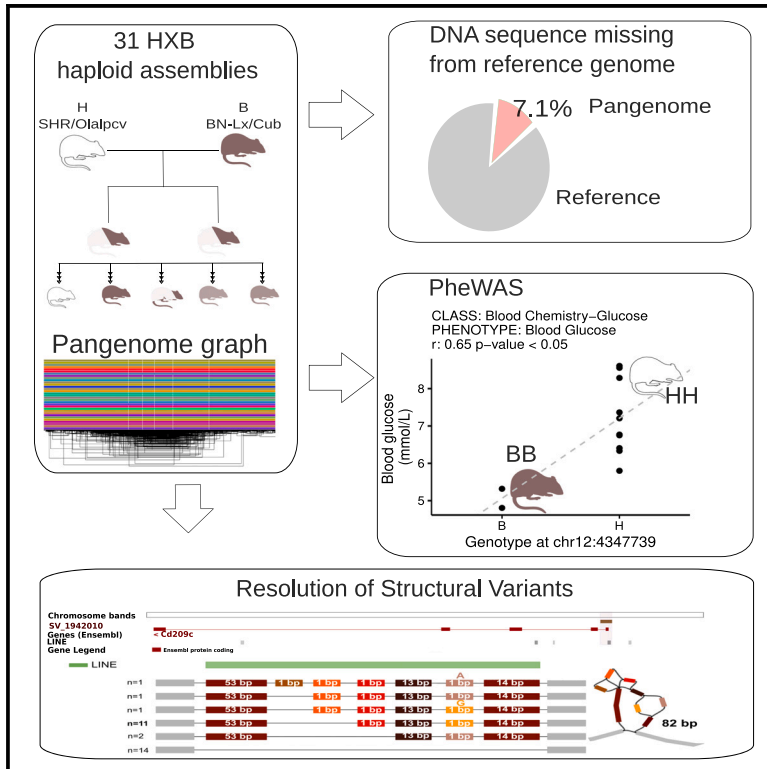


Pangenome reconstruction in rats enhances genotype-phenotype mapping and variant discovery

Graphical abstract



Authors

Flavia Villani, Andrea Guarracino, Rachel R. Ward, ..., Robert W. Williams, Hao Chen, Vincenza Colonna

Correspondence

vcolonna@uthsc.edu

In brief

Genomics; Quantitative genetics; Association analysis; Model organism

Highlights

- Rat pangenome reveals 200 million base pairs (7.1%) missing from reference genome
- Graph-based approach identifies 36% more simple variants and 99.95% more complex variants
- Pangenome finds variants linking glucose regulation to chromogranin protein levels
- Pangenome resolves complex structural variants



Article

Pangenome reconstruction in rats enhances genotype-phenotype mapping and variant discovery

Flavia Villani,¹ Andrea Guarracino,¹ Rachel R. Ward,² Tomomi Green,² Madeleine Emms,³ Michal Pravenec,⁴ Burt Sharp,¹ Piotr Prins,¹ Erik Garrison,¹ Robert W. Williams,¹ Hao Chen,² and Vincenza Colonna^{1,3,5,*}

¹Department of Genetics, Genomics, and Informatics, University of Tennessee Health Science Center, Memphis, TN, USA

²Department of Pharmacology, Addiction Science, and Toxicology, University of Tennessee Health Science Center, Memphis, TN, USA

³Institute of Genetics and Biophysics, National Research Council, 80111 Naples, Italy

⁴Institute of Physiology, Czech Academy of Sciences, 14200 Prague, Czech Republic

⁵Lead contact

*Correspondence: vcolonna@uthsc.edu

<https://doi.org/10.1016/j.isci.2025.111835>

SUMMARY

The HXB/BXH family of recombinant inbred rat strains is a unique genetic resource that has been extensively phenotyped over 25 years, resulting in a vast dataset of quantitative molecular and physiological phenotypes. We built a pangenome graph from 10x Genomics Linked-Read data for 31 recombinant inbred rats to study genetic variation and association mapping. The pangenome includes 0.2Gb of sequence that is not present in the reference mRatBN7.2, confirming the capture of substantial additional variation. We validated variants in challenging regions, including complex structural variants resolving into multiple haplotypes. Phenome-wide association analysis of validated SNPs uncovered variants associated with glucose/insulin levels and hippocampal gene expression. We propose an interaction between *Pir111*, chromogranin expression, *TNF- α* levels, and insulin regulation. This study demonstrates the utility of linked-read pangenomes for comprehensive variant detection and mapping phenotypic diversity in a widely used rat genetic reference panel.

INTRODUCTION

Rattus norvegicus has a long history of scientific research and has been used as a model organism to study various human diseases.¹ One notable example is the HXB/BXH (referred to as HXB in general hereafter) family of recombinant inbred (RI) strains of rats. The HXB family started with reciprocal crosses between the SHR/Olalpcv (H) and BN-Lx/Cub (B) strains, with the goal of creating distinct genetic lines. Currently, there are a total of 32 RI strains, all having undergone more than 50 generations of inbreeding. Among them, 21 belong to the HXB/lpcv group and 11 to the BXH/Cub group, with DNA samples preserved from almost all the original strains for the purpose of constructing genetic maps.²

The HXB is currently the most widely used RI family of laboratory rats and therefore a great resource for the identification and mapping of quantitative trait loci (QTLs) associated with complex phenotypes, motivating efforts to produce a comprehensive catalog of genetic variants. HXB have been used for studying the genetics of a wide range of phenotypes related to human diseases, such as blood pressure,³ cardiac conduction,⁴ Pavlovian conditioning,⁵ ethanol metabolism,⁶ and hippocampal neurogenesis.⁴

Pangenomic methods directly compare all genomes to each other, enabling a comprehensive analysis of genomic diversity and relationships.⁷ These methods have previously proven successful in representing human genetic variation⁸ and addressing long-standing evolutionary questions.⁹ The design, implementa-

tion, and application of pangenomic models constitute an active area of research. Ideally, pangenomes are constructed from long read data, but this technology is not always available, and the accuracy and utility of pangenome construction from suboptimal sequence data have been insufficiently explored.

In this study, we build a pangenome graph from 10x chromium Linked-Reads sequence data from 31 HXB rats plus BN/NHsdMcwi (mRatBN7.2 reference genome), and use newly identified/discovered genetic variation to perform phenome-wide association analysis and explore complex genetic variability within structural variants. We demonstrate the impact of using pangenomes in the mapping of complex traits through examples of genetic association of novel variants discovered from the pangenome and traits related to insulin and glucose metabolism.

RESULTS

Pangenome building from Linked-Reads

We performed whole genome sequencing using the 10x chromium Linked-Reads technology (average coverage depth 109) and *de novo* genome assembly of 31 inbred strains from the RI rat panel¹⁰ (Figure S1A). Evaluation of genome assembly within genic regions¹¹ shows that the majority of the genes are complete and present in a single-copy configuration (Figure S1), which is indicative of a high quality.

Using classical reference-based approach as implemented in joint-calling (JC) of variants using DeepVariant/GLnexus,¹² we



