads or more across their length, allowing measurement of ARs with a precision of +/- 10%, the vast majority of hetSNPs are interrogated by far fewer sequencing reads, allowing the detection of only extreme allelic skews in individuals (such as those present at imprinted loci) and requiring averaging across many individuals to detect more subtle skews. Enriching RNA-seq libraries for sites of interest can be done with PCR, arrays, or padlock probes¹⁴⁷, but such approaches have tended to distort the relative representation of pairs of alleles or to obscure the extent to which measurements derive from PCR duplicates.

To measure allelic ratios across the transcriptome with high precision and accuracy, we developed an approach, Het-Seq, for enriching RNA-seq libraries for heterozygous sites while preserving allelic ratios and information about the molecular origin of each sequence read. In Het-Seq, we first computationally design thousands of “enrichment probes” to the sequences flanking hetSNPs (**Figure A-2A**). The probes contact only invariant sequence close to the hetSNP, so that the allelic state of the SNP does not influence the avidity of capture. These biotinylated probes are then used to capture and enrich RNA-sequencing libraries for molecular inserts containing the informative SNPs. A custom informatics strategy aligns the resulting sequence reads to a

reference genome on which heterozygous sites have been masked out, making sequence reads with the reference and alternative alleles equally likely to align to that genomic location. Potential PCR duplicates are systematically removed from analysis, ensuring that each allelic count represents a distinct observation of the original first-strand cDNA. In this way, we obtain an integer measurement of the relative abundance of each allele in the original RNA sample. If a read equally matches more than one site (as in the case of CNVs), it is aligned randomly to one site. The digital nature of this count, and its derivation from independent reverse-transcriptase events, allows the significance of AR measurements to be evaluated using binomial statistics.

Het-Seq dramatically enriched RNA-seq libraries for reads that were informative about the states of heterozygous SNPs, allowing thousands of hetSNPs to each be evaluated for AR with more than 100 molecularly independent observations on each one (**Figure A-2B**).

Critical evaluation of Het-Seq

It was important to critically evaluate the extent to which Het-Seq was susceptible to artifacts that would undermine the measurement of allele-specific *cis*-regulatory effects. Such artifacts can be discerned by analyzing genomic DNA, in which each pair of alleles is equally abundant, allowing robust detection of technical influences on measurement.

The first potential problem involves systematic bias toward alleles present in the reference human genome sequence. Such “reference bias” arises wherever pairs of alleles are captured with unequal avidity or are aligned to the reference genome with unequal

efficiency (for example, because the alternative allele is disadvantaged by already containing one mismatch to the reference sequence). Supporting the idea that these are important effects, we found that exome sequence data (from NA12878 and NA19240 in the 1000 Genomes Project) contained the reference allele in 55% of aligned sequence reads. By contrast, in Het-Seq, the reference and alternative alleles were ascertained equally (49.9% reference, 50.1% alternate). About three-fifths of this improvement appeared to arise from Het-Seq's allele-neutral molecular capture strategy, with the rest coming from a custom informatics pipeline that neutralized sources of reference-allele bias (**Appendix B**).

The second potential problem comes from the tendency of PCR to create duplicate copies of the sequences in molecular libraries. If scored and treated as independent observations, PCR duplicates cause measurements of allelic ratio to be overdispersed – to tend toward extreme ratios – relative to a null hypothesis in which allelic counts arise purely from random statistical sampling. The resulting P values would be systematically lower, with the result that the breadth and magnitude of allelic influence would be overestimated. Because Het-Seq involves paired-end sequencing of a molecular library with diverse molecular sizes and offsets relative to each SNP, we can filter all data points that are potential PCR duplicates (those with the same start and end location on the genome) while maintaining those observations that are molecularly independent. This ensures that each allelic count represents a distinct observation of the original first-strand cDNA.

Encouragingly, allelic imbalance P values for genomic DNA showed a uniform distribution similar to the distribution expected under the null hypothesis (of random, 50-

50 sampling of each pair of alleles) after allele-neutral capture and Het-Seq informatics analysis (**Figure A-2C**). Though there is still a minor inflation, the effect sizes of these skews are small and disappear in the RNA libraries, which have a lower read count (**Discussed in Appendix B. Experimental Procedures**).

Because we were analyzing highly manipulated biological samples, it was critical to understand the extent to which AR measurements reflect durable features of the underlying biology. To evaluate this, we grew the cells in distinct experiments months apart, harvested RNA at distinct times (also months apart), and processed the samples separately. AR measurements were reproducible across these biological replicates. Notably, the high correlation of AR measurements was observed not only for genes with extreme measurements of allelic skew, but for genes that showed modest allelic skewing of 10-50% (**Figures A-2D**).

With our precision confirmed, we tested our measurements' accuracy. We selected 20 SNPs covering a range of Het-Seq allelic skews and assayed them with allele-specific droplet digital PCR across the RNA fractions and individuals. This created over 100 independent calls that were broadly concordant with Het-Seq (**Figures A-2E**). Thus, we believe this is the first study of allele-differential expression that has simultaneously met the criteria of (i) deep ascertainment of heterozygous sites, (ii) allelic neutrality during detection, (iii) dispersion statistics that closely match random sampling, (iv) precise, accurate measurements of even modest allelic skew, and (v) independent measurements across the lifecycle of RNA.

An allelic-skew profile acts as a fingerprint of genome identity

These data allowed the detailed profiling of a transcriptome in terms of the quantitative influences of genome sequence variation on thousands of genes. Though the causal sequence variants are unobserved, their direct effects are measured via the systematic use of sequence variation on nearby transcripts to monitor allele-specific regulatory effects in an internally controlled way.

We sought to understand the global properties of these genome-wide profiles of allelic skew. We found that when we used the RNA-seq data to measure absolute gene expression levels in nascent, early, nuclear, cytoplasmic, polyribosome-attached, and mRNA fractions, these genome-wide profiles clustered by the RNA fraction from which they were derived (rather than the individual from whom we derived them), reflecting substantial differences among the fractions in the abundances of different transcripts. However, when we clustered Het-Seq allelic-ratio profiles derived from these same RNA libraries (limiting analysis to 1,795 hetSNPs that were heterozygous in both probands and deeply interrogated by >100 reads in each), AR measurements clustered by the genome of the individual from whom they were obtained, reflecting the unique set of unseen regulatory variants in each individual (**Figure A-3A**).

We sought to determine whether the regulatory variants were genetic, or from other non-genetic forces such as random or programmed monoallelic expression¹⁹⁶, or subtle, technical biases not detected in the above evaluation. To distinguish between these hypotheses, we developed a test that utilizes the mosaic distribution of heterozygosity in a typical human genome. Each diploid human genome is a mosaic of segments of low and high heterozygosity, ranging from regions where one's maternal and paternal haplotypes are very similar, to regions where they have many sequence differences per kilobase. In each sample, we divided all protein-coding genes into ten groups (deciles) based on the level of heterozygosity at each locus, from the most-homozygous loci to the most-heterozygous loci. The heterozygosity present in each decile strongly predicted the tendency of its genes to exhibit definitive allelic skew (**Figure A-3B**). This relationship was linear with a y-intercept of almost zero. This result suggests that allelic skewing in RNA arises overwhelmingly from genome sequence variation, rather than from other mechanisms.

Figure 4. Most allelic skews arise at transcription. (A) Distribution of allelic skew across phases of RNA maturation in GM19240. SNPs included in the analysis were present in all fractions with greater than 50 reads. In order to equalize the distribution-slimming effects of high coverage in some fractions, allelic counts for each SNP were down-sampled to 50 before plotting. (B) Pairwise comparison of each SNP's allelic skew. (C) Principal components analysis of allelic skew profiles.

Allelic skew affects 20-24% of genes and is modest in magnitude.

Het-Seq measurements of allelic ratios in each RNA sample showed a distribution substantially wider than the distribution derived from analysis of genomic DNA or expected from random statistical sampling of pairs of alleles (**Figure A-4**). The observed variance in allelic-ratio measurements is a composite of (i) true regulatory influences, and (ii) the effect of random (“binomial”) statistical sampling upon each measurement. To parse these effects and infer the true distribution of regulatory effect sizes, we used beta-binomial

Figure 5. Allelic skew is common and modest in RNA

Beta-binomial analysis splits the observed distribution into a distribution of real effects and a distribution of null effects. It estimates that 24% of genes exhibit a skew with a median effect size of 1.5-fold (60:40) in GM19240.

statistics, which model the data as arising from both an underlying distribution of real regulatory effects (affecting a subset of genes, with effect sizes exhibiting a beta distribution across these genes), and the influence of binomial sampling upon all measurements. We found that beta-binomial statistics fit the experimental data well, and used the beta-binomial model to estimate two key parameters: the fraction of genes affected by allele-specific influences, and the distribution of regulatory effect sizes.

The model estimated that ~24% of genes in GM19240 displayed allele-differential expression with a median influence of 1.5-fold, corresponding to an allelic ratio of 60:40 (20% of genes at 1.6-fold for GM12878). In fact, 80% of the effects changed expression 2.0-fold or less (2.1-fold for GM12878). This is mostly consistent with a recent study that found the majority of mutations within mammalian enhancers influenced activity less than 1.5 fold, with only 3% of mutations affecting activity by more than two-fold²¹⁵. Our effect sizes are higher, probably due to the additional variation expected to occur within an individual's enhancer, promoter and transcript that may influence expression.

How expression variation affects individual genes.

Once we had characterized genome-wide expression variation, we documented how this affected particular genes. A majority of genes showed no apparent allelic influence – the maternal and paternal alleles were equally abundant in nascent transcripts and at every subsequent stage of RNA maturation, export, and processing. Four examples are shown in **Figure A-6A**.

Figure 6. Examples of different allelic skew profiles. Most genes are like those of (A), with no effect. However, a substantial fraction are like those in (D), where an effect manifests at transcription and persists through RNA maturation. Rarely, an effect manifests in the cytoplasm, presumably from a cytoplasmic-specific RNA maturation step.

We documented extreme allelic skew from known imprinted such as *SNURF*, a paternally imprinted gene residing in the Prader Willi locus on chromosome 15q11, and *MEST* (also known as *PEG1*), a paternally imprinted gene residing on chromosome 7q32²¹⁶. Notably, some imprinted genes, such as *MEST*, showed incomplete inactivation of the non-favored allele, producing as much as 5% of the transcripts from the gene. We also found new genes that fit the pattern of being imprinted (*TMEM173*, *MACC1*, *AL662904.1*, and *FHOD3*), though *FHOD3* and *MACC1* were only skewed in one individual. As expected, most genes on the X chromosome were inactivated. However, *MSL3*, *ZFX*, *RBBP7*, and *KDM6A* escaped inactivation in both individuals. *ZFX* is an important, dosage-dependent cancer gene in non-small cell lung cancer²¹⁷, but it could be an artifact of cell culture so more investigation is needed. In total, cases of overwhelming (>90%:10%) allelic skew were found in only 2.7% and 7.3% of skewed genes in GM12878 and GM19240, respectively – over half of which were on the X chromosome.

