Mutational signatures in GATA3 transcription factor and its DNA binding domain that stimulate breast cancer and HDR syndrome

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Transcription factors (TFs) play important roles in many biochemical processes. Many human genetic disorders have been associated with mutations in the genes encoding these transcription factors, and so those mutations became targets for medications and drug design. In parallel, since many transcription factors act either as tumor suppressors or oncogenes, their mutations are mostly associated with cancer. In this perspective, we studied the GATA3 transcription factor when bound to DNA in a crystal structure and assessed the effect of different mutations encountered in patients with different diseases and phenotypes. We generated all missense mutants of GATA3 protein and DNA within the adjacent and the opposite GATA3:DNA complex models. We mutated every amino acid and studied the new binding of the complex after each mutation. Similarly, we did for every DNA base. We applied Poisson-Boltzmann electrostatic calculations feeding into free energy calculations. After analyzing our data, we identified amino acids and DNA bases keys for binding. Furthermore, we validated those findings against experimental genetic data. Our results are the first to propose in silico modeling for GATA:DNA bound complexes that could be used to score effects of missense mutations in other classes of transcription factors involved in common and genetic diseases.

The family of proteins that code for transcription factors is considered the largest family among all proteins types (about 10%). Specifically, 2600 proteins in the human genome contain DNA-binding domains, and most of them code for transcription factors. The GATA3 TF is encoded in humans by the GATA3 gene and it controls the expression of a wide range of biologically and clinically important genes. GATA3 belongs to the GATA family of zinc finger transcription factors, which are named according to their DNA binding subsequence ‘GATA’.

Many studies confirmed that GATA3 mutations are involved in the development of certain types of breast cancer in humans. GATA3 was shown to be one of the three genes mutated in > 10% of breast cancers. Some studies on mice indicated that GATA3 is critical for the normal development of breast tissue and directly regulates luminal cell differentiation, whereas other studies indicated that it is integral to the expression of estrogen receptor alpha and to the signaling of androgen receptor. Approximately one-half of the GATA3 mutations identified in patients with breast cancer are clustered in exons 5 and 6, which encode ZnF2 and the C terminal domain of the protein. Experimental evidence showed that ZnF2 of GATA3 is required for DNA binding. 15% of the mutations published in male breast cancer are present in GATA3, with hotspots recorded at residues S308 and S407 in luminal A and luminal B subtypes, respectively.

Besides the different associations of GATA3 with different forms of breast cancer, GATA3 has been associated with hypoparathyroidism, deafness, and renal dysplasia (HDR) syndrome. The first described missense mutation (Leu348Arg) in HDR patients does not alter DNA binding or the affinity but likely alters the conformational change that occurs during binding in the DNA major groove. Other mutations of GATA3 TF, causing the HDR dysplasia syndrome, include: Two nonsense mutations Glu-228 to Stop and Arg-367 to Stop, one acceptor splice site mutation that leads to a frameshift from codon 351, a premature termination at codon 367, and two missense mutations: Cys-318 to Arg and Asn-320 to Lys. Mutations involving GATA3 ZnF2 or adjacent basic amino acids result in a loss of DNA binding, but those involving GATA3 ZnF1 either lead to a loss of interaction with FOG2 (Friend of GATA, cofactor) or alter DNA-binding affinity.

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In this work, we studied all possible amino acids and DNA bases missense mutations on two protein-DNA complexes (GATA3:DNA complex models with PDB ID: 3DFV and 3DFX). We applied Poisson–Boltzmann electrostatic study for the analysis of those mutations. The original method has been applied on many protein–protein complexes, and for ionic–only amino acids. In this context, we studied the role of every amino acid, and the role of every DNA base in regard to binding, and that is by mutating each amino acid and mutating each of Subsequence1, GATA_Subsequence, DNA bases (i.e., Subsequence1, GATA_Subsequence, represents the DNA sequence where the GATA3 TF protein binds, and where Subsequence1, and Subsequence2, can be any of the four DNA bases: Adenine (A), Cytosine (C), Thymine (T), or Guanine (G)) that might lead to malfunction in transcription. Unlike previous studies, we hereby assessed the role of all amino acids (ionic and non-ionic) of the GATA3 protein and the role of all DNA bases of the Subsequence1, GATA_Subsequence, DNA sequence during non-specific recognition (between amino acids and DNA backbone) and in specific binding (between amino acids and DNA bases).

This paper is organized as follows: We first described the computational method applied to the study of amino acids and DNA bases mutations in Section II. We then illustrated in details the results of applying the method in Section III; we revealed key amino acids and key DNA bases for complex binding, in addition to revealing the amino acids and DNA bases that play neutral roles in binding. Afterwards, we discussed those results, linked them to disease phenotypes, and validated them with published experimental data. Finally, we drew conclusions in Section IV and presented related future work.