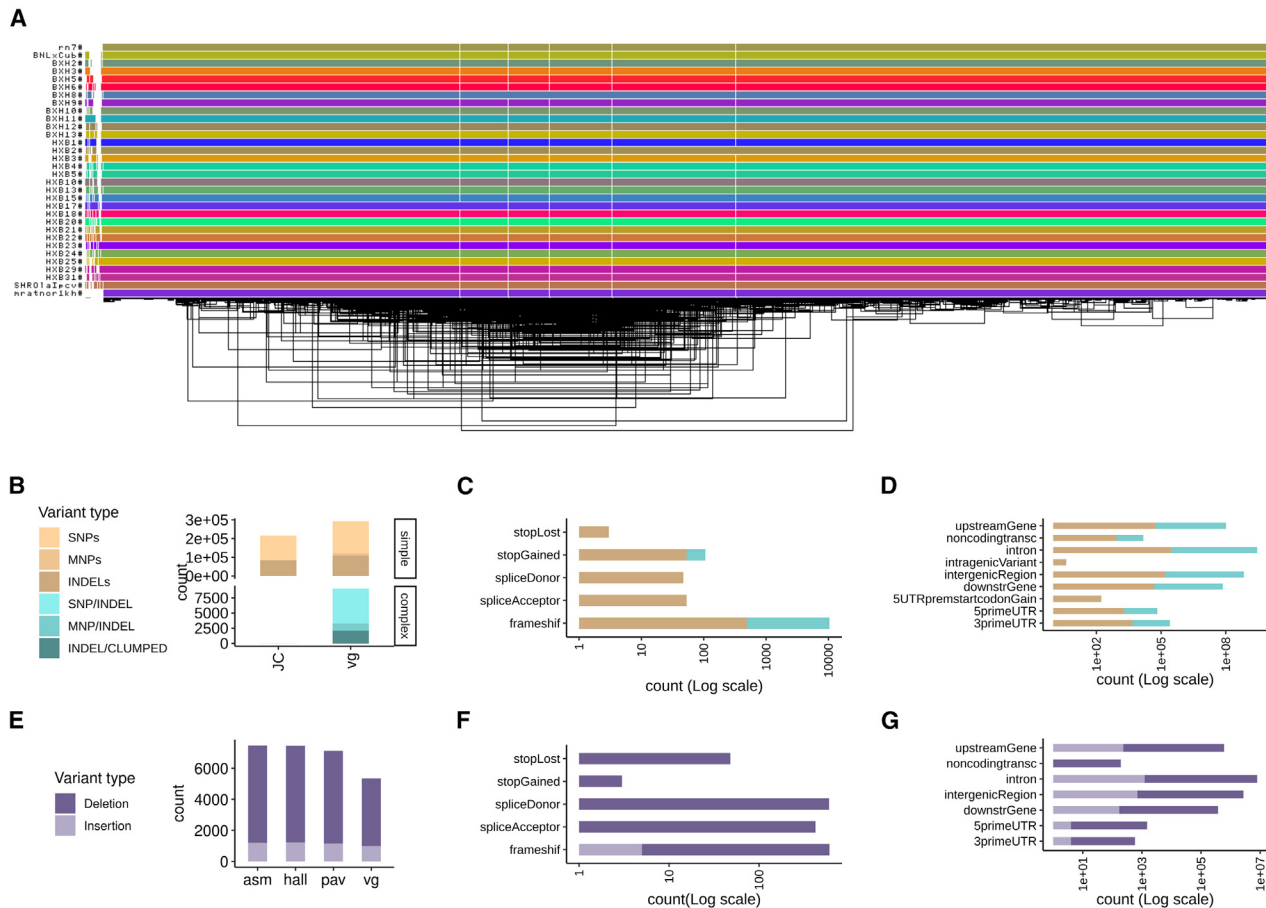


Figure 1. Pangenome graph of chromosome 12 from 31 HXB/BXH rats and genetic variants derived from it

(A) odgi-viz visualization of the pangenome graph for chromosome 12 from 31 rats. Horizontal colored lines denote haplotypes, with vertical white stripes representing insertions/deletions. Includes the mRatBN7.2 reference genome (top line).

(B) Comparison between small variants (<50bp) called from the pangenome graph using (vg) with variants joint-calling directly from 10x data using GLnexus (JC), stratified by genome complexity. vg called 36% and 99.95% more simple and complex variants, respectively, compared to JC.

(C and D) Predicted functional annotations for small variants called from the pangenome graph.

(E) Comparison of three assembly-based methods (asm, hall, pav) and one graph-based method (vg) to call structural variants (SVs, >50bp).

(F and G) Predicted functional annotations for SVs called from the pangenome graph.

identified 7,520,223 variant sites from the HXB/BXH panel, approximately 24.1%–53.5% of the alleles at these sites of each RI strain were derived from SHR/OlaIpcv. We found that the majority of the strains were highly inbred, with close to 98% of the variants being homozygous, one exception was BXH2, in which 7.7% of variants were heterozygous, likely due to a recent breeding error.⁶ Based on these results, we considered the assembly as haploid and applied PGGB¹³ to build pangenome graphs for all chromosomes from *de novo* genome assemblies of the full HXB family (generated from 10x Linked-Reads) and the mRatBN7.2 (rn7) reference genome. The lengths of such chromosome pangenome graphs were calculated as the sum of their node lengths, and after removing uncertain sequence stretches they were, on average, 1.05 times greater than the corresponding chromosome length of the reference mRatBN7.2, suggesting that the pangenome captures genetic variation beyond that contained in the reference (Table S1).

This is expected because the pangenome graphs we built represent the genetic diversity of all the strains they represent, including Single Nucleotide Polymorphisms (SNPs), Insertions-deletions (Indels), and Structural Variations (SVs). In particular, each allele would be represented with a dedicated node, therefore increasing the total graph length. For this reason, pangenome graphs can be longer than the individual genomes they represent. To demonstrate the utility of a pangenomic approach, we focused on chromosome 12 (chr12), one of the shortest chromosomes. The pangenome graph of scaffolds that map to chr12 consists of 1M nodes, 1.6M edges, and 28.5k paths, with a total length of 78M bp (Table S2; Figure 1A).

Small variants analysis and validation in the pangenome

Small variants (<50 bp in length) were called from the pangenome using graph decomposition to identify bubbles corresponding to non-overlapping variant sites, as implemented in

the vg toolkit.¹⁴ We also performed a classical reference-based approach as implemented in joint-calling (JC) of variants using DeepVariant/GLNexus.¹² Variant calling from the pangenome of chr12 identified 302,048 variants (SNPs, multi-nucleotide polymorphisms (MNP), indels, and complex, i.e., nested mixed variants) versus 215,431 variants called by JC. Of these, vg called 36% and 99.95% more simple and complex variants respectively (Figure 1B). With a focus on size up to 50 bp (small variants), we predicted functional consequences of variants on genes and proteins (Table S3). The majority of variants were classified as modifiers (99.0%). A smaller proportion of variants had a high impact (0.1%) and were located within 364 protein-coding genes. For each class of variant impact (high, moderate, low, and modifier) annotations were more abundant for simple variants (Figures 1C and 1D). Finally, with respect to gene structure, the majority of variants were located in intergenic regions and introns (Table S3).

Small variants called from the pangenome using vg were validated over the JC call set (gold standard) in the SHR/Olalpcv sample (Table S4), which represents one of the best assemblies among the strains in our pangenome in terms of quality (Table S5; Figure S1B). Genome-wide accuracy of vg calls was ~90% (Figure S2), with the exceptions of chromosome Y and 16, possibly due to the complexities in assembling chromosome Y and the abundance of complex variants for chromosome 16 (Figure S2C). Further validation by resequencing was restricted to twenty SNPs on chr12. Selection criteria for these small variants were: support by original reads determined by mapping reads against the pangenome with vg giraffe,¹⁵ and location in regions supposed to be particularly challenging (14 were within repetitive regions and 6 within 1500 bp from repetitive regions). Twelve out of the 20 variants were confirmed by Sanger sequencing (Table S6), which translates into a 0.6 rate of validation. Approximately 90% of variants (7 out of 8) that failed the validation were within repeats. While these results cannot be generalized to the entire genome given the targeted Sanger validation we performed, we used these high-confidence variants for subsequent analyses.

Novel pangenome variants associated with glucose, insulin and chromogranin expression

We leveraged phenotypic data present in GeneNetwork¹⁶ to perform genome-wide association analysis (PheWAS) for the twelve SNPs validated through Sanger sequencing. PheWAS was restricted to 30 rat strains with phenotypic data, and 60 phenotype classes with at least two phenotypes per class. Two SNPs were significantly associated with six phenotypes within five phenotype classes (LRS>16, Figures 2A and 2B; Table S7). We further considered three of the six significant associations that were validated using a linear mixed model corrected for kinship implemented in GeneNetwork (Figures 2C–2E).