Modest allelic skew was far more common than extreme allelic skew. Consistent with the genome-wide analyses, the most common pattern was an effect arising at transcription. This pattern involved an allelic skew that appeared for the first time in nascent RNA, then persisted at a constant magnitude across all subsequent fractions (**Figure A-6D**). 61% of classifiably skewed genes exhibited this pattern in GM12878 and 58% in GM19240. This number rises to 85% and 80%, respectively, when genes with an additional post-transcriptional effect are included.

For some genes (7.8% and 7.3% of skewed genes in GM12878 and GM19240), the allelic skew appeared to grow steadily in magnitude as the transcriptome matured (**Figure A-6E**). Such observations were particularly common among genes with different

isoforms, and tended to involve a hetSNP in an alternatively spliced exon. However, some of these could result from different rates of decay.

Other genes (4.7% and 5.0% of skewed genes in GM12878 and GM19240) exhibited allelic balance in all nuclear-resident fractions but strong skew in the two cytoplasmic fractions, suggesting that they are regulated in an allele-specific way by mechanisms that reside in the cytoplasm, such as miRNAs or nonsense mediated decay, or were blocked from the cytoplasm, perhaps by nuclear export mechanisms. Examples of these genes are the complex I assembly protein *NDUFAF1* and the interferon-induced family member *IFI44L* (**Figure A-6F**). Consistent with the hypothesis of cytoplasmic regulation, *IFI44L* had a heterozygous SNP in a predicted binding site for the LCL-expressed microRNA1206, and the abundance of the allele that better matched the mir1206 binding site was reduced in cytoplasmic RNA fractions.

Figure 7. When allele-specific effects arise in the transcriptome of GM19240.

Genes with more than 50 reads in each fraction and at least one significant allelic skew were sorted into 6 different categories of allele-specific regulation (424 genes total). Even though this data is based on a small number of genes, it is concordant with the other genome-wide analyses.

Overall, the classification of genes agrees with the genome-wide assessments of the impact of transcriptional and post-transcriptional effects. Transcriptional effects seem to dominate, yet there is a substantial effect produced by alternative splicing and a few convincing instances of cytoplasmic-based regulation (**Figure A-7**). Given that 20–24% of genes exhibit these skews, there are many ways a variant can affect mRNA expression and thereby influence phenotype.

DISCUSSION

We described an approach for quantifying the effects of cis-acting allelic perturbations across the genome. Data from this approach are highly reproducible and robust to statistical tests of validity, and offer a promising, straightforward way to profile the effects of genome sequence variation upon most genes.

We anticipate that Het-Seq will have many other applications. One set of applications will involve further characterizing and mapping the regulatory perturbations ascertained in genome-wide association studies (GWAS). Critical directions in the biological exploitation of GWAS results include determining which gene(s) are affected at each locus, determining the sign and magnitude of allelic effects on each gene's expression level, and localizing these effects to specific populations of cells. By performing Het-Seq on cell-type-specific populations of RNA, regulatory effects in complex tissues could be systematically mapped to specific cell types, analogous to our mapping of such effects to compartments within cells of a single type. This will be useful for going from these early genetic leads to models of pathophysiology in complex tissues.

Our results suggest that transcriptional regulation predominates among the influences of genome sequence variation on gene expression. Several potential reasons are suggested by the data in this study. First, transcriptional regulation appears to present a substantial mutational target – we estimate from the density of heterozygous sites (about one per 1,250 bp) and the frequency of allelic skew (about 20% of genes) in an individual genome that, even in a single cell type, the average gene contains at least 250 bp of sequence that can mutate to an expression phenotype. Such sequences could include not only enhancers but also others classes of transcriptional regulatory elements, such as insulators, repressors, pause sites, and matrix attachment regions. Second, transcriptional regulation appears to offer many opportunities for mutation to give rise to soft, quantitative effects on the expression level of a gene. We estimate from the mathematical distribution of allelic skews that most regulatory influences involve a less than 1.4-fold difference in the expression levels of the two alleles, and fewer than 10% involve a 1.8-fold difference. Such quantitative changes may be well-tolerated in robust biological systems, allowing such functional variants to become common in populations and to contribute to variation that is generally within the healthy range. It is likely that constellations of many such variants in the same pathway may, in some individuals, push phenotypic variation to extremes within and outside the range defined as normal. The high evolvability of regulatory sequences may help us to understand why they account for the phenotypic variation that arises within species on short time scales.

Appendix B. Experimental Methods

Amylase

Cohort Collection:

Estonian Sample Collection: The Estonian Biobank is the population-based biobank of the Estonian Genome Center of the University of Tartu (EGCUT) (www.biobank.ee). The EGCUT is conducted according to the Estonian Gene Research Act, and all participants are volunteers that have signed broad informed consent¹⁶⁷. The cohort size is currently 51,535 people from 18 years of age and up. All subjects are recruited randomly by general practitioners and physicians in hospitals. A computer-assisted personal interview is conducted at the doctor's office to record personal data, genealogical data, lifestyle data, and the subjects' educational and occupational history.

Medical history and current health status are recorded according to the ICD10 and medication according to the ATC. Anthropometric measurements are taken, along with blood pressure (sitting position at the end of the interview), and resting heart rate. 30-50 mL of venous blood are collected into EDTA Vacutainers. These are transported to the central laboratory of EGCUT at 4-6 degrees Celsius within 6 to 36 hours after collection. Upon arrival, DNA, plasma and WBC are immediately isolated and kept in aliquots in MAPI straws in liquid nitrogen.

A Hamilton Robotics Automated Sample Management system with a 100,000 tube capacity is used for intermediate storage of normalized DNA samples (50–100 ng/ μ L) in tubes with 2D-barcodes. This enables a quick and highly accurate delivery of the samples by cherry-picking according to the selected barcodes.

The 1,000 Estonian samples used in the current study were selected from the BMI extremes of the EGCUT population cohort of 51,535 samples. The lean ($\text{BMI} < 22$) and the obese ($\text{BMI} > 33$) groups were matched on age-at-recruitment and year-of-birth to account for cohort and life-course effects on overall body composition, resulting in the obese being in the 98th percentile (females: 99.5th percentile) of the cohort's BMI and the lean being in the 87th percentile (females: 82nd percentile). The extreme sets included 250 samples from both genders and were limited to only individuals with Estonian nationality and whose self-reported mother tongue was Estonian. Further measures to account for potential population structure are described further down.

GoT2D Sample Collection: The Genetics of Type 2 Diabetes (GoT2D) study aims to characterize type 2 diabetes and related quantitative traits' genetic architecture through low-coverage whole-genome sequencing, deep (~100x) exome sequencing, and 2.5M SNP genotyping of cases and controls from four large European cohorts: Diabetes Genetics Initiative (DGI), Finland-United States Investigation of NIDDM Genetics (FUSION), GoT2D-UK, and Kooperative Gesundheitsforschung in der Region Augsburg (KORA)^{13,218-221}. These larger cohorts are a mixture of smaller ones obtained in Finland, Sweden, the UK, and Germany. Due to the confounding effect of diabetes on BMI, cases and controls were analyzed separately while controlling for the cohort of origin. Only the low-coverage data was used in the current study.

InCHIANTI Cohort Sample Collection: Individuals ($n = 680$) were selected from the InCHIANTI study^{168,222} – a study of aging from the Chianti region in Tuscany, Italy - for low pass whole genome sequencing. Individuals were selected for sequencing

based on the availability of gene expression and circulating biomarker data, though neither were used in this study.

Whole-genome sequencing was performed at the Beijing Genomics Institute (BGI), Shenzhen, China using the Illumina HiSeq 2000 to obtain a minimum read depth of 6X. An average of 240 million paired-end 90bp reads per sample were aligned to the 1000 Genomes implementation of the Genome Reference Consortium's build 37 of the human reference genome²²³, using the burrows-wheeler aligner (BWA) version 1.5.9²²⁴.

GPC Cohort Sample Collection: Headed by the Center for Genomic Psychiatry at USC, the GPC cohort is a collection of individuals with schizophrenia and bipolar disorder, along with controls. Participants are recruited from the United States and selected sites abroad. The subset used in this study consisted of 768 self-reported (and genetically confirmed) European-ancestry patients from the United States that were whole genome sequenced from blood to a depth of 30x. Data on BMI were not available, so this cohort was used in the analyses determining the amylase structural forms and their relationships to SNPs.

Droplet Digital PCR:

General: Droplet digital PCR is similar in concept and preparation to a real-time qPCR reaction but with a few important modifications¹⁵⁴. Before amplification, the DNA is first digested with a restriction enzyme in order physically separate the copies of the CNV that are on the same DNA strand (the enzyme is chosen based on the locus, but common choices include AluI, EcoRII, and RsaI). The PCR reaction mixture is prepared similar to qPCR, with each primer at 900 nM, the fluorescent probes at 250 nM, and the

digested DNA around 1 ng/ul. The reaction (20 µl) is then emulsified into approximately 20,000 water droplets surrounded by oil using a droplet generator (BioRad). The droplets are thermocycled using a standard thermocycler with the BioRad-supplied PCR protocol (with an additional 10 cycles), and the droplets containing the probes' targets then become fluorescent. The fluorescent droplets are then counted by a droplet reader (BioRad). At low DNA input concentrations, each fluorescent droplet likely contains only one PCR target, thus allowing us to count the near-exact number of targets within the reaction – as opposed to comparing amplification curves, like in qPCR. A Poisson correction factor is applied by the software to account for droplets that contain more than one copies.

Control Probes: The standard control probe for ddPCR is targeted to *RPP30*, a non-CNV housekeeping gene. However, since amylase is in a late replicating region¹⁶¹, DNA isolated from replicating cells will naturally have less of it than other parts of the genome – the parts that have already replicated. To counteract this, we used a probe assay targeted to just outside the amylase region, called Near_AMY.

Genotype Calling: The output of the droplet reader is a scatterplot with FAM fluorescence on the y-axis and HEX/VIC on the x axis. Each dot represents a droplet. The QuantaSoft software draws suggested thresholds for positive FAM and HEX droplets. The experimenter checks those thresholds and redraws if needed (while still blinded to sample identity). A CNV copy number call is found by dividing the number of droplets fluorescing FAM, corresponding to the CNV target, by the number of droplets fluorescing VIC, the control target (both numbers being Poisson corrected). Before the

final genotype call, the raw CNV calls of each plate are corrected by a plate-wide correction factor, generally between 0.97-1.05.