Methods
Recognition and binding form the two major steps of electrostatic association between protein and DNA molecules\(^{18}\). Non-specific long-range electrostatic interactions characterize recognition, whereas specific favorable local short-range electrostatic interactions, such as hydrogen bonds, salt bridges, medium-range coulombic interactions, in addition to hydrophobic and van der Waals interactions, characterize binding. An accelerated weak encounter complex is formed during recognition. Contrariwise, the protein and DNA are locked into their final bound conformation during binding, and this occurs after local side change rearrangements, and exclusion of solvent atoms from their binding interface.

Several diseases were revealed through the effect of charged amino acids\(^{20}\). Similar diseases include the eye disease known as Age-related Macular Degeneration (AMD)\(^{23}\), the kidney disease known as atypical Hemolytic Uremic Syndrome (aHUS), the Dense Deposit Disease (DDD), also known as membranoproliferative glomerulonephritis\(^{22}\), and immune system disorder (over-activity or under-activity)\(^{23}\). The electrostatic type of interactions was shown in complexes like C3d–CR2\(^{24-27}\) and C3d–Efb/Cehp association\(^{26-28}\), and in interactions with viral proteins VCP/SPICE\(^{30,31}\) and Kaposica\(^{32}\). The functional properties of each subunit of the E1 heterodimer activating-enzyme for NEDD8, UBA3, and APPBP1 was studied electrostatically in\(^{32}\). Hierarchical clustering analysis of electrostatic potentials and charges of V3 loop of HIV-1, which plays a crucial role in viral entry into cells, was performed in\(^{34}\) and was mainly mediated by electrostatics. Single-alanine mutants of charged residues in the complexes CD46(SCR1-2)-Ad21k and CD46(SCR1-2)-Ad11k were computationally generated to mark out specific interfacial electrostatic interactions that are critical for association\(^{35}\).

SUMO4, a type 1 diabetes susceptibility gene, was found amenable to SENP2—a protease enzyme that processes SUMO into conjugatable form—processing via a single amino acid mutation through electrostatic computational modeling, and a combination of two amino acid mutations makes it highly accessible to SENP2 substrate\(^{36}\). Electrostatic detailed investigation of factor H (FH) complement control protein (CCP) modules, in which mutations are linked to autoimmunity, revealed three binding sites in binding to complement protein C3b, thus increasing the affinity of FH for host surfaces\(^{37}\). Similar to FH, mutations in the MAC complex (C5b6) can lead to autoimmune diseases. Correspondingly, an electrostatic study of the interaction between C5b6 and C6 complement proteins was completed in\(^{38}\). Electrostatic similarity methods applied to perturbed structures of C3d and Cr2 revealed electrostatic “hot-spots” at the two functional sites of C3d and a lack of electrostatic “hot-spots” at the surface of Cr2, despite its excessively positive nature\(^{39}\). Additional electrostatic computational approaches were used to gain insight into the binding mode of the C3d:Cr2 complex\(^{40}\). Theoretical alanine-scan mutagenesis and validation with experimental data was completed on five protein complexes in order to discern the role of individual ionized amino acids to protein association\(^{41}\).

Protein-DNA interactions: framework description. Binding free energy calculations of many protein–protein interactions were implemented using the integrated Analysis of Electrostatic Similarities Of Proteins (AESOP) framework\(^{26,27,29,33-42}\). We based the electrostatic study of protein-DNA interactions on the same framework because protein-DNA interactions and protein–protein interactions share the same type of interactions; they both comprise bonded (bond, angle, torsion) and non-bonded (short-range and long-range electrostatic, van der Waals (vdW), and hydrogen bonds) interactions, as depicted in Table 1.\(^{43}\) Intra-molecular represents interactions within the same molecule, whereas inter-molecular represents interactions between different molecules, like protein and DNA molecules. We then expanded AESOP to study all types of amino acids (ionic and non-ionic), in addition to all DNA bases and DNA backbone.

The Expanded-AESOP framework encompasses the following steps:

**Preparation of mutants from all GATA3 protein amino acids and from all nucleotides of the DNA sequences.** R scripts were implemented to generate all types of mutants. We used as input the two forms of GATA3:DNA crystal structures (3DFV and 3DFX) from the Protein Data Bank (PDB). We replaced every amino acid—expected to be charged at physiological pH—one at a time, with each of the other nineteen amino acids. Along, we also
replaced every base of the DNA sequence Subsequence1\_GATA\_Subsequence2 with each of the other three DNA bases (e.g., G is mutated with A, C, or T).

Calculation of Poisson–Boltzmann electrostatic potentials. The electrostatic potentials were calculated using APBS software\textsuperscript{43}, which is based on the linearized Poisson–Boltzmann equation, as illustrated in previous studies\textsuperscript{25–27,29}. The atomic radii and charges, needed for APBS calculations, were calculated using PDB2PQR\textsuperscript{44} program and AMBER force field parameters\textsuperscript{45,46}.