The first association was between blood glucose concentration and an SNP (chr12_4347739) within the long non-coding RNAs gene *Zfp958L1*. Rats carrying the *H allele* had significantly higher blood glucose concentrations (Figure 2C). chr12_4347739 variant was located within a mapped QTL that controls insulin/glucose ratio, mapped in an independent cohort of 185 F2 rats (Insglur6; LOD score 18.97, *p*-value: 0.001).¹⁷

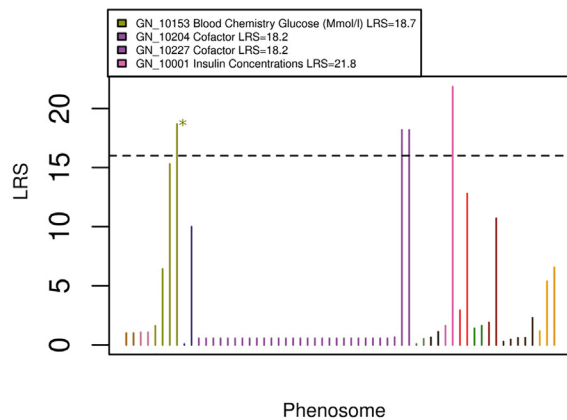
The second relevant association was between a variant and two phenotypes, insulin concentration in blood and gene expression of chromogranin (CGA) in the hippocampus, and an intronic SNP (chr12_18797475) within an annotated locus (*LOC685157*). Rats with the *H allele* at chr12_18797475 had higher expression of CGA in the hippocampus and lower blood insulin concentration (Figures 2D and 2E). The relationship between increased CGA expression, and insulin concentration aligns with previous findings, chromogranin A regulates catecholamine storage and release, variation affecting CGA levels could impact catecholamines that can suppress insulin secretion and affect glucose metabolism.^{18,19} CGA expression is ubiquitous throughout the central nervous system,²⁰ especially in the hypothalamus and amygdala regions, and stimulates the production of tumor necrosis factor alpha (TNF- α)²¹ a key inflammatory molecule that promotes insulin resistance and glucose accumulation. We hypothesize that in rats with the *H allele*, increased CGA expression and subsequent TNF- α production leads to insulin resistance, impairing cellular glucose uptake and resulting in reduced blood insulin levels (Figure 2F). This proposed mechanism is consistent with the observed reduction in insulin concentration associated with the other significant PheWAS variant (Figure 2C).

Remarkably, *LOC685157* is similar to the paired immunoglobulin-like type 2 receptor beta 1 like 1 gene (*Pilrb111*, Ensembl: *LOC681182*) predicted to code for a protein putatively located on the membrane. The associated intronic variant chr12_18797475 was located just 0.7kb upstream of *Pilrb111*. *Pilrb111* orthologs to human paired immunoglobulin like type 2 receptor alpha (*PILRA*) code for a lectin that recognizes both O-glycan and aglycon²⁰ and a missense variant (rs1859788, p.Arg78Gly) in the functional immunoglobulin like domain seems to be protective for Alzheimer's disease.²¹ In mice, *Pilrb111* is abundantly expressed on myeloid cells, and required for cell movement out of the circulatory system and toward the site of tissue damage or infection.²² *Pilrb-/-* mice have reduced serum or bronchoalveolar lavage fluid levels of tumor necrosis factor alpha (TNF- α)²³ suggesting that increased CGA expression and consequent TNF- α production might compensate for the lack of function of *Pilrb111*. Finally, chr12_18797475 is located 0.8 Mb from a mapped QTL that controls hyperglycemia related to diabetes and increased susceptibility to autoimmune disorder (Iddm2; LOD 18.97).¹⁷

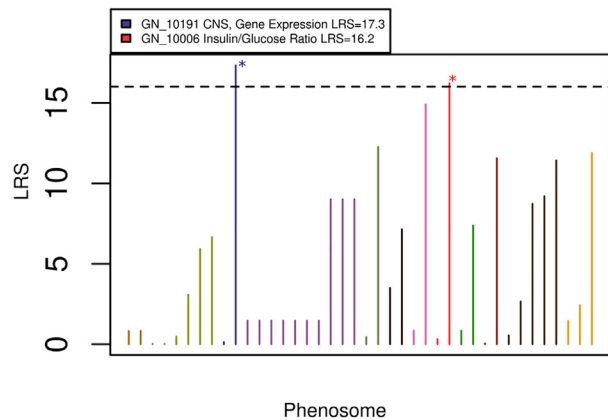
Novel complex structural variants discovered from the graph pangenome

SVs (>50bp in length) of chr12 from 31 HXB were called using four assembly-based methods (pav, svim, hall, vg),⁸ one of which was a graph-based method (vg). We retained 7,811 SVs supported by at least two methods, out of 9,405 identified by at least one method (Figure S3). Contrary to small variants, here the graph-based method called less SVs than other methods (Figure 1E). Out of the 7,811 SVs, 1,282 were insertions and 6,526 deletions. We predicted the functional consequences of variants on genes and proteins (Table S8; Figures 1F and 1G). The majority of variants were classified as modifiers (56.0%). A big proportion of variants had a high impact (44%) and were located within 918 protein-coding genes. Notably, the majority of variants were located in exon regions and introns (Table S8).

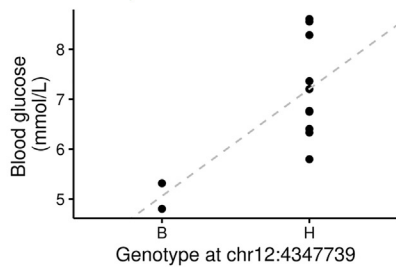
A Chr12:4347739 (*Zfp958l1*)



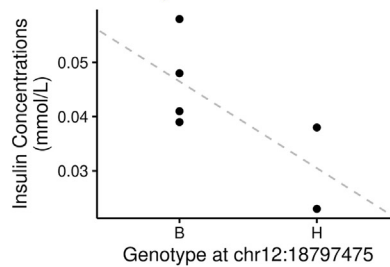
B Chr12:18797475 (*LOC685157*)



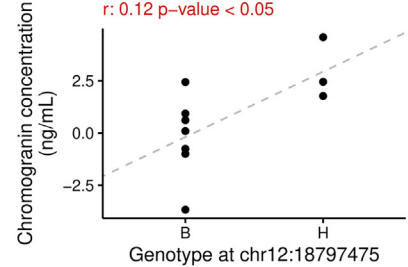
C CLASS: Blood Chemistry–Glucose
PHENOTYPE: Blood Glucose
r: 0.65 p–value < 0.05



D CLASS: Insulin/Glucose Ratio
PHENOTYPE: Insulin Concentrations
r: –0.83 p–value < 0.05



E CLASS: CNS/Gene expression
PHENOTYPE: Chromogranin in hippocampus
r: 0.12 p–value < 0.05



F

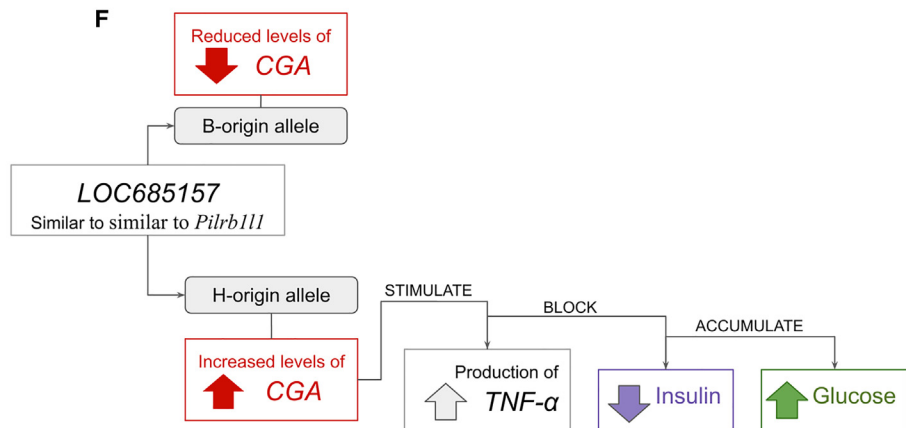


Figure 2. Mapping of validated pangenomic variants against phenotypes in GeneNetwork

(A and B) PheWAS and results for two variants in the long non-coding RNAs gene *Zfp958l1* and the annotated gene *LOC685157*, which is similar to *Ptilrb111*. Colors refer to phenotype classes, the dashed horizontal line indicates threshold for significance in the PheWAS. Stars mark genotype-phenotype associations that were validated also through Spearman correlation analysis (adjusted p -value < 0.05) reported in (C–E). In (F) we propose a hypothesis that reconciles our genotype-specific findings with what is known in literature about these genes and their relationship.

