Quantifying Evolutionary Importance of Protein Sites: A Tale of Two Measures

Short title: *Quantifying Evolutionary Importance of Protein Sites*

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Abstract

A key challenge in evolutionary biology is the accurate quantification of selective pressure on proteins and other biological macromolecules at single-site resolution. The evolutionary importance of a protein site under purifying selection is typically measured by the degree of conservation of the protein site itself. A possible alternative measure is the strength of the site-induced conservation gradient in the rest of the protein structure. However, the quantitative relationship between these two measures remains unknown. Here, we show that despite major differences, there is a strong linear relationship between the two measures such that more conserved protein sites also induce stronger conservation gradient in the rest of the protein. This linear relationship is universal as it holds for different types of proteins and functional sites in proteins. Our results show that the strong selective pressure acting on the functional site in general percolates through the rest of the protein via residue-residue contacts. Surprisingly however, catalytic sites in enzymes are the principal exception to this rule. Catalytic sites induce significantly stronger conservation gradients in the rest of the protein than expected from the degree of conservation of the site alone. The unique requirement for the active site to selectively stabilize the transition state of the catalyzed chemical reaction imposes additional selective constraints on the rest of the enzyme.

Introduction

The evolutionary importance of protein sites under purifying selection can be quantified in two very different ways. The classical, “intrinsic” measure for the evolutionary importance of a protein site is the degree of conservation or evolutionary rate of the protein site itself. Protein residues experience different degrees of selective pressure as a result of the different roles they play in protein stability and function(1,2). For example, residues in a protein core are generally under stronger selective pressure than surface residues due to their importance in stabilizing the protein. Indeed, structural determinants such as solvent exposure(3–7) and degree of packing (8–10) were shown to explain a large portion of the variability in the observed site-specific evolutionary rates. In addition, residues in functional sites such as catalytic sites(11) and ligand-binding sites are also under stronger selective pressure than non-functional residues.

An alternative, “extrinsic” measure for the evolutionary importance of a protein site is the conservation gradient the site exerts on the rest of the protein. Rather than quantifying evolutionary conservation of the protein site itself, this measure captures how the evolutionary conservation of other residues surrounding the site gradually decreases with distance from the site in the tertiary structure. Several studies have indicated the possibility for selective pressure to propagate from the functional site to the rest of the protein via physical interactions between neighboring residues in the three-dimensional structure (12–15). While it is clear that the two measures of the evolutionary importance of protein sites are substantially different, it remains unknown how the two measures relate to each other.

68 In this paper, we addressed the fundamental question whether there is a direct relationship
69 between the intrinsic and extrinsic measures of the evolutionary importance of protein sites. We
70 have based our study on a dataset of homology-based structural models of the yeast
71 proteome(5,7). Despite their major differences, we show here that there is a strong linear
72 relationship between the degree of conservation of a protein site and the conservation gradient
73 induced from it. In other words, more conserved protein sites tend to induce stronger
74 conservation gradient in the rest of the protein, as selective pressure acting on the protein site
75 percolates via residue-residue interactions. This linear evolutionary conservation-percolation
76 relationship is universal in that it holds for different types of proteins as well as for different types
77 of functional sites in proteins. Remarkably however, catalytic sites in enzymes are the only
78 exception to this universal rule, as catalytic sites induce significantly stronger long-range
79 conservation gradient than other types of functional sites with similar degrees of conservation.
80 We conclude that for many different types of functional sites, site-induced conservation gradient
81 can be explained by the percolation of site-specific selective pressure through the rest of the
82 protein via residue-residue contacts. However, catalytic sites in enzymes induce significantly
83 stronger long-range conservation gradient in the rest of the protein than expected from the
84 percolation theory. This is likely due to the unique requirement for the enzyme active site to
85 selectively bind to and stabilize the transition state of the catalyzed chemical reaction(16).

86 Overall, we show that a more complete understanding of the selective pressure on protein sites
87 can be achieved by integrating the intrinsic measure of site-specific evolutionary conservation
88 with the extrinsic measure of site-induced conservation gradient, with potential implications in
89 protein design, study of disease mutations and better phylogenetic inference(17).