HapMap Samples: Plates containing the HapMap¹⁵⁸ DNA samples for CEU and YRI were subjected to ddPCR in three reactions for *AMY2B*, *AMY2A*, and *AMY1* using the “assay1” assays listed in **Table B-1**. All except half the CEU individuals on the *AMY2B* run were done using the control assay Near_AMY. DNA inputs varied, due to the variation of DNA concentration across the plate, but the ideal DNA concentration strived for was 1 ng/ul for *AMY1* reactions and 0.5 ng/μl for *AMY2B* and *AMY2A* reactions. All copy numbers reported are from a single reaction for each gene. However, we do have multiple runs on file for these assays and others, runs that were done during the optimization stage.

Table B-1**a.**

ddPCR Assays:

	Primer	Primer	Probe	Annealing temperature used
AMY2B_assay1	GGATGCGATCATCTTGA TCTT	CCCAGACCATCTTCAACTC C	GTAGGCCAACACGGTGCTAT	60C
AMY2B_assay2	GCAGAGGAGAGAAGAC AGTGGT	CAAGGAATCTTCTCTGAAC TCCA	ACAGGAGTGTGGTGATCAA CCTGTTGT	60C
AMY2A_assay1	AACATCAAAAAGTCTCTC ATGGAA	CAAATTTTGGTTTCTACTG TTATGTG	GGCCCCAGCAACAGGTCAC T	57C
AMY1_assay1	AAAAACCCAAGAATTAG GAATGG	CCTGGAAGGATTTCTGGT G	TGCTCTCATTTTAGATGACT TGTG	58C
AMY1_assay2	TGTTTGCAAGGAGGTCT TCTC	TTGGCCTTTCATCTGTGATT T	AAATGATTCCCAGAACTGTA GC	58C

Controls:

RPP30	GATTTGGACCTGCGAGC G	GCGGCTGTCTCCACAAGT	CTGACCTGAAGGCTCT
Near_AMY	AAATTTATTGGAGGGAT GTTGG	TTCAAGTTTGACTGCTAAC TCCTG	TGGAATAAAGAATCATTGG GCACAGGT

b.Read-depth bins
(hg19):

	Bin 1	Bin 2	Bin 3
AMY2B	chr1 104103087- 104137966	NA	NA
AMY2A - first part	chr1 104153652- 104161927	NA	NA
AMY2A - pseudogene	chr1 104161928- 104210498	chr1 104256097- 104304649	NA
AMY1	chr1 104161928- 104210498	chr1 104210499- 104256096	chr1 104256097- 104304649
AMY1, InCHIANTI cohort	chr1 104181165- 104209159	chr1 104228052- 104256049	chr1 104275307- 104303297
Intergenic	chr1 104137967- 104153344	chr1 104211074- 104226763	NA
IntergenicA	chr1 104137967- 104144300	chr1 104220425- 104226763	NA
IntergenicB	chr1 104144301- 104153344	chr1 104211074- 104220426	NA

Estonian Samples: The Estonian DNA was aliquoted into 96 well plates, with a random distribution of under- and overweight samples (KS test, $P = 0.51$). The ddPCR runs were done within a three week period in the same lab, using the same machines, with an experimenter blind to the case-control status of the samples – thus reducing the risk of batch effects and biases. Each sample had one genotyping run of each of these

assays: AMY2B_assay1, AMY2A_assay1, AMY1_assay1, and AMY1_assay2 (with the exception of Plate 1, which did not have an AMY1_assay2 run).

AMY2B: Initially, 28 Estonians had copy number calls less than 2 for *AMY2B* (CN of 0 or 1), a call that should be impossible given *AMY2B*'s copy number distribution. We hypothesized that an Estonian-specific SNP may be interfering with the assay and ran those samples again using AMY2B_assay2. All the samples, except for 3, then had calls consistent with the known *AMY2B* distribution (CN of 2 or 3). In the association analysis, the AMY2B_assay2 genotype calls were used for these samples.

AMY1: Two different assays targeting *AMY1* were used in order to reduce the noise a single assay may have. Two different DNA input concentrations were used with the *AMY1* assays to ensure that each sample had at least one genotype call acquired when it was within the optimal concentration range for ddPCR. In the concentrated reaction, each sample of the plate was pre-calculated to have > 0.2 ng/ul DNA input concentration at a fixed input volume and were genotyped with the *AMY1_assay1*. However, given the wide distribution of sample concentrations on each plate, the concentrated run resulted in many of the samples oversaturating their reactions. In the diluted run (*AMY1_assay2*), each sample input was pre-calculated to produce >10% probe-negative droplets, thus diluting the previously over-saturated samples.

AMY2A-adjusted averaging of the *AMY1* copy number calls: To avoid biases that may arise from sample DNA concentration differences between cases and controls, we did not filter or clean the data based on concentration and used every genotype call the BioRad QuantaSoft software provided. However, a straight average of these two *AMY1* replicate genotype calls was not ideal, since many of the samples had one genotype call

obtained when the sample was too dilute or over-concentrated, thus adding noise to their better genotype call. Instead, an *AMY2A*-adjusted average was used, as described below.

Given that *AMY1* and *AMY2A* share parity (that is, their copy numbers are either both odd or both even), we can check the correctness of the *AMY1* copy number call using the *AMY2A* call. In practice, this means checking each individual's two replicate *AMY1* calls for concordance with their *AMY2A* call. If both *AMY1* calls were concordant, they were averaged (70% of samples). If only one was concordant, only the concordant *AMY1* genotype was used (24% of samples). If both calls were not concordant, they were averaged (6% of samples). This resulted in better clustering at integers (average deviation from integer 0.152, compared to straight averaging 0.179) despite having nothing to do with either DNA concentration, distance from an integer, or confidence intervals. It should be noted that a BMI association was done separately with all three arrangements – *AMY2A* adjusted average, straight average, and each run separately – all resulting in $P > 0.05$.

Read-depth genotyping:

Algorithm: As a second method for determining the integer copy number of the CNV segments, we used recent versions of the Genome STRiP software¹⁵⁶ to determine copy number from whole-genome sequencing data. Briefly, for each CNV, the number of unique sequencing reads falling within the target CNV were counted for each individual and compared to the expected number of reads. We required a minimum mapping quality of 10 and that the reads were aligned to a unique position on the reference genome, except in cases where the target CNV is duplicated in the reference genome (such as

AMY1). The expected number of reads per copy was estimated based on the genome-wide sequencing coverage for each individual, correcting for the alignability of the CNV segment and for sequencing bias due to GC content. Alignability was estimated by mapping overlapping k-mers from the reference genome back to the reference. For the HapMap cohort (from 1000 Genomes Phase 1), we used a k-mer length of 36 and for the GoT2D, InCHIANTI, and GPC cohorts (which have longer reads), we used a k-mer length of 101. GC-bias was estimated by counting the number of aligned reads in overlapping 400bp windows binned by GC fraction compared to a set of selected reference windows having no evidence of copy number variability.

The vectors of observed and expected read counts were fitted to a constrained Gaussian mixture model with two parameters (m_1 and m_2) and a site-specific number of genotype classes corresponding to the potential copy numbers. The number of copy-number classes was based on the individual with the highest observed to expected read count ratio (rounding up to the nearest integer and adding one extra copy number class). The means of each genotype class were constrained to be proportional (m_1) to the copy number and the variances were constrained to be proportional (m_2) to the copy number (or to a small constant $k = 0.2$ for the copy number zero class). After using an expectation maximization (EM) algorithm to determine the most likely values for m_1 , m_2 and the proportional weighting of each copy-number class, the relative likelihood of the observed read depth given each potential genotype class was calculated for each individual. Fractional copy-number estimates for each individual used in plotting are computed as the observed to expected ratios scaled by m_1 .

Determining the locations and boundaries of the copy-number variable

genomic segments: We created an initial map of the potentially copy number variable segments at the amylase locus based on the paralogous gene annotations from the reference genome, annotated segmental duplications, and results from previous studies (Groot *et al* 1989, Perry *et al* 2007). ddPCR measurements were used to confirm copy number variability at specific primer amplification sites, while measurements from sequencing read depth were used to determined variability (or lack thereof) by interrogating the average copy number per individual across longer genomic segments.

Segmentation was further guided by building an alignability map of the locus by aligning k-mers (k=36, k=70 and k=100) from the reference genome back to the reference genome using BWA²²⁴ and using this alignability map to generate hypotheses about the extent of the copy-number variable segments. The segment boundaries were refined based on prospective genotyping of multiple candidate segments using sequencing read depth and Genome STRiP and then optimizing for segments that yielded integer copy numbers in all samples and high posterior genotype likelihoods (similar to the automated method used in recent versions Genome STRiP for optimizing boundaries in non-repetitive sequence). When some individuals were observed to cluster at mid-integer copy number estimates, suggesting the presence of additional copy-number variable sub-segments, we applied this procedure recursively down to the length scale resolvable from the available sequencing data sets. The variability of all segments, except for the intergenic region, was confirmed by designing ddPCR assays to these segments and carrying out ddPCR experiments to confirm the sequencing-based results

The bins used for the read-depth analysis are in **Table B-1**. Even though the bins for *AMY1* are substantially larger than the *AMY1* repeated segment, most of the signal Genome STRiP used to call genotypes arose from the *AMY1* repeated segment.

Genotyping InCHIANTI cohort with alternative read-depth method:

We analyzed 657 samples after quality control checks. Average depth was 7x. We aligned the reads to a repeat masked GRC build 37 reference genome using the mrsFAST ultra version 3.3.1 algorithm, which can align single reads to multiple positions in the genome and so is optimal for regions of variable copy number²²⁵. Repeats were detected and masked using both RepeatMasker Open-3.0²²⁶ and Tandem Repeats Finder 4.07b²²⁷. Reads were mapped in single-read mapping mode with a hamming distance threshold of < 4 bp. We derived GC corrected absolute copy number in 100bp windows using mrCaNaVaR version 0.51²²⁸, a program that predicts from read depth and GC enrichment an absolute copy number. We calculated a mean copy number value for the three combined *AMY1* regions, *AMY2A* and *AMY2B*.

BioNano Genomics, optical mapping:

Optical mapping experiments were performed by BioNano Genomics. In brief, optical mapping can be thought of as next-generation restriction mapping. Strands of whole, labeled DNA (nick labeled and backbone stained with YoYo1) are electrophoresed through a NanoChannel array. The array straightens the DNA and the fluorescence left at the nick sites creates patterns that can be used to assemble a whole genome, or pieces thereof, in a manner similar to restriction mapping. Each amylase gene

has its own restriction pattern, so we can determine the order and orientation of the genes from these patterns. Long, whole strands of DNA (~300kb) are spread on a slide. A restriction enzyme nicks the DNA, and the nicks are labeled with a fluorophore. The strands are photographed, and assemblies are built by aligning the patterns of nicks to a reference genome. De novo assembly is also possible. A mix of *de novo* and reference-assembling was used for this project.