We further validated SVs of the SHR/OlaIpcv sample called from the pangenome. We retained 2,481 variants supported by at least two methods out of 4,853 identified by at least one method (Figure S3). We selected ten variants with high impact and sized between 50 and 500bp, that were examined using Ox-

ford Nanopore long-read data from SHR/OlaIpcv rat. Two insertions and four deletions were confirmed, suggesting a validation rate of 0.6 (Figure S4).

All six validated SVs were detected in additional non-SHR strains, with variant frequency varying from 0.35 to 1 (Table S9).

A deletion of 300bp in the *Lmtk2* gene was found in SHR/Olalpcv and all other samples (Figure 3A). A second deletion of 357bp in the *Mcemp1* gene found in SHR/Olalpcv was also found in 17 rats (Figure 3B). This G-rich SV contained a non-LTR retrotransposon that belongs to the long interspersed nuclear elements (LINEs). It also contained two B1F repetitive elements, or *Alu*, that belong to the class of retroelements called short interspersed elements (SINEs, Table S9).

One deletion and one insertion found in SHR/Olalpcv represent examples of complex variation that were fully resolved by the pangenome graph that captured all the realized haplotypes. The first complex deletion SV (117 bp, supported by two methods) was found in the *LOC679924* gene, predicted to enable ATP binding activity, and overlapped for 80bp with ab SINE element (Figure 3C). The second complex insertion SV (82 bp, supported by all four methods) fell within the *Cd209c* gene, and, despite its shorter length, was made of seven variable blocks, which combined into six haplotypes, four of which were seen less than two times (Figure 3D). It contained a Lx8b_3end, a non-LTR retrotransposon in rodents (LINE). The Human ortholog of *Cd209c* is implicated in Coronavirus infectious disease,²² aspergillosis,²³ dengue disease,²⁴ and tuberculosis.²⁵

DISCUSSION

In this study, we built the first rat pangenome to explore the genetic variation landscape of the rat HXB/BXH family. We conduct association mapping with emphasis on novel variants identified by our pangenomic approaches on chr12. The pangenome graph we built enabled the discovery of novel small variants and SVs. Although the sequencing data we used (i.e., 10x Linked-Reads) were not ideal, our research highlights the potential for adequate pangenomic reconstruction. One key finding of this study lies in the significant increase in the number and complexity of variants identified by the pangenome.

After removing uncertain sequence stretches (Ns) the ratio between pangenome and reference sequence lengths is 1.05. Variants in the same genomic regions across multiple samples lead to multiple representations of that region, and the graph accounts for all the different alleles. For this reason, pangenome graphs can be longer than the individual genomes they represent. In particular, we observed in the pangenome 0.18 Gb of non-reference sequence with respect to mRatBN7.2. This figure becomes 3.9 Gb when including Ns: such high value overestimates the real non-reference bases in our pangenome and reflects the technological limitation of applying Linked-Reads in *de novo* assembly. Most of the novel pangenomic variation is complex, confirming that the pangenome captures genetic variation beyond that contained in the reference genome.

Validation in SHR/Olalpcv shows high rates of concordance and, except for complex repeated regions, we validated through resequencing novel variants in challenging regions. We provide validated examples of how the pangenome fully resolves complex nested variants within repetitive regions. We identified and validated novel complex SVs falling within genes implicated in immunity and enriched for transposable elements, a major source of genetic polymorphism, known to act as regulatory elements within immune regulatory networks, and facilitate rapid

adaptive evolution of immune responses.²⁶ Mutations in the *Lmtk2* gene have been linked to multiple neurological disorders, including Alzheimer's disease, Parkinson's disease, and schizophrenia in humans. *Mcemp1* seems to be involved in the regulation of mast cell differentiation and immune responses. Its human orthologous *MCEMP1* is one of the top inducible genes in many inflammatory diseases, such as asthma,²⁷ idiopathic pulmonary fibrosis,²⁸ cancer,²⁹ sepsis,³⁰ and stroke.³¹ *MCEMP1* is a critical factor in allergic and inflammatory lung diseases,³² with the level of *MCEMP1* expression positively correlating with disease severity. These examples provide crucial insights into regions of the genome that play a key role in adaptation, but have been poorly investigated so far due to their challenging resolution.

We demonstrate that novel variants identified by the pangenome are valuable for mapping quantitative traits, demonstrating that access to this fullest spectrum of genetic diversity is key for studying genotype-phenotype connections. We used validated SNPs to conduct phenome-wide association analysis, to discover two significant associations: blood glucose concentration linked to a variant (chr12_4347739) within the *Zfp958L1* gene, and insulin levels in the blood and the expression of chromogranin in the hippocampus, mediated by an intronic variant (chr12_18797475) within a gene (*LOC685157*) similar to *Pilrb111*.

Overall, this pangenomic study enhances our understanding of the rat HXB/BXH family and sets a new standard for genomic research in model organisms, underscoring the importance of comprehensive genetic analysis for revealing the complexity of genotype-phenotype connections.

Limitations of the study

Although this pioneering rat pangenome analysis demonstrates the capability to capture complex variation beyond the reference genome and enable more complete genotype-phenotype mapping, we note a few limitations. The Linked-Reads technology is not ideal for *de novo* assembly and leads to an overestimation of non-reference sequences due to unresolved regions. Additionally, complex regions remain challenging to fully resolve despite validation efforts. The combination of Linked-Reads and long-read sequencing technologies offers the promise of constructing higher-quality assemblies and then pangenomes. As sequencing technologies and algorithms continue advancing, we expect to achieve complete pangenome assembly and whole genome structural variation characterization that will reveal the fullest extent of genome variation across rat strains. Despite current limitations, our study demonstrates the capability of pangenomes to enable more accurate, even more complete genotype-phenotype mapping.

In the PheWAS analysis, we did not assess the influence of sex due to the limited sample size. Consequently, this limitation affects the generalizability of our research findings.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and data should be directed to and will be fulfilled by Hao Chen (hchen3@uthsc.edu) and Vincenza Colonna (vincenza.colonna@uthsc.edu).