90 # Results

91 ***Evolutionary conservation gradient induced from a protein residue in the proteins structure is***
92 ***linearly correlated with the conservation of the residue***

93 We based our study on a dataset of homology-based structural models of the *S. cerevisiae*
94 proteome. This basic dataset contains structural templates from the Protein Data Bank (PDB)(18)
95 mapped to ORFs of *S. cerevisiae* via sequence alignment (see Methods). We had 1274 yeast ORFs
96 with structural models from the PDB for which residue conservation scores are available in
97 ConSurf-DB (19,20). Each residue in the dataset was ranked according to its relative conservation
98 within the protein (the residue's rank of conservation divided by the total number of residues).
99 This normalized conservation rank of a residue ranges from 0 to 1, with higher rank
100 corresponding to higher conservation within the protein. We have also calculated for each
101 residue the Pearson correlation between the conservation scores of all other residues in the
102 protein and their distance from that reference residue. This Pearson correlation between
103 conservation and distance describes the degree of percolation of the evolutionary conservation
104 from a residue throughout the protein tertiary structure, i.e., the 'long-range conservation
105 gradient'. All residues were then binned into 20 equally spaced bins of conservation rank and the
106 average conservation gradient for each bin was calculated.

107 A clear negative linear trend is observed between the conservation rank of a residue and the
108 strength of the evolutionary conservation gradient induced from it (Fig 1a). The more a residue
109 is conserved within the protein (higher conservation rank), the stronger the evolutionary
110 conservation gradient it induces. The distribution of overall per-protein Pearson correlations

111 between conservation ranks and conservation gradients (per-protein conservation-percolation
112 trend) is mainly between -0.2 and -0.7, and the average correlation in the dataset is -0.5 (Fig 1b),
113 indicating that the linear relationship between site-specific conservation and site-induced
114 conservation gradient is high for all types of proteins.

115 We have also examined this relationship between site-specific conservation and site-induced
116 conservation gradient specifically for functional sites. We identified different functional sites in
117 the dataset: catalytic sites, non-catalytic ligand-binding sites (on enzymes and on nonenzymatic
118 proteins), protein-protein interaction sites, and allosteric sites. We identified catalytic sites using
119 the Catalytic Site Atlas(21), ligand-binding sites using BioLip(22), allosteric sites using the
120 Allosteric Database (ASD)(23,24) and protein-protein interaction sites using our previous
121 protocol of identifying protein-protein interfaces in the yeast proteome(5). Relative solvent
122 accessibility (RSA) of residues was calculated and residues were classified as buried if RSA=0.0,
123 exposed if RSA>0.8 and middle for 0.0<RSA≤0.8. The linear relationship between site-specific
124 conservation rank and site-induced conservation gradient holds regardless of the residue's
125 location within the protein or its functional role (Fig 2). Overall, our results reveal the existence
126 of a conservation–percolation relationship in which higher residue conservation leads to stronger
127 long-range percolation of selective pressure to adjacent sites in the tertiary structure.
128 Furthermore, this relationship holds for residues in different types of functional sites.

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131 ***Conservation gradient induced from a protein site is linearly correlated with the evolutionary***
132 ***rate of the site***

133 The linear trend between conservation and percolation shown above is based on conservation
134 ranks of residues, which are relative within a protein. In these calculations, we have lumped
135 together residues from different proteins having similar conservation rank, however these
136 residues, being from different proteins, can be under different selective pressure. To address this
137 caveat, we examined whether the conservation-percolation linear trend still holds when
138 conservation is measured in terms of absolute evolutionary rate (dN/dS). The evolutionary rate
139 is calculated for the yeast proteins in *S. cerevisiae* compared with its orthologs in four closely
140 related yeast species (see Methods). Conservation score annotations are transferred from
141 structural homologs onto yeast proteins using sequence alignment. Then, we binned all residues
142 into 100 equally spaced bins of conservation rank and calculated their average evolutionary rate
143 (dN/dS). The average evolutionary rate increases for residues with increasing conservation rank
144 (decreasing conservation) (S1 Fig), showing high correlation between the evolutionary rate of
145 yeast protein sites and the conservation scores of their structural homologs. The conservation-
146 percolation linear trend is shown to still hold here, where conservation is measured in terms of
147 absolute evolutionary rate (Fig 3). The lower the average evolutionary rate of a protein site is,
148 the stronger percolation of evolutionary conservation is induced from it.