Calculation of electrostatic free energies of complex binding. The calculations of the electrostatic potentials were fed into the calculations of the electrostatic free energies of binding based on a thermodynamic cycle, as described in\textsuperscript{26,27,29}, and in the form of the following equations:

\[
\Delta \Delta G_{\text{association solution}} = \Delta G_{\text{GATA : DNA solution}} - \Delta G_{\text{GATA solvation}} - \Delta G_{\text{DNA solvation}} \tag{1a}
\]

\[
\Delta \Delta G_{\text{association solution}} = \Delta G_{\text{association solution}} - \Delta G_{\text{association reference}} \tag{1b}
\]

\[
\Delta G_{\text{X solution}} = \Delta G_{\text{X solution}} - \Delta G_{\text{X reference}} \tag{2}
\]

\[
\Delta G_{\text{association Y}} = \Delta G_{\text{GATA : DNA Y}} - \Delta G_{\text{GATA Y}} - \Delta G_{\text{DNA Y}} \tag{3}
\]

where Eq. (1a) presents the binding free energy of the complex in solvent. Equation (1b) presents the binding free energy of the complex after eliminating artifacts. Equation (2) presents the energy of the solvent after subtracting artifacts. Equation (3) presents the energy of the complex after subtracting individual components. Looking backwards, Eqs. (2) and (3) feed into Eqs. (1a) and (1b), whereas Eq. (1a) presents the final form of the complex GATA3:DNA binding free energy calculation.

Data visualization. We used data visualization to help scientists understand the significance of data by placing it in a visual context, such as patterns, trends, and correlations that might go undetected in text-based forms. Swiss PDB Viewer and Chimera represent the visualization software we used\textsuperscript{47,48}.

Results and discussion

Real data. We studied the binding free energy calculations of GATA3:DNA complex (Fig. 1a, b). GATA3:DNA is available under two different conformations with PDB IDs: (a) 3DFX (GATA3 binding to DNA in an opposite manner) and (b) 3DFV (GATA3 binding to DNA in an adjacent manner)\textsuperscript{16}.

The two crystal models share the same types of interactions. Accordingly, we used the available interactions of the Opposite model (OPP) to elaborate on the role of some amino acids hubs in the Adjacent model (ADJ). For electrostatic calculations, we used the crystal structure 3DFV, in which GATA3 comprises the coordinates of amino acids Arg311-Arg366 for each of Chain-D and Chain-C, and in which the DNA module comprises the coordinates of nucleic acids from T1 to C20 for Strand-Y and from A1 to G20 for Strand-Z, as follows:

DNA Strand-Z: A\textsubscript{1}A\textsubscript{2}G\textsubscript{3}C\textsubscript{4}T\textsubscript{5}A\textsubscript{6}G\textsubscript{7}C\textsubscript{8}G\textsubscript{9}T\textsubscript{10}A\textsubscript{11}G\textsubscript{12}T\textsubscript{13}C\textsubscript{14}T\textsubscript{15}A\textsubscript{16}T\textsubscript{17}C\textsubscript{18}A\textsubscript{19}G\textsubscript{20}.

Subsequence\textsubscript{1}: AAGCA

Subsequence\textsubscript{2}: AGTGATATCAG

where

DNA Strand-Z: C\textsubscript{20}G\textsubscript{19}T\textsubscript{18}C\textsubscript{17}G\textsubscript{16}T\textsubscript{15}A\textsubscript{14}T\textsubscript{13}C\textsubscript{12}A\textsubscript{11}G\textsubscript{10}A\textsubscript{9}T\textsubscript{8}A\textsubscript{7}C\textsubscript{6}T\textsubscript{5}C\textsubscript{4}T\textsubscript{3}T\textsubscript{2}T\textsubscript{1}.

where

Subsequence\textsubscript{1}: TTCT

Subsequence\textsubscript{2}: AGCGTATCTGC

As shown in Fig. 1b, Chain-D binds to ‘GATA’ subsequence on Strand-Z and Chain-C binds to ‘GATA’ subsequence (in reverse) on Strand-Y.

| Table 1. Types of protein-DNA interactions. Non-bonded Specific refers to interactions between Amino Acid (AA) and DNA bases and Non-bonded Non-specific refers to interactions between AA and DNA backbone. |
|---|---|---|---|
| **Intra-molecular** | | | |
| Bonded | Bond | Angle | Torsion |
| Non-bonded | vdW | H-bond | Ionic |
| **Inter-molecular** | | | |
| Non-bonded specific | H-bond | | |
| Non-bonded non-specific | vdW | H-bond | Ionic |
After some measurements and based on prior calculations, we set the parameters for GATA3:DNA calculations specifically as follows:

- The probe radii for defining the dielectric: 1.4 Å
- The probe radii of the ion accessibility surface: 2.0 Å
- The dielectric coefficient for the protein interior: 2
- The dielectric coefficient for the solvent: 78.54
- The grid used in the APBS calculations: $129 \times 161 \times 161$ grid points
- The coarse grid lengths: $82 \times 97 \times 104$ Å
- The fine grid lengths: $68 \times 77 \times 81$ Å
- The grid resolution: ≤ 1 Å

**Mutational analysis from computational results.** In order to detect the effect of each mutated perturbation on the overall binding ability of the complex GATA3:DNA, we performed the following steps: First, we generated a family of GATA3 mutants and a family of DNA mutants from the crystallographic structure GATA3:DNA at atomic detail, and that is by replacing each amino acid with each of the other nineteen amino acids and replacing each base of the DNA sequence Subsequence GATA Subsequence, with each of the other three DNA bases. Second, we performed Poisson–Boltzmann electrostatic calculations on each of those mutant
wild structure (sequence2 DNA composing a total of 60 mutants for each strand (Strand-Y and Strand-Z are complementary), or 120 mutants per both strands.