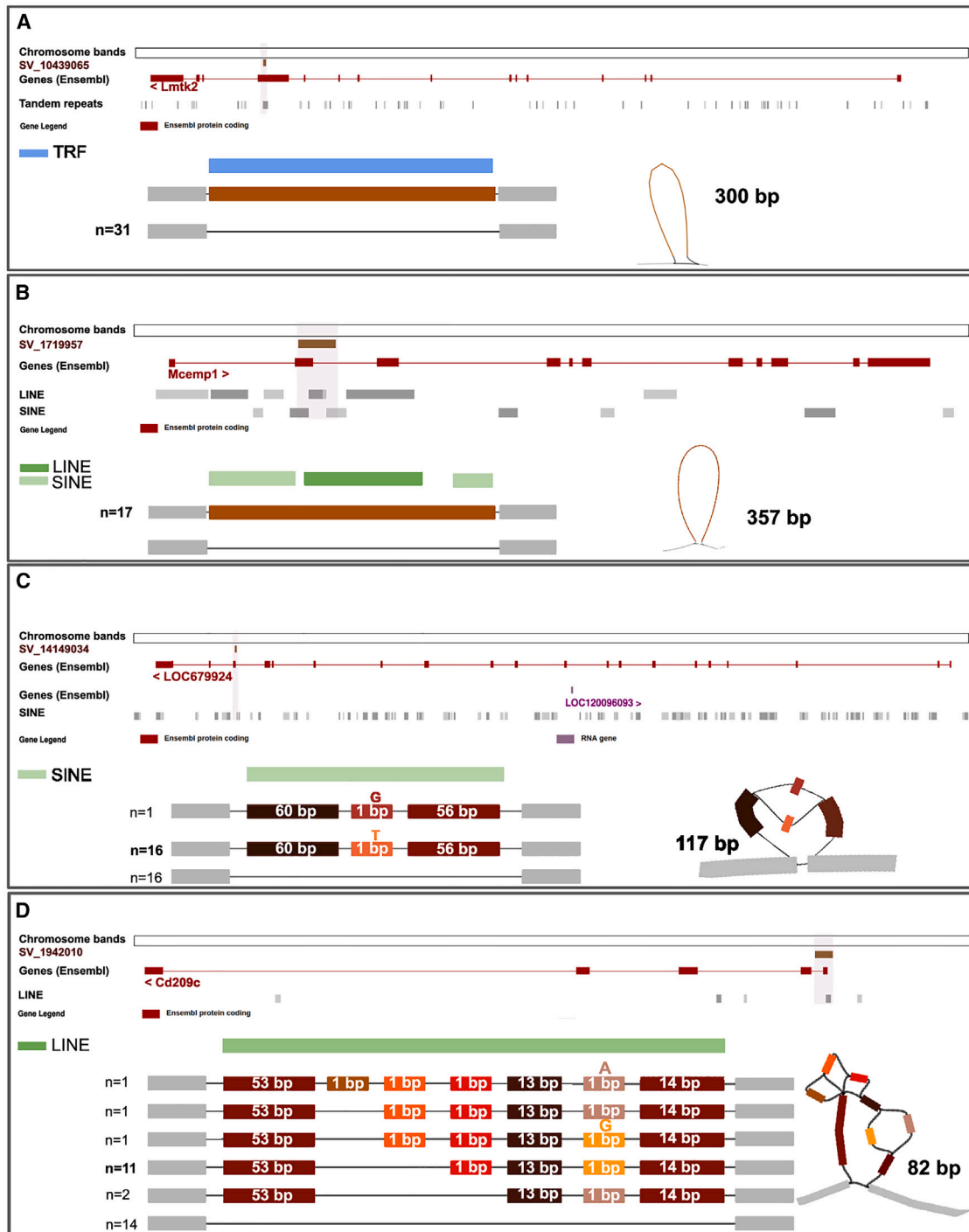


Figure 3. Resolution of complex haplotypes in validated structural variants (SVs) discovered using pangenome graphs
Visualization using ODGI and Bandage of four SVs predicted to have high impact and validated with Oxford Nanopore sequencing technology.
(A) A deletion of 300bp in the *Lmtk2* gene supported by all four methods of identification, is found in all 31 samples and contains a Tandem Repeats element (TRF).
(B) A deletion of 357bp in the *Mcemp1* gene is supported by all four methods and is found in 17 rats. The gene contains SINE and LINE elements.
(C) A deletion of 117 bp in the *LOC679924* gene is supported by two methods, and includes a single nucleotide variant. The SV overlaps for 80 bp with an SINE element.
(D) A complex insertion of 82 bp in the *Cd209c* gene, supported by all four methods. The insertion resolves in six haplotypes of which two are most abundant, and falls completely in a non-LTR retrotransposon (LINE).

Materials availability

Classic inbred strains (BXH/HXB) discussed in the prior sections are included in the Hybrid Rat Diversity Panel (HRDP).³³ These rats are available from the Medical College of Wisconsin (https://rgd.mcw.edu/rgdweb/hrdp_panel.html).

Data and code availability

- The Linked-Reads WGS data have been deposited in NIH SRA and is publicly available.¹⁰ The accession numbers are listed in [Table S10](#).
- All original code used to perform the analyses has been deposited at GitHub (https://github.com/Flavia95/HXB_rat_pangenome_manuscript/) and is publicly available.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

ACKNOWLEDGMENTS

V.C. was supported by Ministero dell'Istruzione, dell'Università e della Ricerca of the Italian Republic (PRIN 2020J84FAM). The work of H.C., E.G., and R.W.W. is supported by NIH NIDA U01DA047638. M.P. was supported by a program from the National Institute for Research of Metabolic and Cardiovascular Diseases (Program EXCELES, ID Project No. LX22NPO5104) funded by the European Union - Next Generation EU and by grant LUAUS25164 within the INTER-EXCELLENCE program of the Ministry of Education, Youth, and Sports of the Czech Republic. B.S. was supported by U01DA057530.

AUTHOR CONTRIBUTIONS

Conceptualization, F.V., A.G., M.E., E.G., R.W.W., H.C., and V.C.; formal analysis, F.V., A.G., M.E., H.E., and V.C.; visualization, E.G., R.W.W., H.C., and V.C.; investigation, R.W., T.G., and M.P.; resources, P.P., R.W.W., H.C., and B.S.; data curation, F.V., A.G., R.W., T.G., E.G., R.W.W., H.C., and V.C.; writing - review and editing, F.V., A.G., R.W., T.G., E.G., P.P., R.W.W., H.C., and V.C.; supervision, E.G., R.W.W., H.C., and V.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- [KEY RESOURCES TABLE](#)
- [EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS](#)
- [METHOD DETAILS](#)
 - Supernova assembly
 - Pangenome generation and evaluation
 - Variant calling
 - Sanger resequencing for variant validation
 - PheWAS analyses and functional annotation
 - SVs validation with nanopore adaptive sequencing
- [QUANTIFICATION AND STATISTICAL ANALYSIS](#)

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2025.111835>.

Received: February 8, 2024

Revised: April 16, 2024

Accepted: January 15, 2025

Published: January 23, 2025

REFERENCES

1. Gibbs, R.A., Weinstock, G.M., Metzker, M.L., Muzny, D.M., Sodergren, E.J., Scherer, S., Scott, G., Steffen, D., Worley, K.C., Burch, P.E., et al. (2004). Genome sequence of the Brown Norway rat yields insights into mammalian evolution. *Nature* 428, 493–521. <https://doi.org/10.1038/nature02426>.
2. Printz, M.P., Jirout, M., Jaworski, R., Alemayehu, A., and Kren, V. (2003). Invited Review: HXB/BXH rat recombinant inbred strain platform: a newly enhanced tool for cardiovascular, behavioral, and developmental genetics and genomics. *J. Appl. Physiol.* 94, 2510–2522. <https://doi.org/10.1152/jappphysiol.00064.2003>.
3. Pravenec, M., Churchill, P.C., Churchill, M.C., Viklicky, O., Kazdova, L., Aitman, T.J., Petretto, E., Hubner, N., Wallace, C.A., Zimdahl, H., et al. (2008). Identification of renal Cd36 as a determinant of blood pressure and risk for hypertension. *Nat. Genet.* 40, 952–954. <https://doi.org/10.1038/ng.164>.
4. Adriaens, M.E., Lodder, E.M., Moreno-Moral, A., Šilhavý, J., Heinig, M., Glinge, C., Belterman, C., Wolswinkel, R., Petretto, E., Pravenec, M., et al. (2018). Systems Genetics Approaches in Rat Identify Novel Genes and Gene Networks Associated With Cardiac Conduction. *J. Am. Heart Assoc.* 7, e009243. <https://doi.org/10.1161/JAHA.118.009243>.
5. Bielavská, E., Kr, V., and Pravenec, M. (2002). Genome Scanning of the HXB/BXH Sets of Recombinant Inbred Strains of the Rat for Quantitative Trait Loci Associated with Conditioned Taste Aversion. *Behav. Genet.* 32, 51–56.
6. Lusk, R., Saba, L.M., Vanderlinden, L.A., Zidek, V., Silhavy, J., Pravenec, M., Hoffman, P.L., and Tabakoff, B. (2018). Unsupervised, Statistically Based Systems Biology Approach for Unraveling the Genetics of Complex Traits: A Demonstration with Ethanol Metabolism. *Alcohol Clin. Exp. Res.* 42, 1177–1191. <https://doi.org/10.1111/acer.13763>.
7. Eizenga, J.M., Novak, A.M., Sibbesen, J.A., Heumos, S., Ghaffaari, A., Hickey, G., Chang, X., Seaman, J.D., Rounthwaite, R., Ebler, J., et al. (2020). Pangenome Graphs. *Annu. Rev. Genom. Hum. Genet.* 21, 139–162. <https://doi.org/10.1146/annurev-genom-120219-080406>.
8. Liao, W.-W., Asri, M., Ebler, J., Doerr, D., Haukness, M., Hickey, G., Lu, S., Lucas, J.K., Monlong, J., Abel, H.J., et al. (2023). A draft human pangenome reference. *Nature* 617, 312–324. <https://doi.org/10.1038/s41586-023-05896-x>.
9. Guarracino, A., Buonaiuto, S., de Lima, L.G., Potapova, T., Rhie, A., Koren, S., Rubinstein, B., Fischer, C., Human Pangenome Reference Consortium, Gerton, J.L., et al. (2023). Recombination between heterologous human acrocentric chromosomes. *Nature* 617, 335–343. <https://doi.org/10.1038/s41586-023-05976-y>.
10. De Jong, T.V., Pan, Y., Rastas, P., Munro, D., Tutaj, M., Akil, H., Benner, C., Chen, D., Chitre, A.S., Chow, W., et al. (2024). A revamped rat reference genome improves the discovery of genetic diversity in laboratory rats. *Cell Genom.* 4, 100527. <https://doi.org/10.1016/j.xgen.2024.100527>.
11. Huang, N., and Li, H. (2023). compleasm: a faster and more accurate reimplementation of BUSCO. *Bioinformatics* 39, btad595. <https://doi.org/10.1093/bioinformatics/btad595>.
12. Yun, T., Li, H., Chang, P.-C., Lin, M.F., Carroll, A., and McLean, C.Y. (2021). Accurate, scalable cohort variant calls using DeepVariant and GLnexus. *Bioinformatics* 36, 5582–5589. <https://doi.org/10.1093/bioinformatics/btaa1081>.
13. Garrison, E., Guarracino, A., Heumos, S., Villani, F., Bao, Z., Tattini, L., Haggmann, J., Vorbrugg, S., Marco-Sola, S., Kubica, C., et al. (2024). Building pangenome graphs. *Nat. Methods* 21, 2008–2012. <https://doi.org/10.1038/s41592-024-02430-3>.
14. Garrison, E., Sirén, J., Novak, A.M., Hickey, G., Eizenga, J.M., Dawson, E.T., Jones, W., Garg, S., Markello, C., Lin, M.F., et al. (2018). Variation graph toolkit improves read mapping by representing genetic variation in the reference. *Nat. Biotechnol.* 36, 875–879. <https://doi.org/10.1038/nbt.4227>.