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152 ***Catalytic sites induce stronger conservation gradients than predicted by the conservation-***
153 ***percolation trend***

154 The annotation of functional and structural site residues was transferred to yeast proteins from
155 their structural models using sequence alignment. The average conservation gradient induced
156 from them is shown in Fig 3. As expected, functional residues evolve more slowly (low dN/dS)
157 than other residues. The average conservation gradients induced from ligand binding sites and
158 protein-protein interaction sites can all be predicted reasonably well from the linear
159 conservation-percolation trend. Protein-protein interaction sites induce slightly weaker
160 conservation gradient than expected, possibly due to their tendency to rewire during evolution.
161 Exposed non-functional residues induce slightly stronger conservation gradient than expected,
162 possibly due to hidden functional sites on protein surfaces. Remarkably, catalytic sites are the
163 only functional sites with the most significant deviation from the linear conservation-percolation
164 trend. The average conservation gradient induced from catalytic site residues is significantly
165 stronger than expected by the linear conservation-percolation trend. This can also be seen from
166 the stronger conservation gradients from catalytic sites compared with other sites with similar
167 conservation rank (Fig 2). These results suggest that the strong long-range conservation gradient
168 induced from catalytic residues cannot be solely attributed to the percolation of the strong
169 selective pressure on them.

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172 ***Catalytic site residues induce stronger conservation gradients than non-catalytic functional site***
173 ***residues with similar evolutionary rates***

174 We have shown that on average, catalytic site residues induce stronger conservation gradients
175 than expected by their average evolutionary rate. Next, we further reinforce this result by
176 comparing the conservation gradient induced from subsets of catalytic and non-catalytic site
177 residues with similar evolutionary rates. We have sampled 1,000 random subsets of residues
178 from each type of functional site. For each such subset (represented by a circle in Fig 4), we
179 calculated the average evolutionary rate and average conservation gradient. As expected,
180 catalytic site residue subsets span the lowest dN/dS values (x-axis), followed by non-catalytic
181 ligand binding sites and allosteric sites, protein-protein interaction sites, ligand binding sites in
182 non-enzymes and finally buried residues. The linear trend between evolutionary rate and
183 conservation gradient holds for each of these functional site types (Fig 4). This result indicates
184 the robustness of the conservation-percolation linear trend regardless of the functional or
185 structural role of the residues. Interestingly, residue subsets from different functional sites with
186 similar evolutionary rates induce conservation gradients with different magnitudes. In particular,
187 catalytic sites induce significantly stronger long-range conservation gradients than all other non-
188 catalytic sites with similar evolutionary rates, including non-catalytic ligand binding sites (Fig 4).
189 Finally, protein-protein interaction site residues induce lower conservation gradients than most
190 other functional site residues with similar evolutionary rates, possibly due to the tendency for
191 protein-protein interactions to rewire during evolution.

192 ***Catalytic site residues often induce stronger conservation gradients than more conserved non-***
193 ***catalytic functional site residues within the same protein***

194 We have shown that conservation gradients induced by functional site residues in general
195 correlate linearly with the evolutionary rates of these functional site residues. We have also
196 shown that catalytic site residues are special in that they induce the strongest conservation
197 gradients among subsets of different functional site residues with similar average evolutionary
198 rates. However, our analyses have so far been carried out by grouping together functional site
199 residues from different proteins. In this section, we perform a stringent, per-protein analysis by
200 focusing on multi-functional proteins with at least two distinct functional sites and comparing
201 the evolutionary properties of different functional site residues within the same protein. Some
202 proteins in our dataset contain both catalytic sites as well as non-catalytic functional sites,
203 whereas other proteins in our dataset contain two distinct non-catalytic functional sites. We
204 found that for the majority of the functional site residue pairs within the same multi-functional
205 protein, the more conserved functional site residue indeed induces stronger conservation
206 gradient (86% if the more conserved residue is catalytic, Fig 5A; 67% if both residues are non-
207 catalytic, Fig 5B). These results agree with the hypothesis that induced conservation gradients
208 are largely driven by the percolation of selective pressure acting on functional sites.

209 Remarkably, in cases where the catalytic site residue is less conserved than the non-catalytic
210 functional site residue within the same multi-functional protein, the catalytic site residue still
211 induces stronger conservation gradient than the non-catalytic functional site for many of these
212 cases (discordance of 49%, Fig 5C). This large discordance shown in cases where the lower

213 conservation residue is a catalytic site residue, is significantly higher compared to the cases
214 where the lower conservation residue is a non-catalytic site residue (binomial test $P<<0.01$).
215 These results clearly show that within the same protein, less conserved catalytic site residues
216 often induce stronger conservation gradient than the more conserved non-catalytic site.
217 Therefore, the strong conservation gradients from catalytic sites cannot be entirely explained by
218 the percolation of the strong selective pressure acting on the catalytic sites.