Clustering of SNP haplotypes (spiderplot):

All unrelated individuals in the 1000 Genomes European populations (CEU, TSI, GBR, FIN, IBS)²⁰⁷ that had amylase genotypes where the two structural haplotypes could be unambiguously determined (phased) were selected for SNP clustering in the spiderplot. These individuals' amylase haplotypes and SNP genotypes (downloaded from the 1000 Genomes website, Omni chip data, (ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/technical/working/20120131_omni_genotypes_and_intensities/) were combined in a .bgl file and phased as a group using BEAGLE version 3.3.2²²⁹ under the default conditions with no reference panel. The spiderplot created was from the 23 closest SNPs with a minor allele frequency greater than 1% and were outside of the variable region (resulting in 9 SNPs upstream, 14 downstream). The spiderplot was created by traversing the set of SNP haplotypes in both directions from the target variant (amylase) and grouping the haplotypes according to their state at each successive SNP to form two tree structures representing the left and right flanks. At each split, the branch corresponding to the minor allele was plotted above the branch corresponding to the major allele. The color of each

horizontal segment indicates the allele frequency of the next SNP on the branch and the thickness corresponds to the number of haplotypes sharing that segment.

Association of SNPs to haplotypes and imputation:

The individuals of the GPC cohort that had genotypes where the two amylase haplotypes could be unambiguously determined were used to search for tag SNPs for each haplotype. SNPs under 1% minor allele frequency and those within the copy number variable region were not used. Every remaining SNP was correlated with every haplotype, in turn, using a pearson test. During the test, all amylase haplotypes were recoded as 0 or 1, with the target haplotype being 1. P values were permuted by shuffling the amylase haplotypes 1 million times to create a distribution of possible r^2 values for each SNP.

The efficiency of imputation was calculated based on leave-one-out trials. Briefly, each individual's amylase haplotypes were masked, in turn, within the unphased data and phased using BEAGLE²²⁹ under default conditions using no reference panel. The amylase haplotypes that BEAGLE assigned were extracted and compared to the true values of the masked individuals. R-squared values were obtained with a pearson correlation and P values were calculated from 1 million permutations, creating an r^2 distribution.

Association of SNPs to diploid copy number of the amylase genes:

Separately, the GPC cohort, the GoT2D cohort, and the Europeans of 1000 Genomes were genotyped with read-depth analysis, and the diploid copy number calls were combined with the individuals' SNP genotypes (recoded as 0, 1, or 2 for the number

of alternative alleles present). Linear regression using each SNP genotype, in turn, as the predictor for *AMY1* diploid copy number gave the effect size (slope of the line/coefficient of the regression) and r^2 for the association of each SNP. P values were permuted by shuffling the amylase genotypes at least ten times (and up to a million times for the best SNPs) to create a distribution of effect sizes to which we compared the ‘true’ effect size. The permuted P values, r^2 values, and effect sizes replicated across cohorts.

Searching GWAS for associated SNPs:

Given the GPC cohort’s greater sample size and larger set of SNPs genotyped, we chose this cohort to display in **Figure 4-1**. We downloaded the publicly available GIANT consortium (<http://www.broadinstitute.org/collaboration/giant>) SNP P values for BMI association⁹⁴ and compared each SNP’s BMI P value (as determined by GIANT) with its association to *AMY1* copy number (as determined by this study).

SNP Genotyping of Estonians, along with the polygenic score and ancestry analysis:

The Estonian extremes had been previously genotyped with ExomeChip-v1.1 (Illumina Inc). As several replating events occurred between the array and ddPCR genotyping, the samples from the ddPCR batch were further genotyped by using Sequenom’s MassARRAY system (which allows a single base extension with allele-specific masses). A multiplex pool of 24 SNPs was used for BMI association in the Estonian cohort with 10 SNPs selected from the *AMY* locus (the best associated SNPs from 1000 Genomes) and 14 previously identified SNPs associated with BMI⁹⁴

(**Supplementary Table 10**). The latter set of SNPs was assayed to estimate the statistical power in the Estonian cohort to validate BMI linked genetic associations. Genotypes were called by mass spectrometry. Samples with less than an 85% genotype success rate and SNPs with less than an 85% genotype success rate and/or poor Hardy-Weinberg P value (<0.001) were excluded from the analysis. 10 AMY locus SNPs and 11 BMI SNPs passed the QC and were used in subsequent analysis. We observed 100% genotype concordance between the MassARRAY and ExomeChip-v1.1 SNP calls.

Table B-2. SNPs for polygenic score in Estonians

Spelotes et al	SNP	Effect Allele	Beta (SE)	EAf	VarExp	NPC
FTO	rs1558902	A	0.39 (0.02)	0.42	0.34%	14.03
MC4R	rs6567160	C	0.23 (0.03)	0.24	0.10%	4.149
SEC16B	rs543874	G	0.22 (0.03)	0.19	0.07%	2.905
FLJ35779	rs2112347	T	0.10 (0.02)	0.63	0.02%	0.82 1
BDNF	rs11030104 (rs10767664)	A	0.19 (0.03)	0.78	0.07%	2.855
MTCH2	rs3817334	T	0.06 (0.02)	0.41	0.01%	0.412
FAIM2	rs7138803	A	0.12 (0.02)	0.38	0.04%	1.65
MAP2K5	rs16951275	T	0.13 (0.02)	0.78	0.03%	1.228
GPRC5B	rs12446632 (rs12444979)	G	0.17 (0.03)	0.87	0.04%	1.628
SH2B1	rs3888190	A	0.15 (0.02)	0.4	0.05%	2.062
NEGR1	rs3101336	C	0.13 (0.02)	0.61	0.04%	1.643
				SUM	0.81%	34.02

The software program PLINK²³⁰ *--score* was used to build a single quantitative index of genetic susceptibility load for obesity. For that, the 11 BMI SNPs' allele dosages were weighted against the effect-sizes reported in Speliotes *et al.* and added up to a single polygenic score. Based on the estimated total trait variation explained reported in Speliotes *et al.*, the constructed polygenic score is capturing roughly 0.8% of BMI variation.

ExomeChip data was also used to account for potential population stratification in the extremes sample. ExomeChip genotype data was quality controlled using PLINK²³⁰ and standard quality control parameters – 1) sample call-rate >95%; 2) marker call-rate >95%; 3) marker allele frequency > 1% and 4) Hardy-Weinberg *P* value <1x10⁻⁶. Cleaned data was combined with HapMap2 genotypes (downloaded from the PLINK resources page) and subsequently analyzed for population structure using the multidimensional scaling (MDS) function in PLINK. Resulting MDS plots show that, although Estonian samples cluster tightly with the CEU cluster, slight structuring is present within the cohort. For that reason, three first MDS vectors were used as covariates in the subsequent association analysis.

Phenotype normalization in the Estonian and GoT2D cohort:

The standard GIANT Consortium protocol for normalizing the measures-of-obesity phenotype normally consists of adjusting BMI scores in a gender-stratified way for age, age-squared and genetic ancestry vectors (usually three and obtained through PCA or MDS analysis of genome-wide genotype) by fitting a linear regression model.

Next, the residuals from the model are transformed using an inverse normal transformation and used in subsequent association analyses.

This protocol was slightly modified for normalizing the 1,000 Estonians to account for the extreme-design and to more precisely capture the underlying trait distribution in the whole sampling cohort of ~51,000. First, the previously described trait normalization (except only age and age-square were used as covariates) was separately performed in females (N:32,724) and males (N:17,352), resulting in normalized BMI statistics for the 1,000 extreme samples. In subsequent analyses, both genders were analyzed together and sex and three MDS genetic vectors (estimated using ExomeChip data) were used as covariates to account for both gender differences and population stratification.

Power Analysis Estonian Cohort:

In order to make sure that the Estonian Extremes design has sufficient statistical power to find the associations reported in Falchi *et al.*, we used the *Genetic Power Calculator* (at <http://pngu.mgh.harvard.edu/~purcell/gpc/>)²³¹, since it has the option to account for threshold-selected quantitative trait design. Falchi *et al.* reports that *AMY1* copy number explains 0.66% to 4.40% (95% CI) of the genetic variance in BMI. By using the same calculations as Falchi *et al.* used to estimate the total variance explained for obesity, we back-calculated the mean total BMI variance explained and estimated it as 1.11% (95% CI = 0.461–1.79). The phenotypic thresholds in standard deviation units for defining the case and control sets were obtained from the normalized BMI scores

described in the previous paragraph and were as follows in standard deviation units – 1) Case thresholds +2.0 and +4.0; 2) Control thresholds -1.2 and -4.2.

The genetic effect sizes, trait-increasing allele frequencies, and respective total trait variation explained for the SNPs were obtained from the latest GIANT Consortium full report¹⁶. For the GIANT Consortium based power analysis, the stage 1 sample size of 124,000 was used. For estimating our power with the polygenic-scores, all individual SNP-based total trait variation explained were summed into one estimate (0.81%) and a conservative trait increasing allele frequency of 10% was used.

Association analysis in the Estonian sample cohorts:

Both logistic and linear regression models were used to detect association between BMI and the 21 directly genotyped SNPs using PLINK²³⁰. In the linear regression model, the normalized BMI scores were used, and in both models, gender and three genetic vectors were used as covariates. We used the integer genotypes of the copy number of all three amylase genes obtained through ddPCR in the same 1,000 individuals. For the *AMY1* gene, four different copy number estimates were used – *AMY2A* adjusted average, straight average, and both genotyping runs separately (as described in section *AMY2A-adjusted averaging*). Again both logistic and linear regression models were fitted using the same phenotype and covariates in R²³². A similar analytical framework was used to detect the association between BMI and the constructed polygenic score. No association between the AMY genes' copy number and obesity was observed by either model.

Association analysis in the InCHIANTI cohort:

We regressed the copy number values against body mass index corrected for age and sex. We used all copy number calls regardless of quality. Our analyses did not provide any evidence of an association between amylase copy number and BMI in *AMY1* ($P = 0.53$), *AMY2A* ($P = 0.37$) and *AMY2B* ($P = 0.49$). Using the more refined method of classifying *AMY1* copy number using the triplicated regions of *AMY1* alone, we still did not see any association with BMI ($P = 0.50$).