15. Sirén, J., Monlong, J., Chang, X., Novak, A.M., Eizenga, J.M., Markello, C., Sibbesen, J.A., Hickey, G., Chang, P.-C., Carroll, A., et al. (2021). Pangenomics enables genotyping of known structural variants in 5202 diverse genomes. *Science* 374, abg8871. <https://doi.org/10.1126/science.abg8871>.
16. Mulligan, M.K., Mozhui, K., Prins, P., and Williams, R.W. (2017). GeneNetwork: A Toolbox for Systems Genetics. In *Systems Genetics Methods in Molecular Biology*, K. Schughart and R.W. Williams, eds. (Springer), pp. 75–120. https://doi.org/10.1007/978-1-4939-6427-7_4.
17. Marissal-Arvy, N., Heliès, J.-M., Tridon, C., Moisan, M.-P., and Mormède, P. (2014). Quantitative Trait Loci Influencing Abdominal Fat Deposition and Functional Variability of the HPA Axis in the Rat. *Horm. Metab. Res.* 46, 635–643. <https://doi.org/10.1055/s-0034-1383574>.
18. Jirout, M.L., Friese, R.S., Mahapatra, N.R., Mahata, M., Taupenot, L., Mahata, S.K., Křen, V., Zídek, V., Fischer, J., Maatz, H., et al. (2010). Genetic regulation of catecholamine synthesis, storage and secretion in the spontaneously hypertensive rat. *Hum. Mol. Genet.* 19, 2567–2580. <https://doi.org/10.1093/hmg/ddq135>.
19. Zhang, K., Mir, S.A., Hightower, C.M., Miramontes-Gonzalez, J.P., Maihofer, A.X., Chen, Y., Mahata, S.K., Nievergelt, C.M., Schork, N.J., Freedman, B.I., et al. (2015). Molecular Mechanism for Hypertensive Renal Disease: Differential Regulation of Chromogranin A Expression at 3'-Untranslated Region Polymorphism C+87T by MicroRNA-107. *J. Am. Soc. Nephrol.* 26, 1816–1825. <https://doi.org/10.1681/ASN.2014060537>.
20. Schafer, M.K.-H., Mahata, S.K., Stroth, N., Eiden, L.E., and Weihe, E. (2010). Cellular distribution of chromogranin A in excitatory, inhibitory, aminergic and peptidergic neurons of the rodent central nervous system. *Regul. Pept.* 165, 36–44. <https://doi.org/10.1016/j.regpep.2009.11.021>.
21. Ciesielski-Treska, J., Ulrich, G., Taupenot, L., Chasserot-Golaz, S., Corti, A., Aunis, D., and Bader, M.-F. (1998). Chromogranin A Induces a Neurotoxic Phenotype in Brain Microglial Cells. *J. Biol. Chem.* 273, 14339–14346. <https://doi.org/10.1074/jbc.273.23.14339>.
22. Nagozir, S., Shakouri Khomartash, M., Parsania, M., Vahidi, M., and Ghorbani, M. (2023). Association between genetic variants in the CD209 gene and susceptibility to COVID-19 in Iranian population. *Hum. Gene* 38, 201215. <https://doi.org/10.1016/j.humgen.2023.201215>.
23. Morton, C.O., Fliesser, M., Dittrich, M., Mueller, T., Bauer, R., Kneitz, S., Hope, W., Rogers, T.R., Einsele, H., and Loeffler, J. (2014). Gene Expression Profiles of Human Dendritic Cells Interacting with *Aspergillus fumigatus* in a Bilayer Model of the Alveolar Epithelium/Endothelium Interface. *PLoS One* 9, e98279. <https://doi.org/10.1371/journal.pone.0098279>.
24. Sakuntabhai, A., Turbpaiboon, C., Casadémont, I., Chuansumrit, A., Lowhnoo, T., Kajaste-Rudnitski, A., Kalayanarooj, S.M., Tangnaratchakit, K., Tangthawornchaikul, N., Vasanawathana, S., et al. (2005). A variant in the CD209 promoter is associated with severity of dengue disease. *Nat. Genet.* 37, 507–513. <https://doi.org/10.1038/ng1550>.
25. Vannberg, F.O., Chapman, S.J., Khor, C.C., Tosh, K., Floyd, S., Jackson-Sillah, D., Crampin, A., Sichali, L., Bah, B., Gustafson, P., et al. (2008). CD209 Genetic Polymorphism and Tuberculosis Disease. *PLoS One* 3, e1388. <https://doi.org/10.1371/journal.pone.0001388>.
26. Ivancevic, A., and Chuong, E.B. (2020). Transposable elements teach T cells new tricks. *Proc. Natl. Acad. Sci. USA* 117, 9145–9147. <https://doi.org/10.1073/pnas.2004493117>.
27. Bosco, A., McKenna, K.L., Firth, M.J., Sly, P.D., and Holt, P.G. (2009). A Network Modeling Approach to Analysis of the Th2 Memory Responses Underlying Human Atopic Disease. *J. Immunol.* 182, 6011–6021. <https://doi.org/10.4049/jimmunol.0804125>.
28. Herazo-Maya, J.D., Noth, I., Duncan, S.R., Kim, S., Ma, S.-F., Tseng, G.C., Feingold, E., Juan-Guardela, B.M., Richards, T.J., Lussier, Y., et al. (2013). Peripheral Blood Mononuclear Cell Gene Expression Profiles Predict Poor Outcome in Idiopathic Pulmonary Fibrosis. *Sci. Transl. Med.* 5, 205ra136. <https://doi.org/10.1126/scitranslmed.3005964>.
29. Zeng, D., Wu, J., Luo, H., Li, Y., Xiao, J., Peng, J., Ye, Z., Zhou, R., Yu, Y., Wang, G., et al. (2021). Tumor microenvironment evaluation promotes precise checkpoint immunotherapy of advanced gastric cancer. *J. Immunother. Cancer* 9, e002467. <https://doi.org/10.1136/jitc-2021-002467>.
30. Chen, J.X., Xu, X., and Zhang, S. (2019). Silence of long noncoding RNA NEAT1 exerts suppressive effects on immunity during sepsis by promoting microRNA-125-dependent MCEMP1 downregulation. *IUBMB Life* 71, 956–968. <https://doi.org/10.1002/iub.2033>.
31. Wood, H. (2016). MCEMP1 — a new prognostic and diagnostic biomarker for stroke? *Nat. Rev. Neurol.* 12, 127. <https://doi.org/10.1038/nrneuro.2016.17>.
32. Choi, Y.J., Yoo, J.-S., Jung, K., Rice, L., Kim, D., Zlojutro, V., Frimel, M., Madden, E., Choi, U.Y., Foo, S.-S., et al. (2023). Lung-specific MCEMP1 functions as an adaptor for KIT to promote SCF-mediated mast cell proliferation. *Nat. Commun.* 14, 2045. <https://doi.org/10.1038/s41467-023-37873-3>.
33. Pattee, J., Vanderlinden, L.A., Mahaffey, S., Hoffman, P., Tabakoff, B., and Saba, L.M. (2022). Evaluation and characterization of expression quantitative trait analysis methods in the Hybrid Rat Diversity Panel. *Front. Genet.* 13, 947423. <https://doi.org/10.3389/fgene.2022.947423>.
34. Weisenfeld, N.I., Kumar, V., Shah, P., Church, D.M., and Jaffe, D.B. (2017). Direct determination of diploid genome sequences. *Genome Res.* 27, 757–767. <https://doi.org/10.1101/gr.214874.116>.
35. A. Guarracino, E. Garrison 2021 wfmash: a pangenome-scale aligner, <https://github.com/waveygang/wfmash>.
36. Danecek, P., Bonfield, J.K., Liddle, J., Marshall, J., Ohan, V., Pollard, M.O., Whitwham, A., Keane, T., McCarthy, S.A., Davies, R.M., and Li, H. (2021). Twelve years of SAMtools and BCFtools. *GigaScience* 10, giab008. <https://doi.org/10.1093/gigascience/giab008>.
37. Tan, A., Abecasis, G.R., and Kang, H.M. (2015). Unified representation of genetic variants. *Bioinformatics* 31, 2202–2204. <https://doi.org/10.1093/bioinformatics/btv112>.
38. Cleary, J.G., Braithwaite, R., Gaastra, K., Hilbush, B.S., Inglis, S., Irvine, S.A., Jackson, A., Littin, R., Rathod, M., Ware, D., et al. (2015). Comparing Variant Call Files for Performance Benchmarking of Next-Generation Sequencing Variant Calling Pipelines. Preprint at bioRxiv. <https://doi.org/10.1101/023754>.
39. Tarailo-Graovac, M., and Chen, N. (2009). Using RepeatMasker to identify repetitive elements in genomic sequences. *Curr. Protoc. Bioinform. Chapter 4*, 4.10.1–4.10.14. <https://doi.org/10.1002/0471250953.bi0410s25>.
40. Martin, F.J., Amode, M.R., Aneja, A., Austine-Orimoloye, O., Azov, A.G., Barnes, I., Becker, A., Bennett, R., Berry, A., Bhai, J., et al. (2023). Ensembl 2023. *Nucleic Acids Res.* 51, D933–D941. <https://doi.org/10.1093/nar/gkac958>.
41. Wheeler, T.J., Clements, J., Eddy, S.R., Hubley, R., Jones, T.A., Jurka, J., Smit, A.F.A., and Finn, R.D. (2013). Dfam: a database of repetitive DNA based on profile hidden Markov models. *Nucleic Acids Res.* 41, D70–D82. <https://doi.org/10.1093/nar/gks1265>.
42. Guarracino, A., Heumos, S., Nahnsen, S., Prins, P., and Garrison, E. (2022). ODGI: understanding pangenome graphs. *Bioinforma. Oxf. Engl.* 38, 3319–3326. <https://doi.org/10.1093/bioinformatics/btac308>.
43. Wick, R.R., Schultz, M.B., Zobel, J., and Holt, K.E. (2015). Bandage: interactive visualization of *de novo* genome assemblies. *Bioinformatics* 31, 3350–3352. <https://doi.org/10.1093/bioinformatics/btv383>.
44. Garrison, E., Kronenberg, Z.N., Dawson, E.T., Pedersen, B.S., and Prins, P. (2022). A spectrum of free software tools for processing the VCF variant call format: vcfliib, bio-vcf, cyvcf2, hts-nim and slivar. *PLoS Comput. Biol.* 18, e1009123. <https://doi.org/10.1371/journal.pcbi.1009123>.
45. English, A.C., Menon, V.K., Gibbs, R.A., Metcalf, G.A., and Sedlazeck, F.J. (2022). Truvari: refined structural variant comparison preserves allelic diversity. *Genome Biol.* 23, 271. <https://doi.org/10.1186/s13059-022-02840-6>.
46. Cingolani, P., Platts, A., Wang, L.L., Coon, M., Nguyen, T., Wang, L., Land, S.J., Lu, X., and Ruden, D.M. (2012). A program for annotating and

- predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w¹¹¹⁸; iso-2; iso-3. *Fly* 6, 80–92. <https://doi.org/10.4161/fly.19695>.
47. Arends, D (2017) BXDtools. <https://github.com/DannyArends/BXDtools/>.
 48. Smith, J.R., Hayman, G.T., Wang, S.-J., Laulederkind, S.J.F., Hoffman, M.J., Kaldunski, M.L., Tutaj, M., Thota, J., Nalabolu, H.S., Ellanki, S.L.R., et al. (2020). The Year of the Rat: The Rat Genome Database at 20: a multi-species knowledgebase and analysis platform. *Nucleic Acids Res.* 48, D731–D742. <https://doi.org/10.1093/nar/gkz1041>.
 49. Gel, B., and Serra, E. (2017). karyoploteR: an R/Bioconductor package to plot customizable genomes displaying arbitrary data. *Bioinformatics* 33, 3088–3090. <https://doi.org/10.1093/bioinformatics/btx346>.
 50. Sayers, E.W., Bolton, E.E., Brister, J.R., Canese, K., Chan, J., Comeau, D.C., Connor, R., Funk, K., Kelly, C., Kim, S., et al. (2022). Database resources of the national center for biotechnology information. *Nucleic Acids Res.* 50, D20–D26. <https://doi.org/10.1093/nar/gkab1112>.
 51. Li, H. (2018). Minimap2: pairwise alignment for nucleotide sequences. *Bioinformatics* 34, 3094–3100. <https://doi.org/10.1093/bioinformatics/bty191>.
 52. Thorvaldsdottir, H., Robinson, J.T., and Mesirov, J.P. (2013). Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Briefings Bioinf.* 14, 178–192. <https://doi.org/10.1093/bib/bbs017>.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
DNEasy Blood and Tissue Kit	Qiagen	69504
Phusion High-Fidelity PCR kit	ThermoFisher	F553L
Ligation Sequencing Kit	Oxford Nanopore Technologies	SQK-LSK114
Other		
g-TUBE	Covaris	520079
Experimental models: Organisms/strains		
Sample metadata for whole RatCollection	De Jong et al. ¹⁰	https://doi.org/10.5281/zenodo.10552790
Software and algorithms		
Supernova	Weisenfeld et al. ³⁴	
Compleasm	Huang and Li ¹¹	
wfmash	Guarracino and Garrison ³⁵	
pqgb	Garrison et al. ¹³	
GLNexus	Yun et al. ¹²	
bcftools	Danecek et al. ³⁶	
Vt peek	Tan et al. ³⁷	
RTG	Cleary et al. ³⁸	
RepeatMasker	Tarailo-Graovac and Chen ³⁹	
Ensembl Genome Browser	Martin et al. ⁴⁰	
Dfam	Wheeler et al. ⁴¹	
vg giraffe	Sirén et al. ¹⁵	
ODGI	Guarracino et al. ⁴²	
Bandage	Wick et al. ⁴³	
vg	Garrison et al. ¹⁴	
Deepvariant	Yun et al. ¹²	
vcfware	Garrison et al. ⁴⁴	
truvari	English et al. ⁴⁵	
SnEff	Cingolani et al. ⁴⁶	
GeneNetwork.org	Mulligan et al. ¹⁶	
BXDtools	Arends ⁴⁷	
Rat Genome Database	Smith et al. ⁴⁸	
karyotypeR	Gel and Serra ⁴⁹	
Integrative Genomics Viewer	Gel and Serra ⁴⁹	

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

All rat inbred strain and substrain used in this study belong to the species *Rattus norvegicus*. Further details, including the RRIDs are provided in [Table S10](#). All the strains are included in the HRDP.³³

The panel is being cryo-resuscitated and cryopreserved at the Medical College of Wisconsin for use by the scientific community. The study involving HRDP is approved by the Institutional Animal Care and Use Committee at the University of Tennessee Health Science Center.