Comparison of every mutant’s free energy calculation with the parent/wild complexes. Third, we performed electrostatic free energy calculations on each of those mutant complexes. Comparison of every mutant’s free energy calculation with the parent/wild GATA3:DNA complex free energy calculation, reveals the alteration effect of each mutated protein amino acid and of each mutated GATA3 DNA binding, and subsequently, gives an indication of key amino acids and key DNA bases for binding.

The current dataset consists of one family of GATA3 protein mutants and one family of DNA sequence mutants. Since each chain consists of 56 amino acids (numbered 311–366) and each amino acid is mutated to 19 other amino acids, we have a total of 1064 protein mutants per one chain of the 3DFV structure, or 2128 per both chains (Chain-C and Chain-D are symmetrical). The dataset also consists of a family of Subsequence1_GATA_sequence2 DNA sequence mutants, where each DNA base is mutated to each of the three other DNA bases, composing a total of 60 mutants for each strand (Strand-Y and Strand-Z are complementary), or 120 DNA mutants per both strands.

We superimposed the structures of the GATA3 protein mutants and those of the Subsequence1_GATA_Subsequence2 DNA sequence using the backbone Cα atoms and centered them on the same grid used for the parent/wild structure (GATA3:DNA). Figures 2, 3, 4, 5, 6, 7, 8, 9 and 10 present the electrostatic free energy calculations at 150 mM ionic strength of major mutants of the 3DFV complex. Plot presents the solvated binding free energy calculations (in KJ/mol) of GATA3 Arg amino acid mutants in both of Chain-D and Chain-C of the crystal structure 3DFV. Basic and acidic mutants are presented in blue and red colors respectively. Mutants shown above the parent/wild are predicted to enhance binding, whereas mutants shown below it are predicted to reduce binding. Due to its positive charge, Arg/R amino acid does not lead to acidic mutants. The x-axis (index) represents the order of the mutant (mutants are numbered and ordered sequentially). The amino acids shown with labels reflect the ones associated with diseases, whereas the amino acids presented with different colors of ‘X’ symbolize critical amino acids identified through the strong hubs of interactions they make.

As shown in Figs. 2, 3, 4, 5, 6, 7, 8, 9 and 10, the strength of each perturbation (mutation) is illustrated in terms of ΔΔG free energy calculation (Eq. 1a). Mutations of acidic GATA3 residues or DNA bases have free energy values higher than the parent/wild free energy and are called inhibitors because they enhance binding, whereas mutations of basic GATA3 residues or DNA bases have free energy values lower than the parent/wild free energy and are called enhancers because they inhibit binding. The lower the computed ΔΔG for a specific mutation, the more crucial the corresponding residue or DNA base is to binding, compared to other residues or DNA bases respectively, and the reasoning behind this is as follows: If the computed solvation binding free energy ΔΔG of a mutated residue or DNA base is lower than the initial solvation binding free energy ΔΔG of the same residue or DNA base before mutation, this implies that this specific residue/DNA base binds better before being mutated, and so this residue/DNA base has a significant impact on binding and is considered a hotspot (case of an inhibitor). Conversely, if the computed solvation binding free energy ΔΔG of a mutated residue or DNA base is higher than the initial solvation binding free energy ΔΔG of the same residue or DNA base before mutation, this implies that this specific residue/DNA base binds better after being mutated, and so this residue/DNA base does not have a significant impact on binding (case of an enhancer).
In Fig. 10, all mutants of all bases of the double-stranded Subsequence1_GATA_Subsequence2 DNA sequence are studied. Since each strand (-Y or -Z) of the DNA sequence is numbered from 1 to 20 (in the .pdb file), we are focusing on the impact of each base of the DNA sequence where GATA3 protein actually binds. Accordingly, we are looking at bases located at positions 4–5–6–7–8–9 on Strand-Y where Chain-C binds, corresponding to nucleotides T
GATA
A, and to complementary subsequence TTA TCA on Strand-Z at positions 14–15–16–17–18–19. On the other hand, we are looking at bases located at positions 5–6–7–8–9–10 on Strand-Z where Chain-D binds, corresponding to nucleotides AGA TAA, and to complementary subsequence of TTA TCT on Strand-Y at positions 13–14–15–16–17–18 (as shown below with underlines and blue highlights).

DNA Strand-Z:
A1A2G3C4A5G6A7T8A9A10G11T12C13T14T15A16T17C18A19G20.

DNA Strand-Y:
C20G19T18C17T16A15T14T13C12A11G10A9A8T7A6G5T4C3T2T1.