Association analysis in the GOT2D cohort:

The dataset was first divided into T2D-cases (N: 1,437) and controls (N: 1,374). The BMI phenotype was transformed using the GIANT protocol (described above – adjusting BMI scores in a gender-stratified way for age and age-squared by fitting a linear regression model and subsequently applying inverse normal transformation on resulting residuals from the model). Cases and controls were analyzed separately and the copy number values were regressed against normalized BMI scores while adjusting for gender and source-cohort. All copy number calls were used, regardless of quality. The latter variable was included in order to correct for population stratification as the GOT2D sample consists of 9 separate cohort-collections (Botnia, Diabetes-Registry, FUSION, Helsinki, KORA, Malmö, MPP, STT and WTCCC). Whereas both sub-cohorts (cases and controls) had >95% power to replicate the Falchi *et al* result (total variance explained 1.11%), we did not observe nominally significant associations with any of the AMY locus copy numbers in either sub-cohort.

To test for BMI-dependent effects of amylase that could have been masked in this relatively high BMI cohort, we also tested the tails of the distributions for association.

Table B-3. BMI-to-amylase associations in GoT2D using select ranges of BMI score				
Controls				
Individuals selected for association	Sample Size	P value	Odds ratio	Beta
Normal BMI (18.5-24.9)	175	0.3		0.01 (-0.018 - 0.038)
Non-obese (<30 BMI)	620	0.11		0.015 (-0.005 - 0.035)
10% tails (the most vs. least obese)	274	0.53	1.046 (0.908-1.204)	
20% tails	550	0.75	1.017 (0.919-1.124)	
30% tails	824	0.15	1.061 (0.980-1.148)	
Cases				
Individuals selected for association	Sample Size	P value	Odds ratio	Beta
Normal BMI (18.5-24.9)	489	0.03		0.021 (0.019 - 0.023)
Non-obese (<30 BMI)	1092	0.75		0.002 (-0.002 - 0.006)
10% tails (the most vs. least obese)	288	0.29	1.162 (0.882-1.531)	
20% tails	574	0.36	1.052 (0.944-1.173)	
30% tails	862	0.54	1.026 (0.945-1.115)	

Het-Seq

EXPERIMENTAL PROCEDURES

Isolation of Transcriptome Compartments

Nascent and early transcripts were isolated using described methods^{209,233-235}.

Briefly, lymphoblastoid cells were pulse-labeled with 200 μ M of 4-thiouridine (4SU) for 5 and 60 minutes, respectively, with DMSO as a negative control. RNA was extracted using Trizol (Invitrogen), purified with RNeasy columns (Qiagen), and eluted to a concentration >0.4 ng/ μ l. Purified RNA was denatured, and the 4SU-incorporated sites were biotinylated with 1 mg/ml EZ-link biotin-HPDP by incubation at 65°C for 1.5 hours, then at 25°C for an additional 1.5 hours. Unincorporated biotin-HPDP was removed by two washes with chloroform-isoamyl alcohol (24:1) and centrifugation in phase-lock-gel tubes (Eppendorf). Biotinylated RNA was captured and purified using MyOne streptavidin C1 beads (Invitrogen) before elution in 5% β -mercaptoethanol. RNA-seq libraries were then constructed using TruSeq library preparations (Illumina).

Nuclear and cytoplasmic fractions were harvested from 20×10^6 lymphoblastoid cells by dounce homogenization and centrifugation as described²³⁶. In short, harvested cells were washed in cold phosphate buffered saline (PBS) before washing in cold hypotonic buffer (20 mM K-HEPES pH 7.8, 5 mM potassium acetate, 1.5 mM magnesium chloride, 1 mM dithiothreitol, 1 mM PMSF, and 50 U/ml RNase inhibitor). Cells were then resuspended in 0.25 ml of cold hypotonic buffer and dounce homogenized (roughly 20 strokes or until $>90\%$ of cells were fragmented as assessed by Trypan blue staining, which marks dead cells) on ice. Lysates were centrifuged to pellet the intact nuclei, and RNA was extracted using Trizol to yield the nuclear fraction. The

cytoplasmic fraction in the supernatant was supplemented with 400 mM potassium acetate, 20 mM potassium HEPES, 15 mM magnesium acetate, and 0.5% deoxycholate. Lysates were clarified and extracted prior to RNA-seq library construction.

Polyribosomes were isolated using methods previously described²¹⁰. Briefly, 20×10^6 cells were exposed to 0.1 mg/ml cycloheximide (or puromycin as a control) for 45 minutes, then washed, homogenized, and centrifuged as described for the cytoplasmic fraction except the hypotonic buffer was supplemented with either 0.1 mg/ml cycloheximide or puromycin. The supernatant was layered on top of 11.2 ml linear 15-50% sucrose gradients, which were centrifuged at 35,000 rpm at 4°C in a pre-cooled SW41 rotor. Fractions of 350 μ l were collected for a total of 30-32 fractions per gradient, and polyribosome-containing fractions were identified using a combination of spectroscopy and quantitative RT-PCR to detect *GAPDH* and *SLBP*. Polyribosome fractions were pooled, depleted of ribosomal RNA, and subjected to RNA-seq library construction.

Control RNA fractions were created by two methods: total RNA, by ribosomal RNA depletion (Invitrogen); and mRNA, by poly(A) purification followed by TruSeq library construction (Illumina).

Enrichment for heterozygous sites using Het-Seq

We identified candidate SNPs by selecting those that had at least three reads per half-lane of sequence data in each fraction in each individual as judged by our preliminary RNA-seq data (13,406 in NA19240; 11,402 in NA12878) and excluding those that came from highly expressed genes in LCLs (including Immunoglobulin, *HLA*,

and *ACTB* genes). We then designed Het-Seq probes that would flank the hetSNP on both sides, excluding the SNP within a five base pair loop roughly in the center of the 120bp probe (no closer than 30bp from either end). Whenever a hetSNP resided near an annotated splice site, we included additional probes to account for alternative splicing. This was not done by hand, but by an R algorithm. These custom probe sets were ordered from Agilent as in-solution hybridization probes.

Sequencing

All libraries were molecularly barcoded and sequenced (to a half-lane of coverage) on an Illumina HiSeq2000 using paired-end 75 bp reads.

Alignment pipeline and other analysis details, in brief

Reads were obtained in FASTQ format from the Illumina HiSeq2000, then adapter-trimmed by Picard's *MarkIlluminaAdapters* (<http://picard.sourceforge.net>). They were aligned by BWA using the paired-read method with a read-trim score of 5. They were aligned to a "neutral reference", in which all SNP locations known in that individual were changed to N. In addition, they were aligned to a neutral, junction reference that spanned exon-exon junctions. The genomic alignment reads and junction alignment reads were then simultaneously compared for each read pair, and the most parsimonious alignment was selected. These aligned reads were then combined with the original data using *MergeBamAlignment* and PCR duplicates were flagged using *MarkDuplicates*. We normalized GC content bias by empirically measuring GC content for all SNPs in a library, then running a linear regression against the observed SNP alleles to determine a

correction factor for the binomial test. We filtered out SNPs that aligned nowhere, with low quality, to the wrong location, or multiple locations in an *in silico* alignment experiment. In addition, SNPs overlapping known pseudogenes and SNPs with P values $<10^{-3}$ in genomic DNA were removed. Details on logic and reference design are in the extended experimental procedures.

Allele-neutral alignment

The two samples used in this study are GM19240 and GM12878, which have both been deeply sequenced by 1000 Genomes (phase 2), giving us knowledge of the variants present. We used this information to construct a “neutral reference” – one in which all SNP locations are changed to “N”. Without a neutral reference genome, reads containing an alternative allele will always have at least one mismatch. This will reduce the number of correctly aligned alternative allele-containing reads, thus biasing the allele count in favor of the reference allele. Setting each allele position to “N” in the reference genome removes this alignment bias by assigning the same number of mismatches to every allele-containing read.

Avoidance of double-counting

Potential PCR duplicates were systematically identified using the Picard package’s *MarkDuplicates* program and removed from analysis. When a single set of paired-end reads made two observations of the same SNP (by sequencing from both ends of the RNA-seq molecule), the two SNP calls were compared. The two reads of the paired end sequencing generally agreed about the allelic state of the SNP, and the allele

was counted once; if the two read pairs were discordant (rare), then the read was discarded.

Analysis of GC bias

Construction of sequencing libraries has a well-known “GC content effect” in which inserts with extremely high or low GC content are under-represented. Since reads containing different alleles of the same SNP may have different GC content, there can be a bias in the ascertainment of one allele over another by as much as 5%. For SNPs that have a C or G allele, there is a bias towards whichever allele moves the GC content towards 50%. As expected, A/T and G/C SNPs do not exhibit this bias. We normalized GC content by empirically measuring it for all SNPs in a library, then running a linear regression against the observed SNP alleles to determine a correction factor. This correction factor was used as the null within the binomial test (i.e. replacing 0.50 with 0.493). It had a very small effect on P values for individual SNPs, but contributed to the calibration of global measurements.

Filtering SNPs in difficult-to-align regions

Some regions of the genome are inherently more difficult to align, potentially affecting two SNP alleles at different rates and biasing observed allelic ratios. To overcome this problem, we screened SNPs *in silico* for alignment problems. We extracted 40 bp of sequence 5' and 3' to each SNP and constructed a synthetic FASTQ file with the bases set to be high quality. These reads were then mapped using our mapping pipeline, and the locations of reads compared to their known locations. If sequences containing a

particular SNP did not map at all, mapped at low quality, mapped to the wrong location, or mapped to multiple locations, then that SNP was removed from further analysis. While all four categories had errors, the majority of the errors were due to SNPs mapping to multiple places on the genome. In addition, SNPs that overlapped known or reported pseudogenes were also removed. After all normalization and filtering methods were applied, a final filter on SNPs with extreme P values (10^{-3}) in genomic DNA were removed.

Junction Library

Because we aligned RNA to a genomic reference (and most of the sequencing was on spliced RNA), we also constructed a custom reference – a “junction library”- that spanned exon-exon junctions using sequences from a GenCode GTF file (gencode.v3b.annotation.NCBI36.gtf). Each junction was encoded as 10 bp of “N” sequence followed by the last 50 bp of the first exon attached to the first 50 bp of the second exon, then 10bp of N. Reads were processed as in the genomic alignment with the same parameters.

After alignment with the junction reference, reads were converted back to their locations in genomic coordinates with gaps in their cigar strings to represent where the reads spanned introns. The genomic alignment reads and junction alignment reads were then simultaneously compared for each read pair, and the most parsimonious alignment was selected by comparing the mapping quality of the reads, their edit distance to their respective references, and whether the resulting read pair would be chimeric. This

method produced results similar to those of the Tophat²³⁷ alignment strategy, while allowing the inclusion of neutral bases at hetSNP sites for allele-neutral alignment.

Pervasive, inflated *P* values

Though Het-Seq capture with custom bioinformatics greatly reduces allelic bias in genomic DNA, visualizing empirical *P* values against those expected by chance with a QQ-plot reveals that a pervasive inflation remains. This seems to be caused by a small bias in all SNPs, as opposed to a few SNPs of large effect, and the biases can be traced back to both biological and technical reasons.