219 Discussion

220 In this paper we have shown a linear relationship between two measures of evolutionary
221 importance of protein sites under purifying selection. These are the degree of evolutionary
222 conservation of the site itself, as well as the percolation of evolutionary conservation induced
223 from the site via neighboring residues in the protein tertiary structure. Despite major differences
224 between these two measures, we have shown that the linear relationship between the two
225 measures is universal as it holds for different types of proteins as well as for different types of
226 functional sites in proteins. However, catalytic sites in enzymes are the principal exception to this
227 rule. We have shown here that catalytic sites in enzymes induce significantly stronger
228 conservation gradients in the rest of the protein than expected from the degree of conservation
229 of the site alone. Catalytic sites have a unique and complex functionality as they both bind a
230 substrate as well as reduce the free energy barrier required for a chemical reaction to occur.
231 These catalytic sites were shown to be under stronger selective pressure compared with other
232 functional sites such as protein-protein interaction sites and ligand binding sites(13,15). It was
233 also shown that they induce a significantly stronger long-range evolutionary rate gradient than
234 other functional sites. One hypothesis regarding the origin of the strong conservation gradient

235 from catalytic sites is that it is simply due to the strong selective pressure acting on these sites
236 percolating through the rest of the protein via residue-residue contacts. However, we have
237 shown here that the strong selective pressure acting on catalytic sites cannot entirely explain the
238 strong conservation gradients induced from catalytic sites.

239 The main determinant of the long-range conservation gradient from catalytic sites is still not
240 completely understood. Local structural constraints (such as residue burial and packing) are
241 usually potential contributors as they are known to generally have a significant effect on residue
242 evolutionary rate(1,2,5,10). However, it was shown that local structural constraints are not the
243 main determinants of long-range conservation gradients in enzymes(13,25,26). Moreover, the
244 fact that non-catalytic ligand binding sites and allosteric binding sites induce significantly weaker
245 evolutionary rate gradients implies that the ligand binding and allosteric function are not the
246 main determinants of the long-range conservation gradients in enzymes either(15). We are
247 therefore left with the hypothesis that the uniquely strong conservation gradient in enzymes is
248 imposed by the special requirement for catalytic sites to differentially bind the transition state of
249 a chemical reaction rather than the reactants or products with very similar properties (16), a
250 function which is unique to catalytic sites compared with other functional binding sites. A recent
251 physical model of residue evolutionary rates in enzymes introduced an activation term in addition
252 to a stability term and showed improved predictive ability(27). The model attributed the activity
253 to the free energy required to transform a distorted catalytic site upon mutation to its native
254 conformation. The improved ability of the model supports the hypothesis that the main
255 determinant of the observed long-range conservation gradient in enzymes is a functional rather
256 than structural constraint. Overall, our results suggest that the stringent requirement for the

257 catalytic site to differentially bind to and stabilize the transition state of the catalyzed chemical
258 reaction imposes extensive evolutionary constraints on a large portion of the enzyme beyond
259 just the catalytic site, all of which play key roles in maintaining the catalytic function.

260 The results in this paper provide a complete, quantitative picture of evolutionary conservation
261 patterns within proteins induced by functional sites. The linear relationship between the degree
262 of conservation of a protein functional site and the induced conservation gradient in the rest of
263 the protein suggests that the strong selective pressure acting on the functional site percolates
264 through the rest of the protein via residue-residue contacts. Our results also clearly show the
265 unique evolutionary behavior of enzymes in which the catalytic site induces significantly stronger
266 evolutionary constraints on their surroundings than can be explained by the percolation theory
267 alone. Moreover, our results emphasize that catalysis requires the participation of a much larger
268 set of residues than just the few key catalytic residues. Finally, our proposed measure of the
269 evolutionary importance of protein sites can assist in better phylogenetic inference, functional
270 site prediction and protein design.