The results showed the following DNA bases mutants to impact binding (inhibitors shown below with green highlights). The lowest free energy values correspond to inhibitors with highest impact (strongest inhibitors), and those are: G6T, G6A, and G6C on Strand-Z, and G5T, G5A, and G5C on Strand-Y. Accordingly, we can see how the bases in the ‘GATA’ subsequence are definitely the first to impact binding, and then a few bases in the neighborhood of the ‘GATA’ subsequence, which are not necessarily the first adjacent base on the left and/or the first adjacent base on the right of the ‘GATA’ subsequence. The rest of the DNA bases mutants that lay around the parent/wild region (Fig. 10) do not have a major impact on binding.

DNA Strand-Z: A1A2G3C4A5G6A7T8A9 (Chain-D – Chain-C).
DNA Strand-Y: C20G19T18C17T16A15T14T13C12A11G10A9A8T7A6G5T4C3T2T1 (Chain-D – Chain-C).

ZnF2

Figure 3. Electrostatic free energy differences of Asparagine (Asn/N) within GATA3:DNA complex. Plot presents the solvated binding free energy calculations (in KJ/mol) of GATA3 Asn amino acid mutants in both of Chain-D and Chain-C.

ZnF2

Figure 4. Electrostatic free energy differences of Aspartic Acid (Asp/D) within GATA3:DNA complex. Plot presents the solvated binding free energy calculations (in KJ/mol) of GATA3 Asp amino acid mutants in both of Chain-D and Chain-C.
Comparatively, all inhibitors are presented together in order to reveal the ones with the highest impact on protein-DNA binding. Hence, Fig. 11 shows that Arg and Lys are the most influential amino acids for efficient binding. It also shows the minor impact of mutated DNA bases, which lay around the parent/wild region, when compared to amino acid mutants. This result uncovers the crucial role of DNA backbone in the interactions with the GATA3 protein amino acids, unlike the specific role of the DNA bases, which appears to be minimal in comparison. Similarly, Fig. 12 shows all enhancers of amino acids, highlighting the crucial role of Gln mutants in binding.

Computationally, we detected the hydrogen bonds (listed in detail in Supplementary Figure SF12 and Supplementary Table ST12) between Chain-C and the DNA, revealing the vital role to binding of the following amino acids: Arg312, Arg329, Arg330, Arg364, Arg366, Lys346, and Asn339. In addition, we computationally detected the salt bridges (listed in detail in Supplementary Figure SF13 and Supplementary Table ST13) between Chain-C and the DNA for the following amino acids: Arg312, Arg330, Arg352, and Lys358.

Mutating amino acids to Arg or Lys make them enhancers due to adding more positive to the binding with DNA, which is initially more negatively charged. Figures 2, 3, 4, 5, 6, 7, 8 and 9 show the effect on binding when mutating any amino acid to Arg or Lys. Conversely, when any amino acid is mutated to Asp/Glu, it is implied as an inhibitor that impedes binding. In particular, when Arg or Lys are changed to Asp/Glu (Fig. 2/Table 2 or SF6/ST6 respectively), those mutants are called strong inhibitors and the impeding effect on binding is multiplied. In this case, we are losing more positive charges (property of Arg and Lys) and adding more negative charges.
(property of Asp and Glu), and that is not favorable for binding to DNA which has the property of being negatively charged on the backbone.

**Experimental validation.** Our computational results are further validated against experimental evidence, based on experimental published papers. The experimental information, listed in Supplementary Tables ST14, ST15, ST16, and ST17, substantiates our mutational results in Figs. 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 and in SF1-SF11. The crucial effect of the predicted key amino acids and key DNA bases on binding are verified through: (1) The different GATA3 crystal structure models available, (2) the hub of protein–protein and/or protein-DNA interactions they are engaged in, and (3) The association in diseases and manifestation in phenotypes.

**ADJacent and OPPosite GATA3:DNA models.** The complex GATA3:DNA exists in two forms: The Opposite model (OPP), as shown in Fig. 1a, and the Adjacent model (ADJ), as shown in Fig. 1b. The OPP model (PDB: 3DFX) has GATA3 factors Chain-A and Chain-B binding to opposite ends of the double stranded DNA (Strand-X and -Y) and the ADJ model (PDB: 3DFX) has GATA3 factors Chain-C and Chain-D binding to same ends of the double stranded DNA (Strand-Y and -Z).

In the actual binding of GATA3 to DNA lies in the second chain from the N-terminal, which is Chain-B in the OPP model and Chain-C in the ADJ model. In both models, Chain-A (OPP model) and Chain-D (ADJ model) bind to Friends of GATA (FOG); their role is only to enhance binding of GATA3 to DNA.
**Figure 9.** Electrostatic free energy differences of Threonine (Thr/T) within GATA3:DNA complex. Plot presents the solvated binding free energy calculations (in KJ/mol) of GATA3 Thr amino acid mutants in both Chain-D and Chain-C.