Though it is often assumed that there are always two copies of a locus within a cell, replicating cells have more copies, which can cause an allelic skew if the replication timing differs between two alleles. A chromosome-wide example would be the X chromosome²³⁸. The cells divide soon after replicating the inactive X, but the active X already has two copies by early S phase, causing a skew towards the active X's alleles. We see this skew in our genomic DNA library with a *P* value of $6.2e^{-13}$ (with all the X loci, collectively) but the skew disappears when we measure an autosome. Though the X chromosome is the only known large area of effect, there may be smaller pockets of replication differences affecting our results.

Though HetSeq probes were carefully filtered and designed to prevent allelic bias in capture, it was later found that a small subset of probes bound alleles differentially due to microhomology. As well as forming a five base pair loop containing the SNP, they could shift the loop to invariant sequence by binding one allele of the SNP, causing preferential capture. We found that 750 sites in NA12878 and 1178 sites in NA19240

contained sequence similarity overlapping a targeted SNP, and they were more likely to be called significant.

Since we are not confident we have found all sources of bias, we instead focused on their impact on RNA allelic ratios. The vast majority of skews in DNA were less than 0.10 away from the 0.50 proportion expected for ref/alt allele, and those that were significant were few in number. In addition, with the fewer reads that RNA libraries contain, many of the small skews disappear with the lack of power. So, even though there is a pervasive bias, it is small in effect and should not affect our analyses.

Beta-binomial Modeling

We observe an allelic ratio $\frac{a_i}{a_i + b_i}$ for each gene, where a_i and b_i are the number of paternal and maternal reads, respectively. We think of a_i as binomial successes out of $n_i = a_i + b_i$ total reads, with a hidden probability p_i of success for gene i . The underlying distribution of p_i represents a mixture of genes that are homozygous in their active regulatory regions, and therefore have $p_i \approx 0.5$, and genes that have SNPs in regulatory regions, where p_i may be shifted from 0.5. We modeled this composite distribution of p_i as a mixture of two symmetric $Beta(\alpha_k, \alpha_k)$ distributions ($k = 1, 2$) with mixing weights w_k . α_k was fixed using the best fit $Beta(\alpha_1, \alpha_1)$ to gDNA downsampled to the median of RNA library of interest to model the homozygous genes, and the effect sizes of imbalanced genes are distributed $Beta(\alpha_2, \alpha_2)$. The generative model for expected data is thus: for each $i = 1, 2, \dots, N$, select k with probability w_k . Then generate $p_i \sim Beta(\alpha_k, \alpha_k)$, $a_i \sim Binomial(n_i, p_i)$, and set $b_i = n_i - a_i$.

We fit w and a_2 using a standard expectation-maximization (EM) approach:

For observation (a_i, b_i) the likelihood under the k th Beta distribution is:

$$p(a_i|n_i, \alpha_k) = L(k, i) = \frac{B(\alpha_k + a_i, \alpha_k + b_i)}{B(\alpha_k, \alpha_k)}$$

Thus the posterior probability that k was selected is:

$$\gamma_{ik} = \frac{w_k L(k, i)}{\sum_k w_k L(k, i)}$$

and the log likelihood is:

$$\Lambda(k, i) = \log \left(\sum_k w_k L(k, i) \right)$$

Further the mean posterior of $\log(p_i)$ if k is selected is:

$$E[\log p_i | a_i, n_i, \alpha_k] = X(k, i) = \psi(\alpha_k + a_i) - \psi(2\alpha_k + n_i)$$

Reestimates are obtained by computing:

$$X_k = \frac{\sum_i \gamma_{ik} X(k, i)}{\sum_i \gamma_{ik}}$$

$$N_k = \sum_i \gamma_{ik}$$

Set new weights:

$$w_k = \frac{N_k}{N}$$

and solve:

$$X_k = \psi(\alpha_k) - \psi(2\alpha_k)$$

for α_2 .

ddPCR Confirmations

We selected 24 SNPs for confirmation by ddPCR, with 20 assays passing basic quality control tests for off-target activity. Each assay was composed of two probes (FAM and HEX) that were specific for either allele of the target SNP. We used the

official Bio-Rad protocol for their QX100 Droplet Digital PCR System. Due to the digital nature of ddPCR, it was possible to count the number of cDNA molecules containing each SNP and to calculate an allelic ratio with 95% confidence intervals. These assays were performed on the same RNA samples that were sequenced and were judged to agree with Het-Seq when the confidence intervals from both methods overlapped.

Appendix C. Protocols for amylase genotyping using ddPCR

In the lab:

1. Determine the approximate DNA concentration of the sample.

Restriction Digest:

2. Put together the following reaction:

1 μ l of 10 $\times$ CutSmart Buffer (New England Biolabs)

0.5 μ l of EcoRI (20,000 units/ml, New England Biolabs)

80 ng of DNA

Water, bringing the reaction volume up to 10 μ l

3. Incubate at 37°C for 1 hour.

4. Add 10 μ l of water

AMY2B genotyping:

5. Prepare the following reaction:

11 μ l of 2 $\times$ ddPCR SuperMix for Probes (Bio-Rad)

0.55 μ l of Near_AMY1 assay (40 $\times$ concentration, ordered from IDT, 12.5

nMoles probe, 45 nMoles of each primer, resuspended in 1250 μ l water)

0.55 µl of AMY2B_assay1 assay (40× concentration, ordered from IDT,
12.5 nMoles probe, 45 nMoles of each primer, resuspended in 1250 µl water)

4 µl of digested DNA

5.9 µl of Water

6. Generate droplets using a QX200 droplet generator (Bio-Rad) or QX200 Auto-DG (Bio-Rad) according to manufacturer directions. Seal the plate with an aluminum slip, such as TempPlate (USA scientific).

7. Amplify in a thermocycler following this protocol: 10 min at 95°C, 50 cycles of 30 sec at 94°C and 60 s at 60°C, then 10 min at 98°C.

8. Run the droplets on a QX100 droplet reader (Bio-Rad) according to manufacturer directions.

AMY2B analysis

9. With the whole plate selected in the Quantasoft analysis software (Bio-Rad), draw the thresholds for FAM- and VIC-positive calls. These thresholds will be between the clusters of droplets, and the software will have likely called half the samples itself.

Download the .csv file

10. Using the “R scripts” part of this chapter, calculate the average DNA concentration of the PCR reaction (script is for a 96-well plate).

AMY2A Genotyping

11. If the bulk of samples had a 1 ng/μl DNA concentration in the *AMY2B* ddPCR reaction, as calculated by the script above, then genotype *AMY2A* in the same manner as *AMY2B*. Use the *AMY2A_assay1* mix (mixed the same way as the *AMY2B_assay1*) and *Near_AMY1* mix. The PCR protocol is as follows: 10 min at 95°C, 50 cycles of 30 sec at 94°C and 60 s at 57°C, then 10 min at 98°C.

11a. If the bulk of samples did not have a 1 ng/μl DNA concentration, adjust the digested-DNA input into the *AMY2A* ddPCR reaction mixture and continue with the *AMY2A* genotyping described above.

AMY2A Analysis

12. Using the Quantasoft software (Bio-Rad), set the thresholds for FAM- and VIC-positive calls, as in the *AMY2B* analysis. Download the .csv file.

13. Using the “R scripts” part of this chapter, use the concentration slider to evaluate the average DNA concentration of the PCR reaction (script is for a 96-well plate).

AMY1 First Genotyping Run

14. Use the concentration slider to determine the optimum digested-DNA input for the first *AMY1* run. The goal of this run is to “capture” most of the DNA samples, such that most will be at a concentration higher than 0.5 ng/μl. Select an input that will capture nearly all samples on the plate. Then, genotype as described above. Use the

AMY1_assay1 or 2 mix (mixed the same way as the AMY2B_assay1) and Near_AMY1 mix. The PCR protocol is as follows: 10 min at 95°C, 50 cycles of 30 sec at 94°C and 60 s at 58°C, then 10 min at 98°C.

AMY1 First Run Analysis

15. Using the Quantasoft software (Bio-Rad), set the thresholds for FAM- and VIC-positive calls, as in the *AMY2B* analysis. Download the .csv file.

16. Using the “R scripts” part of this chapter, use the percent slider to evaluate the average concentration of double-negative droplets (script is for a 96-well plate).

AMY1 Second Genotyping Run

17. Use the percent slider to determine the optimum digested-DNA input for the second *AMY1* run. The goal of this run is for every sample to have more than 10% double-negative droplets (this is to capture the samples that oversaturated their first run with PCR products). Then, genotype as described above. Use the AMY1_assay1 or 2 mix (mixed the same way as the AMY2B_assay1) and Near_AMY1 mix. The PCR protocol is as follows: 10 min at 95°C, 50 cycles of 30 sec at 94°C and 60 s at 58°C, then 10 min at 98°C.

R script:

#Import these functions. Code for running is at very bottom

```

#Loads in the csv file QuantaSoft spits out

load.in = function(file,graph=F,use.well="Well")

{

  if(graph!=F)

  {

    dev.new()

    dev.off()

    dev.new()

    par(mfrow=c(3,1))

    plate1=plate.plot(file,plate="None")

  }

  if(graph==F){plate1=plate.plot(file,plate="None",graph="no")}

  #correction factor calculation with the best samples

  conf.width = as.numeric(plate1[, "PoissonCNVMax"])-

  as.numeric(plate1[, "PoissonCNVMin"])

  percent=as.numeric(plate1[, "Negatives"])/(as.numeric(plate1[, "Negatives"])+as.numeric

  (plate1[, "Positives"]))

  select = which(conf.width<0.75 & as.numeric(plate1[, "Linkage"])<200 & percent>0.1)

  if(graph!=F){plate.cor.graph(plate1[select,])}

  #correction factor application and final plate spit-out

  if(graph!=F){corr.amy=plate.corrected(plate1[select,],file="BMI",min=0.9,max=1.2,inte

```

```

rval=0.001,plot="yes"))}

if(graph==F){corr.amy=plate.corrected(plate1[select,],file="BMI",min=0.9,max=1.2,interval=0.001,plot="yes",graph="no")}

plate1[, "CNV"]=as.numeric(plate1[, "CNV"])*corr.amy

plate1[, "PoissonCNVMax"]=as.numeric(plate1[, "PoissonCNVMax"])*corr.amy

plate1[, "PoissonCNVMin"]=as.numeric(plate1[, "PoissonCNVMin"])*corr.amy

conc.ng.ul=(Num(plate1[, "Concentration"])/Num(plate1[, "Ratio"]))/290

conc.ng.ul[is.na(conc.ng.ul)]=-0.25

if(graph!=F)

{

dev.new()

plot(plate1[order(as.numeric(plate1[, "CNV"])),"CNV"],xlab="individual",ylab="copy number",main=paste("Corrected Copy Number \nCorrection Factor =",corr.amy,"\\n",strsplit(file,"/")[1][5]),pch=20)

abline(h=c(1:15), col="gray")

segments(1:nrow(plate1),as.numeric(plate1[order(as.numeric(plate1[, "CNV"])),"PoissonCNVMax"]),1:nrow(plate1),as.numeric(plate1[order(as.numeric(plate1[, "CNV"])),"PoissonCNVMin"])),col="dark gray")

par(mfrow=c(1,1))

}

return(cbind(plate1[,c(use.well, "CNV", "PoissonCNVMin", "PoissonCNVMax", "Linkage")],conc.ng.ul,percent))