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274 # Methods

275 ***Protein dataset collection and functional site annotations***

276 The current dataset is based on a dataset of structural homologs of yeast proteins(15). The
277 dataset was created first by using gapped BLAST(28) searches between protein subunit
278 sequences with solved structure from the Protein Data Bank(18) and 5,861 translated open
279 reading frames (ORFs) of the yeast *Saccharomyces cerevisiae*(29). The ORF–subunit pairs were
280 chosen such that both the subunit sequence and the ORF sequence had coverage of ≥50% in the
281 alignment and E-value $<10^{-5}$ and could be paired with their orthologs in four other closely-related
282 yeast species *S. paradoxus*, *S. mikatae*, *S. bayanus and S. pombe*. This way, 1,555 yeast ORFs were
283 mapped to homologs in the PDB. The procedure included the following steps:

284 First, if one of the homology-based structural models of a yeast ORF had an annotated allosteric
285 site, this model was chosen. For 115 yeast proteins the structural model was identified with a
286 known allosteric site as well as pre-calculated conservation scores in ConSurf-DB(19). For all other
287 yeast ORFs, if they had structural models with known ligand binding sites that do not overlap
288 with catalytic sites and the closest catalytic site residue is at least 6Å away (non-catalytic ligand
289 binding site), the model with the lowest E-value out of them was chosen. Overall, 54 non-
290 enzymes with 57 ligand binding sites and 41 enzymes with 47 non-catalytic ligand-binding sites
291 were part of the dataset. For all other yeast ORFs, the structural model with the lowest E-value
292 was chosen. In this manner, 1064 more ORFs for which the best structural model had pre-
293 calculated conservation scores in ConSurf-DB were added to the dataset. Overall, 1,274 ORF-
294 subunit pairs were included in the study. Out of them, 148 yeast proteins were identified with
295 280 protein-protein interactions sites and 348 proteins were identified with catalytic sites. Full

296 list of yeast proteins, their structural models along with identified functional sites can be found
297 in Supplementary Table S1.

298 ***Functional site annotations***

299 Allosteric sites within the structural subunits were found and their residues annotated using the
300 Allosteric Database (ASD)(23,24). Biologically-relevant ligand-binding sites were found using
301 Binding MOAD (Mother of all Databases)(30,31), a database of biologically significant protein-
302 ligand binding in the PDB. Using MOAD, sites with bound crystallographic additives, buffers, salts,
303 metals and sites with covalently linked ligands are excluded. Ligand-binding residues were
304 identified using the BioLip(22) database. Catalytic sites within the protein structural subunits
305 were found using the Catalytic Site Atlas(21). In order to find proteins that participate in protein-
306 protein interactions in our dataset, we identified structural subunits where each subunit is both
307 in physical contact with another subunit and the corresponding modelled ORFs are reported as
308 interacting by at least one physical experiment in the BioGRID (32,33). Our dataset contains 148
309 proteins with 280 protein-protein interaction interfaces. Interfacial residues were identified as
310 residues with different solvent accessibility values when in complex compared to when the
311 interacting partner is manually deleted from the tertiary structure. Distances between residues
312 were calculated as distances between their respective Cα atoms. All functional site annotations
313 of the chosen structural subunits were transferred to the yeast ORF sequence according to the
314 sequence alignment.

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318 ***Evolutionary conservation and rate calculations***

319 In this study we calculated both evolutionary conservation scores for the residues of the
320 structural subunits as well as average absolute evolutionary rates (dN/dS) for the yeast ORFs.
321 Evolutionary conservation scores were taken from ConSurf-DB(19), which is a database of pre-
322 calculated conservation scores of residues in proteins with known structures in the Protein Data
323 Bank (PDB). ConSurf-DB conservation scores are based on collected sequence homologs of the
324 PDB structure and using the Rate4Site algorithm(34).

325 To calculate the average evolutionary rates (dN/dS) for residues of *S. cerevisiae*, we first used the
326 orthology assignment of the protein-coding genes of *S. cerevisiae* with four other closely-related
327 yeast species (*S. paradoxus*, *S. mikatae*, *S. bayanus, and S. pombe*), according to the Fungal
328 Orthogroup Repository(35). We then aligned the ORFs using MAFFT(36). Then, evolutionary rates
329 were calculated using the program codeml within the PAML software package(37). The tree was
330 specified as ((((*S. cerevisiae*, *S. paradoxus*), *S. mikatae*), *S. bayanus*), *S. pombe*). Codon
331 frequencies were assumed equal (CodonFreq=0) and other parameters in codeml were left to
332 their default values. The codon alignments can be found in Supplementary S2 File.

333 ***Statistical analysis***

334 1000 random sample of 250 residues were collected from each type of functional site residues
335 for Fig 4.