**Figure 10.** Electrostatic free energy differences of the DNA sequence within GATA3:DNA complex. Plot presents the solvated binding free energy calculations (in KJ/mol) of Subsequence1_ GATA _Subsequence2 mutations in Strand-Y and Strand-Z. Blue colors signify basic mutants. Those mutants are predicted to reduce binding since they are shown below the parent/wild. DNA bases illustrated with labels signify the strongest inhibitors.

**Table 2.** Arginine (Arg/A) amino acid mutants.
| Enhancer     | Inhibitor     | Enhancer     | Inhibitor     |
|--------------|---------------|--------------|---------------|
| N333R (Asn #333 to Arg) | N333D (Asn #333 to Asp) | N331K (Asn #331 to Lys) | N331E (Asn #331 to Glu) |
| N333K (Asn #333 to Lys)  | N333E (Asn #333 to Glu)  | N349R (Asn #349 to Arg)  | N349D (Asn #349 to Asp)  |
| N351R (Asn #351 to Arg)  | N351D (Asn #351 to Asp)  | N349K (Asn #349 to Lys)  | N349E (Asn #349 to Glu)  |
| N351K (Asn #351 to Lys)  | N351E (Asn #351 to Glu)  | N365R (Asn #365 to Arg)  | N365D (Asn #365 to Asp)  |
| N339R (Asn #339 to Arg)  | N339D (Asn #339 to Asp)  | N319K (Asn #319 to Lys)  | N319E (Asn #319 to Glu)  |
| N339K (Asn #339 to Lys)  | N339E (Asn #339 to Glu)  | N319R (Asn #319 to Arg)  | N319D (Asn #319 to Asp)  |
| N319R (Asn #319 to Arg)  | N319D (Asn #319 to Asp)  | N365K (Asn #365 to Lys)  | N365E (Asn #365 to Glu)  |
| N339K (Asn #339 to Lys)  | N339E (Asn #339 to Glu)  | N349R (Asn #349 to Arg)  | N349D (Asn #349 to Asp)  |
| N349K (Asn #349 to Lys)  | N349E (Asn #349 to Glu)  | N319K (Asn #319 to Lys)  | N319E (Asn #319 to Glu)  |

Table 3. Asparagine (Asn/N) amino acid mutants.

| Enhancer     | Inhibitor     |
|--------------|---------------|
| D335R (Asp #335 to Arg) | D335K (Asp #335 to Lys) |

Table 4. Aspartic Acid (Asp/D) amino acid mutants.

| Enhancer     | Inhibitor     |
|--------------|---------------|
| C338R (Cys #338 to Arg) | C338D (Cys #338 to Asp) |
| C338K (Cys #338 to Lys)  | C338E (Cys #338 to Glu)  |
| C320R (Cys #320 to Arg)  | C320D (Cys #320 to Asp)  |
| C320K (Cys #320 to Lys)  | C320E (Cys #320 to Glu)  |
| C317R (Cys #317 to Arg)  | C317D (Cys #317 to Asp)  |
| C317K (Cys #317 to Lys)  | C317E (Cys #317 to Glu)  |
| C341R (Cys #341 to Arg)  | C341D (Cys #341 to Asp)  |
| C341K (Cys #341 to Lys)  | C341E (Cys #341 to Glu)  |

Table 5. Cysteine (Cys/C) amino acid mutants.

| Enhancer     | Inhibitor     |
|--------------|---------------|
| Q321A (Gln #321 to Ala) | Q362A (Gln #362 to Ala) |
| Q321M (Gln #321 to Met)  | Q362M (Gln #362 to Met)  |
| Q321N (Gln #321 to Asn)  | Q362N (Gln #362 to Asn)  |
| Q321P (Gln #321 to Pro ) | Q362P (Gln #362 to Pro ) |
| Q321R (Gln #321 to Arg)  | Q362R (Gln #362 to Arg)  |
| Q321S (Gln #321 to Ser ) | Q362S (Gln #362 to Ser ) |
| Q321W (Gln #321 to Trp ) | Q362W (Gln #362 to Trp ) |
| Q321V (Gln #321 to Val)  | Q362V (Gln #362 to Val)  |
| Q321Y (Gln #321 to Tyr ) | Q362Y (Gln #362 to Tyr ) |
| Q321T (Gln #321 to Thr)  | Q362T (Gln #362 to Thr)  |

Table 6. Glutamine (Gln/Q) amino acid mutants.

| Enhancer     | Inhibitor     |
|--------------|---------------|
| L343D (Leu #343 to Arg) | L343D (Leu #343 to Asp) |
| L343K (Leu #343 to Lys)  | L343E (Leu #343 to Glu)  |
| L327R (Leu #327 to Arg)  | L327D (Leu #327 to Asp)  |
| L327K (Leu #327 to Lys)  | L327E (Leu #327 to Glu)  |
| L347R (Leu #347 to Lys)  | L347D (Leu #347 to Glu)  |
| L347K (Leu #347 to Lys)  | L347E (Leu #347 to Glu)  |
| L354R (Leu #354 to Lys)  | L354D (Leu #354 to Glu)  |
| L354K (Leu #354 to Lys)  | L354E (Leu #354 to Glu)  |