```

```

}

#####

#These functions are needed for the load in function to work:

#This calculates a plate-wide correction factor

plate.corrected <- function(file,plate,min,max,interval,plot="yes",graph="yes")
{
  f2 <- function(x) mean(abs(x-round(x))) #finds the mean deviation away from a round
  number

  a <- as.numeric(plate[, "CNV"]) #imports list of means
  amy <- as.matrix(na.omit(a)) #I think that's what he did
  adj <- seq(min, max, interval) # i know what it does, I don't know what its for
  amy_adj <- outer( adj, amy ) #some array product. creates a multiplication
  table with 2 strings of numbers
  result <- apply( amy_adj, 1, f2 )
  corr = adj[which(result==min(result))]
  plate1=plate[order(Fact.Num(plate[, "CNV"])),]
  if(graph=="yes")
  {
    plot(corr*Fact.Num(plate1[, "CNV"]),main=paste(file,"Correction Factor
of",corr),ylab="Corrected CN")
    abline(h=1:16,col="gray")
  }
  if(plot=="yes")
  {

```

```

segments(1:nrow(plate1),corr*Fact.Num(plate1[, "PoissonCNVMax"]),1:nrow(plate1),co
rr*Fact.Num(plate1[, "PoissonCNVMin"]))

}

}

return(corr)

}

f2 <- function(x)

{

mean(abs(na.omit(x)-round(na.omit(x)))) #finds the mean deviation away from a round
number

}

#This just plots the correction factor

plate.cor.graph <- function(plate,graph="yes")

{

f2 <- function(x) mean(abs(x-round(x))) #finds the mean deviation away
from a round number

a <- as.numeric(plate[, "CNV"]) #imports list of copy numbers

amy <- as.matrix(na.omit(a))

adj <- seq(0.25, 4, 0.0001) # i know what it does, I don't know what its for

amy_adj <- outer( adj, amy ) #some array product. creates a multiplication table
with 2 strings of numbers

result <- apply( amy_adj, 1, f2 ) #applies it over multiplicative matrix over rows

if(graph=="yes"){plot(adj, result, type="l", ylim=c(0,0.5), xlab="Correction

```



```

factor",ylab="Mean deviation from integer")}

}

Fact.Num = function(x){return(as.numeric(as.character(x)))}

Num = function(x){return(as.numeric(as.character(x)))}

#This plots the copy numbers in the csv

plate.plot <- function(file,plate,graph="yes")

{

Plate1.rep1 = read.csv(file=file,header=F,stringsAsFactors=F)

colnames(Plate1.rep1)=Plate1.rep1[1,]

Plate1.rep1 = Plate1.rep1[-1,]

plate1 = Plate1.rep1[c(which(Plate1.rep1[,5]=="FAMUnknown")),]

new.format="no"

if(nrow(plate1)==0)

{
  plate1 = Plate1.rep1[c(which(Plate1.rep1[,5]=="Ch1Unknown")),]

  new.format="yes"

}

rownames(plate1)=plate1[,1]

test.1=plate1

if(plate!="None")

{

rownames(Platemap)=Platemap[,1]

test.1=merge(plate1, Platemap,all.x=T,by="row.names")[,c(colnames(plate1),plate)]

colnames(test.1)[ncol(test.1)]="Individual"

```

```

}

colnames(test.1)[which(colnames(test.1)=="Ch1+Ch2+")]="FAM+VIC+"

colnames(test.1)[which(colnames(test.1)=="Ch1+Ch2-")]="FAM+VIC-"

colnames(test.1)[which(colnames(test.1)=="Ch1-Ch2+")]="FAM-VIC+"

colnames(test.1)[which(colnames(test.1)=="Ch1-Ch2-")]="FAM-VIC-"

if(graph=="yes")

{

plot(plate1[order(as.numeric(plate1[,"CNV"]))],"CNV",xlab="individual",ylab="copy
number",main=paste("Uncorrected Copy Number \nSample ID =",plate),pch=20)

abline(h=c(1:15), col="gray")

segments(1:nrow(plate1),as.numeric(plate1[order(as.numeric(plate1[,"CNV"]))],"Poisson
CNVMax"]),1:nrow(plate1),as.numeric(plate1[order(as.numeric(plate1[,"CNV"]))],"Pois
sonCNVMin"]),col="dark gray")

}

return(test.1)

}

#####

#This calculates the DNA concentrations of the samples in the plate

concentrations=function(X,afford,bins=100,amy,plate=1,graph=F)

{

if(graph!=F)

{

#prints current concentration

```

```

dev.new()

dev.off()

dev.new()

par(mfrow=c(1,3))

hist(X,breaks=bins,col="gray",main="Current Concentrations",xlab="ng/ul")

axis(side=1,at=-0.25,label="NA")

if(amy==1){abline(v=c(0.2,0.4,2.5))}

if(amy==2){abline(v=c(0.10))}

hist(X*afford,breaks=bins,col="gray",main=paste("Predicted Concentrations
with\n",afford,"x more",sep=""),xlab="ng/ul")

axis(side=1,at=-0.25*afford,label="NA")

abline(v=c(0.2,0.4,2.5))

if(amy==1){limit=0.2}

if(amy==2){limit=0.10}

con = X>limit

if(plate==1)

{

plot(x=c(rep(1:12,times=8)),y=rep(8:1,each=12),col=con+2,pch=15,cex=3,axes=F,xlab=
paste("Samples below",limit,"ng/ul"),ylab="")

axis(2,at=8:1,labels=c("A","B","C","D","E","F","G","H"),las=2)

axis(3,at=1:12)

}

if(plate==11)

```

```

{

  plot(x=c(rep(1:7,times=8)),y=rep(8:1,each=7),col=con+2,pch=15,cex=3,axes=F,xla
b=paste("Samples below",limit,"ng/ul"),ylab="")

  axis(2,at=8:1,labels=c("A","B","C","D","E","F","G","H"),las=2)

  axis(3,at=1:7)

}

}

}

#This gives you the percentages of negative droplets for each sample. Important for
  making sure the samples with high AMY1 copy number

#don't blow out their reactions (ie. have so much AMY1 that there are very few negative
  droplets)

Percent.slider=function(x,min=0,max=4,input,graph=T)
{
  dev.new()

  par(mfrow=c(2,2))

  min.per=(x[which.min(x[, "percent"]),])

  new.conc=(min.per["conc.ng.ul"]*-log(0.1))/-log(min.per[, "percent"])

  conc.ratio=new.conc/min.per["conc.ng.ul"]

  predicted.conc=conc.ratio*Fact.Num(x[, "conc.ng.ul"])

  predicted.percent=exp(log(x[, "percent"])*as.numeric(conc.ratio))

  hist(Fact.Num(x[, "conc.ng.ul"])*as.numeric(conc.ratio),main=paste("New concentration

```

```

is ",round(conc.ratio,2),"x of the old one\n Predicted Concentration
Values",sep=""),xlab="Predicted concentrations (ng/ul)",breaks=100,col="gray")
abline(v=0.25)

hist(Fact.Num(x[, "conc.ng.ul"]),main="old concentrations",xlab="concentrations
(ng/ul)",breaks=100,col="gray")
abline(v=0.25)

hist(predicted.percent,main=paste("New concentration is ",round(conc.ratio,2),"x of the
old one\n Predicted Concentration Values",sep=""),xlab="Predicted negative
percentage",breaks=100,col="gray")
abline(v=0.06)

hist(x[, "percent"],main="old percentanges",xlab="negative
percentage",breaks=100,col="gray")
abline(v=0.06)

folds=seq(min,max,by=0.1)

captured=c()

kinda=c()

nope=c()

for(i in 1:length(folds))
{
  test=folds[i]*x[, "conc.ng.ul"]
  test.ratio=test[1]/x[1, "conc.ng.ul"]
  test.predicted.percent=exp(log(x[, "percent"])*as.numeric(test.ratio))
  captured[i]=length(which(test>=0.25 & test.predicted.percent>=0.10))
}

```

```

    nope[i]=length(test)-captured[i]
  }
  if(graph==F)
  {
    dev.off()
  }
  dev.new()
  matplot(folds*input,cbind(captured,nope),type="l",col=c("green","gray","red"),xlab="ul
input")
  return(cbind(folds*input,captured,nope))
}

```

#This gives you the concentration you have to run to make sure every sample is >1ng.

Rather, it doesn't give you a number, but gives

#you graphs that help you make a good decision

```

concentration.slider=function(X,min=0,max=4,input,amy,graph=F)

```

```

{
  folds=seq(min,max,by=0.2)
  captured=c()
  kinda=c()
  nope=c()
  if(amy==1){
    for(i in 1:length(folds))
    {

```

```

test=folds[i]*X

captured[i]=length(which(test>=0.4))

kinda[i]=length(which(test<0.4 & test>=0.2))

nope[i]=length(which(test<0.2))

}

}

if(amy==2){
for(i in 1:length(folds))
{
test=folds[i]*X

captured[i]=length(which(test>=0.15))

kinda[i]=0

nope[i]=length(which(test<0.15))

}

}

if(graph!=F){matplot(folds*input,cbind(captured,kinda,nope),type="l",col=c("green","gray","red"),xlab="ul input",ylab="number of samples",main="Green = good
concentration for gentotype call")
}

return(cbind(folds*input,captured,kinda,nope))

}

```

#Compares the AMY2A copy number calls and the AMY1 copy number calls, giving a
you measure of the discordance between the two. It's a
#good indicator of how many correct copy number calls you are getting

```

concordance=function(AMY2A,AMY1,graph=F)
{
  mod.AMY2A=AMY2A[, "CNV"]
  mod.AMY2A[which(round(mod.AMY2A)==0)]=NA
  odd.evenAMY2A=round(mod.AMY2A)%%2
  odd.evenAMY1=round(AMY1[, "CNV"])%%2
  tots = odd.evenAMY1+odd.evenAMY2A
  for(i in which(round(AMY2A[, "CNV"])==0))
  {
    if(is.na(AMY1[i, "CNV"])){tots[i]=NA}
    if(is.na(AMY1[i, "CNV"])==FALSE)
    {
      if(round(AMY1[i, "CNV"])==4){tots[i]=0} else {tots[i]=1}
    }
  }
  discordance=length(which(tots==1))/nrow(na.omit(cbind(AMY2A[, "CNV"], AMY1[, "CNV"])))
  if(graph!=F)
  {
    plot(AMY2A[, "CNV"], AMY1[, "CNV"], pch=20, main=paste("Potential Discordance of", round(discordance*100, 1), "%", sep=""), xlab="AMY2A Copy Number", ylab="AMY1 Copy Number")
    rect(-0.5, 3.5, 0.5, 4.5, col="light gray")
  }
}