METHOD DETAILS

Supernova assembly

Fastq files were used as the input for the Supernova (version 2.1.1) program provided by 10x³⁴ genomics to generate *de novo* assembly with default settings. Complete information about the depth per sample can be found in.¹⁰ To evaluate genome assembly completeness, we used the Compleasm¹¹ tool to assess representation of universal single-copy orthologs expected to be present across mammals (BUSCO Mammalia gene set). Compleasm searched each Supernova genome assembly for the presence and completeness of these conserved orthologs. The reference mRatBN7.2/rn7 genome was analyzed comparably as a benchmark.¹⁰

Pangenome generation and evaluation

Supernova haploid assemblies obtained by 10x chromium Linked-Reads technology were mapped against the mRatBN7.2/rn7.fa reference genome using wfmash³⁵ to partition the assemblies' contigs by chromosome. Mapped assemblies were used to build the pangenome with PGGB (v.c1c3a1565604fc41f880bccd9f46d0a709f3e774)¹³ using this combination of parameters: -p 98 -s 2000 -n 32 -F 0.001 -k 79 -P asm5 -O 0.03 -G 4001,4507 -V rn7:# -t 48 -T 48. Validation of the call set was performed on SHR/Olalpcv sample, using one true positive set obtained from joint-calling conducted using GLNexus.¹² Prior to comparison, the pangenome-derived and the validated call set were processed to remove missing data, sites where alleles are stretches of Ns, homozygous reference genotypes and variants greater than 50bp before normalization and decomposition using bcftools³⁶ under default parameters. While the pangenome-derived VCF was based on haploid assemblies, for comparison purposes the calls were considered as homozygous diploid in the assumption that SHR/Olalpcv is isogenic. Comparison of the two call sets was performed with RTG tools (v.3.12.1)³⁸ using the -squash-ploidy option. The RTG tool used for the variants' evaluation gives us some information about the variants in common, called by vg and JC. From these results we considered only the vcf file which contains variants called by vg-only. RepeatMasker³⁹ was used to mask complex regions, easy regions that do not contain low complexity regions and repeats. Hard regions contain the whole genome regions. RepeatMasker, Ensembl Genome Browser⁴⁰ and Dfam⁴¹ databases are used to check the abundance of repeats across the genome. We mapped raw reads of SHR/Olalpcv against the pangenome using vg giraffe (v. 1.41.0).¹⁵ For the pangenome graph statistics we used ODGI (v.0.7.3),⁴² we report the non-reference sequence computed by summing the length of the graph nodes that are not traversed by the reference genome. This way we count each non-reference node only once. For the pangenome graph visualization we used ODGI and Bandage.⁴³ To check the length of the rat genome assemblies (mRatBN7.2/rn7 reference) we used the National Center for Biotechnology Information (NCBI) online resource.⁵⁰ For the vcf statistics we used bcftools³⁶ stats and vt peek.³⁷

Variant calling

The variant calling of variants on the pangenome was obtained using vg (v. 1.41.0)¹⁴ and in particular -V rn7:# into the pggg command line. Small variants are variants with length <50 bp, simple variants are SNPs/MNPs/Indels, complex variants are allelic variants that overlap but do not cover the same range. For the variant classification in simple and complex variants we used vt resource (https://genome.sph.umich.edu/wiki/Variant_classification). For variant calling based on the reference genome, fastq files of 10x chromium linked data were mapped against mRatBN7.2 using LongRanger (version 2.2.2) (<https://github.com/10XGenomics/longranger>). Long Ranger code is available at <https://github.com/10XGenomics/longranger>. DeepVariant (ver 1.0.0)¹² was used to call variants for each sample. Joint calling of variants for all the samples was conducted using GLNexus.¹² To optimize a pangenomic perspective and capture SVs that might be missed with traditional reference-based methods, we applied several SV calling pipelines. As a graph-based method, we used PGGB and vg to generate a comprehensive VCF file. Variants were normalized using vcfwawe⁴⁴ and then filtered by keeping only variants >50 bp. As assembly-based methods, we applied 3 pipelines as described in Liao et al.⁸ To evaluate the concordance between all the approaches, we conducted an overlap analysis using truvari (v.4.0),⁴⁵ a tool for assessing SV calls following some criteria 1) we merged SVs using a maximum allowed distance of 1000 bp, as measured pairwise between breakpoints (begin1 vs. begin2, end1 vs. end2) 2) we reported calls supported by at least 2 callers and they have to agree on the type and on the strand of the SV 3) we removed variants with more than 10% of Ns in REF/ALT. For the SV's pangenome graph extraction we used odgi (v.0.7.3) and for the visualization we used Bandage and odgi. Subsequently, we investigated the impact of small variants and SVs using SnpEff (v5.1),⁴⁶ a well established tool for annotating and predicting variant effects. Through SnpEff analysis, we gained insights into the potential functional consequences of the detected genetic variants on the identified genes.

Sanger resequencing for variant validation

Variants only found with the pangenome approach were randomly selected from Chr12 for the SHR/Olalpcv sample. Primers were designed using NCBI primerblast and synthesized by IDT at 25 nm scale with standard desalt purification (Coralville, Iowa). DNA from a male adult SHR/Olalpcv rat was extracted from brain tissue using DNEasy Blood and Tissue Kit (Qiagen, Cat no. 69504) and amplified using Phusion High-Fidelity PCR Kit (ThermoFisher, Cat no. F553L) in a Thermocycler for 30 cycles. PCR products were inspected on 1% agarose gel. PCR products with one distinctive band were submitted to GeneWiz (South Plainfield, NJ) for sequencing. Sequencing results were mapped against the target sequence using the blast2seq application and mutations were manually examined for confirmation.

PheWAS analyses and functional annotation

A Phenotype Wide Association Study (PheWAS) was conducted to identify potential traits related to the validated SNPs. The Phenome of the HXB/BXH family was downloaded from [GeneNetwork.org](https://www.genenetwork.org) (version 2)¹⁶ using an API. Out of 324 phenotypes we included 60 phenotypes, with data from at least 2 observations per phenotype within phenotypic class. Phenotypes were correlated via the 'hxb.phenotypes' object, using an adaptation of BXDtools,⁴⁷ an R package containing various genetic tools to work in particular with model organisms. BXDtools employs a simpler linear model. Spearman correlations between genotype and phenotype were carried out using R, with p -values adjusted (Bonferroni) according to the number of samples each phenotype contains. The statistical significance level was set to p -adj < 0.05. This relationship was visualized in a genotype-phenotype plot. Ensembl Genome Browser⁴⁰ and Rat Genome Database (RGD)⁴⁸ were used to identify genes. Genome assembly (mRatBN7.2/rn7) for *Rattus norvegicus* was used.^{4,34} To enhance the visualization and understand the distribution of these genes and their variant impacts on chr12, we employed a specialized R package called karyotypeR.⁴⁹

SVs validation with nanopore adaptive sequencing

To validate the SVs identified, we employed Adaptive Sampling, a nanopore sequencing-specific software-controlled enrichment method. For Oxford Nanopore Adaptive Sequencing, 4.4 μ g of DNA was extracted from 22 mg of microdissected rat brain tissue using the Qiagen DNeasy Blood & Tissue Kit and quantified using Nanodrop. The extracted DNA was fragmented using Covaris' g-TUBE for 30 s at 9100 \times g at room temperature, for a median fragment size of \sim 3kb. Library prep was performed with ONT's Ligation Kit and libraries were quantified with Qubit. Following ONT's guidelines for the use of their Ligation Kit with Adaptive Sampling and ONT Community input, 100 fmol of library was loaded onto a MinION flow cell and run for 72 h with Adaptive Sampling selected in run setup. Nanopore validated reads are mapped against the mRatBN7.2/rn7.fa reference genome with minimap2,⁵¹ and the bam files are visualized using Integrative Genomics Viewer (IGV).⁵²

QUANTIFICATION AND STATISTICAL ANALYSIS

Phenotypes used for the PheWAS analysis are available through GeneNetwork.¹⁶ Statistical analyses were performed using R and all the original code used to perform the analyses is available on GitHub (https://github.com/Flavia95/HXB_rat_pangenome_manuscript/). p -values in [Figures 2C–2E](#) were obtained from Spearman's rank correlation test; the number of samples is indicated in the figures.