Table 7. Leucine (Leu/L) amino acid mutants.
Table 8. Methionine (Met/M) amino acid mutants.

| Enhancer | Inhibitor |
|----------|----------|
| M365R (Met #356 to Arg) | M365D (Met #356 to Asp) |
| M365K (Met #356 to Lys) | M365E (Met #356 to Glu) |

Table 9. Threonine (Thr/T) amino acid mutants.

| Enhancer | Inhibitor |
|----------|----------|
| T315R (Thr #315 to Arg) | T315D (Thr #315 to Asp) |
| T315K (Thr #315 to Lys) | T315E (Thr #315 to Glu) |
| T322R (Thr #322 to Arg) | T322D (Thr #322 to Asp) |
| T322K (Thr #322 to Lys) | T322E (Thr #322 to Glu) |
| T363R (Thr #363 to Arg) | T363D (Thr #363 to Asp) |
| T363K (Thr #363 to Lys) | T363E (Thr #363 to Glu) |
| T326R (Thr #326 to Arg) | T326D (Thr #326 to Asp) |
| T326K (Thr #326 to Lys) | T326E (Thr #326 to Glu) |

Table 10. DNA bases mutants.

| Enhancer | Inhibitor |
|----------|----------|
| G6T (Gua #6 to Thy) | T8A (Thy #8 to Ade) |
| G6A (Gua #6 to Ade) | T7G (Thy #7 to Gua) |
| G5T (Gua #5 to Thy) | A5G (Ade #5 to Gua) |
| G5C (Gua #5 to Cyt) | T16G (Thy #16 to Gua) |
| G5A (Gua #5 to Ade) | T8C (Thy #8 to Cyt) |
| T8G (Thy #8 to Gua) | G19T (Gua #19 to Thy) |
| A5T (Ade #5 to Thy) | G19C (Gua #19 to Cyt) |
| T8C (Thy #8 to Cyt) | A7G (Ade #7 to Cyt) |
| T8A (Thy #8 to Ade) | C20G (Cyt #20 to Gua) |
| C20A (Cyt #20 to Ade) | A9G (Ade #9 to Gua) |

Both models (OPP and ADJ) are similar in that Chain-B and Chain-C both bind to ‘GATA’ DNA subsequence. The only minor difference between Chain-A and Chain-D lies in that they bind to slightly different DNA subsequences, ‘GATT’ and ‘GATA’ respectively. Hence, we can use some of the experimental results performed on the OPP model to validate some of the computational results performed on the ADJ model. As shown in Fig. 1a, Chain-A binds to ‘GATT’ bases at positions 13, 14, 15, and 16 of the DNA Strand-Y, and Chain-B binds to ‘GATA’ bases at positions 14, 15, 16, and 17 of the DNA Strand-X. Figure 1b shows Chain-D binding to ‘GATA’ bases at positions 6, 7, 8, and 9 of the DNA Strand-Z and Chain-C binding to ‘GATA’ bases at positions 5, 6, 7, and 8 of the DNA Strand-Y.

Interactions and hubs. Based on the results of our Expanded-AESOP method (as shown in Figs. 2, 3, 4, 5, 6, 7, 8, 9 and 10), we validated experimentally the predicted enhancers and inhibitors, described them in details in Supplementary Material, and summarized them in Supplementary Tables ST14, ST15, ST16, and ST17. Experimental evidence showed extensive protein–protein interactions (between the two GATA3 molecules) and protein–DNA interactions (between GATA3 and the DNA molecule) of many enhancers (Ala340, Arg351, Asn349, Asn339, Pro335, Leu345, Leu347, Leu327, Leu343, Glu359, His348, Ile350, Ile361, Thr355, Thr326, Gln362, Met356, Tyr344) and many inhibitors (Ala340, Arg364, Arg329, Arg352, Arg312, Arg330, Lys357, Lys358, Lys346, Asn351, Asn349, Asn339, Pro335, Leu354, Leu347, Leu327, Leu343, His348, Ile350, Ile361, Thr355, Thr326, Met356, Tyr344), revealing strong hubs of interactions comprising all different types of interactions (hydrogen bonds, salt bridges, van der Waals, etc.).

On the other hand, we detected computationally hydrogen bonds between Chain-C amino acids (Arg312, Arg329, Arg330, Arg364, Arg366, Lys346, Asn339) and the DNA (shown in Supplementary Figure SF12 and summarized in Supplementary Table ST12). In addition, we detected computationally salt bridges between...
**Figure 11.** Electrostatic free energy differences of all GATA3 inhibitors within GATA3:DNA complex. Plot presents the solvated binding free energy calculations (in KJ/mol) of all GATA3 amino acid mutants that are inhibitors in both of Chain-C and Chain-D, and that is in comparison with those of the DNA sequence bases in order to elucidate their relative effect on binding.