```



```

oddAMY1=c(3,5,7,9,11,13,15,17,19)
evenAMY1=c(2,4,6,8,10,12,14,16,18)
oddAMY2A=c(1,3,5,7)
evenAMY2A=c(2,4,6,8)
for(i in 1:length(oddAMY1))
{
  rect(oddAMY2A[1]-0.5,oddAMY1[i]-
0.5,oddAMY2A[1]+0.5,oddAMY1[i]+0.5,col="light gray")
  rect(oddAMY2A[2]-0.5,oddAMY1[i]-
0.5,oddAMY2A[2]+0.5,oddAMY1[i]+0.5,col="light gray")
  rect(oddAMY2A[3]-0.5,oddAMY1[i]-
0.5,oddAMY2A[3]+0.5,oddAMY1[i]+0.5,col="light gray")
  rect(oddAMY2A[4]-0.5,oddAMY1[i]-
0.5,oddAMY2A[4]+0.5,oddAMY1[i]+0.5,col="light gray")
}
for(i in 1:length(evenAMY1))
{
  rect(evenAMY2A[1]-0.5,evenAMY1[i]-
0.5,evenAMY2A[1]+0.5,evenAMY1[i]+0.5,col="light gray")
  rect(evenAMY2A[2]-0.5,evenAMY1[i]-
0.5,evenAMY2A[2]+0.5,evenAMY1[i]+0.5,col="light gray")
  rect(evenAMY2A[3]-0.5,evenAMY1[i]-
0.5,evenAMY2A[3]+0.5,evenAMY1[i]+0.5,col="light gray")
}

```

```

    rect(evenAMY2A[4]-0.5,evenAMY1[i]-
0.5,evenAMY2A[4]+0.5,evenAMY1[i]+0.5,col="light gray")
}

segments(x0=AMY2A[, "PoissonCNVMin"],x1=AMY2A[, "PoissonCNVMax"],y0=AM
Y1[, "CNV"],col="pink")

segments(y0=AMY1[, "PoissonCNVMin"],y1=AMY1[, "PoissonCNVMax"],x0=AMY2
A[, "CNV"],col="pink")

points(AMY2A[, "CNV"],AMY1[, "CNV"],pch=20)
}

tots=as.character(tots)

tots[which(tots==0 | tots==2)]=TRUE

tots[which(tots==1)]=FALSE

return(tots)
}

```

#This takes the concordance to the next level by selecting which of your two copy number calls most fit with your AMY2A call. If

#both fit, then it averages. If neither are concordant, then it averages.

Concordance.Averager =

```

function(AMY1.matrix,AMY1.redo.matrix,concordant.AMY1,concordant.AMY1.redo)
{
concordance.average=cbind(AMY1.matrix[, "CNV"],AMY1.redo.matrix[, "CNV"],AMY1
.matrix[, "CNV"],AMY1.redo.matrix[, "CNV"])

colnames(concordance.average)=c("adjusted CNV concentrated","adjusted CNV

```

```

dilute", "CNV concentrated", "CNV dilute")

rownames(concordance.average)=AMY1.matrix[, "Well"]

#Getting rid of discordant samples

concordance.average[which(concordant.AMY1=="FALSE"), "adjusted CNV
concentrated"]=NA

concordance.average[which(concordant.AMY1.redo=="FALSE"), "adjusted CNV
dilute"]=NA

#Average

average=rowMeans(concordance.average[, c("adjusted CNV concentrated", "adjusted
CNV dilute")], na.rm=T)

average[is.na(average)]=rowMeans(concordance.average[is.na(average), c("CNV
concentrated", "CNV dilute")], na.rm=T)

#confidence intervals

conf.dil=concordance.average[, "adjusted CNV dilute"]-
AMY1.redo.matrix[, "PoissonCNVMin"]

conf.conc=concordance.average[, "adjusted CNV concentrated"]-
AMY1.matrix[, "PoissonCNVMin"]

conf=((rowSums(cbind(conf.dil^2, conf.conc^2), na.rm=T)^0.5))/(4-
(as.numeric(as.factor(is.na(concordance.average[, "adjusted CNV
concentrated"]))))+as.numeric(as.factor(is.na(concordance.average[, "adjusted CNV
dilute"]))))))

concordance.average.total=cbind(average, average-conf, average+conf)

colnames(concordance.average.total)=c("CNV", "PoissonCNVMin", "PoissonCNVMax")

```

```
return(concordance.average.total)
```

```
}
```

```
#This is where you do your work
```

```
#Load in your csv from QuantaSoft using this command. The big graph contains your corrected values. The three smaller graphs display the work the programs are doing in order to find a correction factor. This is the plate-wide correction factor that will bring the majority of your calls closer to integers. It is calculated only from those samples that have tight confidence intervals, but is applied to the entire plate
```

```
AMY2B.2=load.in(file="/Users/cusher/Desktop/Dropbox/QLPs/GoT2D_AMY2B_2014-12-30-00-59/GoT2D_AMY2B.csv",graph=T)
```

```
AMY2B.1=load.in(file="/Users/cusher/Desktop/Dropbox/QLPs/GoT2D_Plate1_for_real_2014-12-30-01-02/GoT2D_Plate1_for_real.csv",graph=T)
```

```
#The first histogram contains your DNA concentration values for the ddPCR. The second histogram is a hypothetical one calculated from the 'afford' value. The afford value is how much more DNA you could put in the reaction if you had to (if you put 3ul in this reaction, you could hypothetically afford to put in 3x more into the next one before you hit the limit of 9ul in a reaction. The bin value simply adjusts the histogram. The amy value is saying whether this reaction was for AMY1 or AMY2A/B. The third graph in this series is displaying the well positions of samples that failed to have enough DNA in this reaction. This graph helps to see if an entire row failed, and the graph should remain mostly constant in the other reactions, unless the plate is accidentally flipped.)
```

```
concentrations(X=AMY2B.1["conc.ng.ul"],afford=2.47,bins=100,amy=2,graph=T)
```

```
concentrations(X=AMY2B.2["conc.ng.ul"],afford=2.47,bins=100,amy=2,graph=T)
```

```
concentration.slider(X=AMY2B.1[, "conc.ng.ul"], min=0, max=2.47, input=3.6, amy=2, graph=T)
```

```
concentration.slider(X=AMY2B.1[, "conc.ng.ul"], min=0, max=2.47, input=3.6, amy=1, graph=T)
```

```
concentration.slider(X=AMY2B.2[, "conc.ng.ul"], min=0, max=2.47, input=3.6, amy=1, graph=T)
```

```
concentration.slider(X=AMY2B.2[, "conc.ng.ul"], min=0, max=2.47, input=3.6, amy=2, graph=T)
```

#More of the same for AMY2A reaction

```
AMY2A=load.in(file="~/Desktop/Quantalife Runs  
Amylase/BMI/Plate3_Estonian_BMI_AMY2A/Plate3_Estonian_BMI_AMY2A.csv", graph=T)
```

```
concentrations(X=AMY2A[, "conc.ng.ul"], afford=2.47, bins=100, amy=2, graph=T)
```

```
AMY2A=load.in(file="~/Desktop/Dropbox/qlps/AMY2A_InChianti_2014-10-28-21-  
26/AMY2A_InChianti.csv", graph=T)
```

```
concentrations(X=AMY2A[, "conc.ng.ul"], afford=2.47, bins=100, amy=2, graph=T, plate=1)
```

#This graph helps you to decide how much sample to put into the AMY1 reaction based on the AMY2A reaction. You want to pick an input at where the green line levels off. The input value is how much sample (ul) that you put into the AMY2A reaction. The min and max are the multiples that you could put into the AMY1 reaction (you don't really have to change those). The amy value is 1 if you're using this function to predict for an AMY1 reaction.

```
concentration.slider(X=AMY2A[, "conc.ng.ul"], min=0, max=2.47, input=3.6, amy=1, graph=T)
```

#More of the same for the AMY1 reaction

```
AMY1=load.in(file="/Users/cusher/Desktop/Dropbox/QLPs/AMY1_plate1_2014-12-30-18-36/AMY1_plate1.csv", graph=T)
```

```
concentrations(X=AMY1[, "conc.ng.ul"], afford=1, bins=50, amy=1, graph=T)
```

```
AMY1.2=load.in(file="/Users/cusher/Desktop/Dropbox/QLPs/AMY1_plate2_2014-12-30-19-45/AMY1_plate2.csv", graph=T)
```

```
concentrations(X=AMY1.2[, "conc.ng.ul"], afford=1, bins=50, amy=1, graph=T, plate=11)
```

#Gives you a graph of the concordance between the AMY1 values and the AMY2A values. Gray boxes are expected clusterings. Percentage given at top is a rough approximation of how accurate the assays are being.

```
concordant=concordance(AMY1=AMY1, AMY2A=AMY2A, graph=T)
```

#Now that you've done one AMY1 reaction, it's time to do another one. This time, you're trying to salvage the high copy number samples that botched their last assay because they oversaturated the reaction with FAM. The idea behind this graph is much the same as the other one: pick the concentration with the highest peak.

```
Percent.slider(x=AMY1.2, min=0, max=2.6, input=4.3, graph=T)
```

#Doing much the same with the second AMY1 reaction

```
AMY1.redo=load.in(file="~/Desktop/Quantalife Runs  
Amylase/BMI/Plate3_Estonian_BMI_AMY1_redo/Plate3_Estonian_BMI_AMY1_redo.  
csv", g=T)
```

```
AMY1.redo=load.in(file="~/Desktop/Dropbox/QLPs/Extend_AMY1_2nd_run_2014-10-
```

```

31-11-09/Extend_AMY1_2nd_run.csv",g=T)

concentrations(X=AMY1.redo[, "conc.ng.ul"],afford=1,bins=50,amy=1,graph=T)

concordant.redo=concordance(AMY1=AMY1.redo,AMY2A=AMY2A,g=T)

#Spits out the AMY2A-informed averages of AMY1 copy number

AMY1.Informed.With.AMY2A.Copy.Number=Concordance.Averager(AMY1,AMY1.re
do,concordant,concordant.redo)

```

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