**Figure 12.** Electrostatic free energy differences of all GATA3 enhancers within GATA3:DNA complex. Plot presents the solvated binding free energy calculations (in KJ/mol) of all GATA3 amino acid mutants that are enhancers in both of Chain-C and Chain-D.
Chain-C amino acids (Arg312, Arg330, Arg352, Lys358) and the DNA (shown in SF13 and ST13), again bringing to light the role of those amino acids to binding.

Thus, the experimental and computational validations substantiate the crucial role of the predicted amino acids and of the DNA backbone, in addition to demonstrating the effectiveness of our developed approach Expanded-AESOP.

Gene-disease associations/phenotypes. Identifying gene-disease associations from experimental methods can be expensive and time consuming. Yet, this process is highly needed to design therapeutic strategies against diseases. Accordingly, in silico methods were developed to predict those associations from available experimental data and other types of data. In this section, we validated the results predicted by our computational method by elaborating on the diseases already witnessed as a result of the disruptions caused by our studied mutations.

The enhancer C317R (as listed in the GATA3:DNA.pdb file from the Protein Data Bank, but numbered as C318R in12—with an offset of one position) caused by a missense mutation as shown in Fig. 5, leads to a disruption of the second zinc finger (ZnF2) that is manifested in HDR syndrome, where the loss of ZnF2 coordination was marked as haploinsufficiency (HI)32. Another missense mutation, leading to a disruption of the second zinc finger (ZnF2) and manifesting in HDR syndrome, is the enhancer N319K (numbered as N320K in12), as shown in Fig. 3. Again, this specific missense mutation has been noticeable as HI in the HDR syndrome41. This mutation affects the basic region and is likely to disturb the DNA conformational change. The mutation R352S, despite being in the parent/wild region, has a major effect. As shown in Fig. 2, it is on the far end of the parent/wild region, and that makes it an approximate inhibitor. It is predicted to disrupt the helical turn and thus change the angle between the C-terminal zinc finger and the adjacent C-terminal tail; this phenomenon has been visible in the HDR syndrome within the Chinese population35.

On the other hand, GATA3 TF is one of the most frequently mutated genes in Breast Cancer. The mutation R366L (numbered as R367L in48) which lies in the parent/wild region, turns to be of major effect. As shown in Fig. 2, it is considered an approximate inhibitor due to its distant location from the parent/wild complex. Such missense mutation in Exon 5 of ZnF2 was seen in Breast Tumor Ull-01149, resulting in high expression of GATA3, and leading to Breast Neoplasm disease49. Also, the missense mutation L343F (numbered as L344F in49) in Exon 5 of ZnF2 showed high expression of GATA3 in Breast Tumor BR00-0587, causing the same disease46. On the other hand, the mutations M356K (numbered as M294K in48) and is the only Methionine in ZnF2 and N333K (numbered as N334K in49), in Exon 4 and Exon 5 respectively, were witnessed in Breast Cancer on the molecular and clinical levels30. A heterozygous mutation (frameshift) D335Gfs (numbered as D336Gfs in51) was seen in Breast Cancer51 and the frameshift mutation R329fs (numbered as R330fs in52,53) was associated with Breast Cancer. The previous two results show the importance of the specified amino acids (D335 and R329) which were predicted as strong enhancer and inhibitor respectively in our results (Figs. 2 and 4 respectively). Lastly, the mutational frameshift P490Asfs, which occurred in several Breast Cancer cases48, shows the importance of studying all GATA3 mutants (charged and non-charged amino acids). Yet, we could not verify this specific amino acid (P490) due to its position outside ZnF2 (not covered in .pdb input file range).

Conclusion
We started from the AESOP framework, which predicts ionic residues with major effect to binding, and modified it to the Expanded-AESOP framework, which predicts all types of residues (ionic and non-ionic) and DNA bases, affecting binding in a biomolecular protein-DNA complex. Unlike previous GATA work46–48, where we tackled only charged amino acids, we modified the method here to cover all mutations types of all amino acids and all mutations types of all DNA bases. We applied the new method to the structural information of two models of the GATA3:DNA complex43. After computing the electrostatic potential calculations using APBS, and feeding them into the free energy calculations in view of a two-step model, we detected key residues and key DNA bases crucial for the complex intermolecular interactions, and therefore for binding. Analysis of the corresponding free energy calculations showed that the DNA backbone plays a more critical role in binding than the DNA bases, and that was confirmed by the related interactions listed computationally and experimentally.

The results showed that some non-ionic amino acids do play a major role in binding, and that may be rationalized to many factors, such as the position of the non-ionic amino acid in the complex (i.e., too close to the interface or too close to many other charged amino acids) or the contribution of this non-ionic amino acid to some favorable conformation.

Future work will include studying key amino acids and key DNA bases in the crystal structure of GATA4:DNA. Such studies will form the basis for designing future experiments and biopharmaceutical studies that will assist in understanding better the biochemical pathways involved in GATA:DNA binding, for enhanced regulation of GATA target genes.

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AE developed the R scripts, generated the results, drew the figures, and wrote the manuscript. ZD supervised, guided, and supported the whole effort. GN corrected the manuscript while offering his expertise in the research domain. AK helped with editing. All authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.

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