336 Estimated standard errors in our measurements of conservation gradients (Pearson correlations)
337 and of dN/dS values were done using 50 rounds of bootstrap resampling.

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Figure Legends

Figure 1. **Long-range conservation gradient induced from a protein residue is linearly correlated with its conservation within the protein**. (a) Violin plots and respective average of long-range conservation gradient from a residue as a function of conservation rank for all residues in the dataset binned into 20 equally spaced bins of conservation rank. (b) Distribution of per-protein Pearson correlation between residues' conservation ranks and conservation gradients.

Figure 2. Residues in functional and structural sites exhibit linear correlation between conservation and conservation gradient. Violin plots of conservation gradient of residues as a function of their conservation rank within a protein for residues in different types of structural and functional sites.

Figure 3. **Conservation gradient induced from residues is linearly (negatively) correlated with their evolutionary rate (dN/dS), with catalytic site residues inducing stronger conservation gradient than expected by the linear trend**. Average conservation gradient (calculated as the average Pearson correlation between conservation of residues and their distance from a site) as a function of average evolutionary rate (dN/dS) for all yeast protein residues binned according to their annotated conservation rank into 100 equally spaced bins.

Figure 4. **Catalytic site residues induce stronger conservation gradients than non-catalytic functional site residues with similar evolutionary rates.** Average conservation gradients of subsets of functional residues. Each circle represents a subset of residues, colored by different types of functional sites.

Figure 5. Catalytic site residues often induce stronger conservation gradients than more conserved non-catalytic functional site residues within the same protein. Within the same protein, (A) more conserved catalytic site residues tend to induce stronger conservation gradient than less conserved non-catalytic site residues (binomial test, $P<<0.001$); (B) more conserved non-catalytic site residues tend to induce stronger conservation gradient than less conserved non-catalytic site residues (binomial test, $P<<0.001$); (C) less conserved catalytic site residues often induce stronger conservation gradient than more conserved non-catalytic site residues (discordance for half of the cases, binomial test, $P=0.13$). Functional site residue pairs for which the ordering of residue conservation agrees with the ordering of induced conservation gradient (concordance) are marked in blue. Functional site residue pairs for which the ordering of residue conservation disagrees with the ordering of induced conservation gradient (discordance) are marked in orange.

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Figures

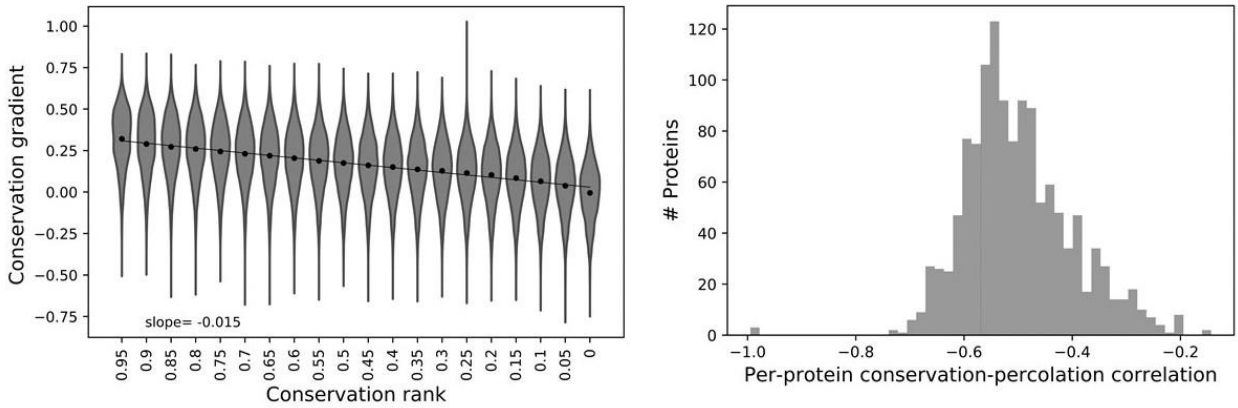


Figure 1. **Long-range conservation gradient induced from a protein residue is linearly correlated with its conservation within the protein**. (a) Violin plots and respective average of long-range conservation gradient from a residue as a function of conservation rank for all residues in the dataset binned into 20 equally spaced bins of conservation rank. (b) Distribution of per-protein Pearson correlation between residues' conservation ranks and conservation gradients.

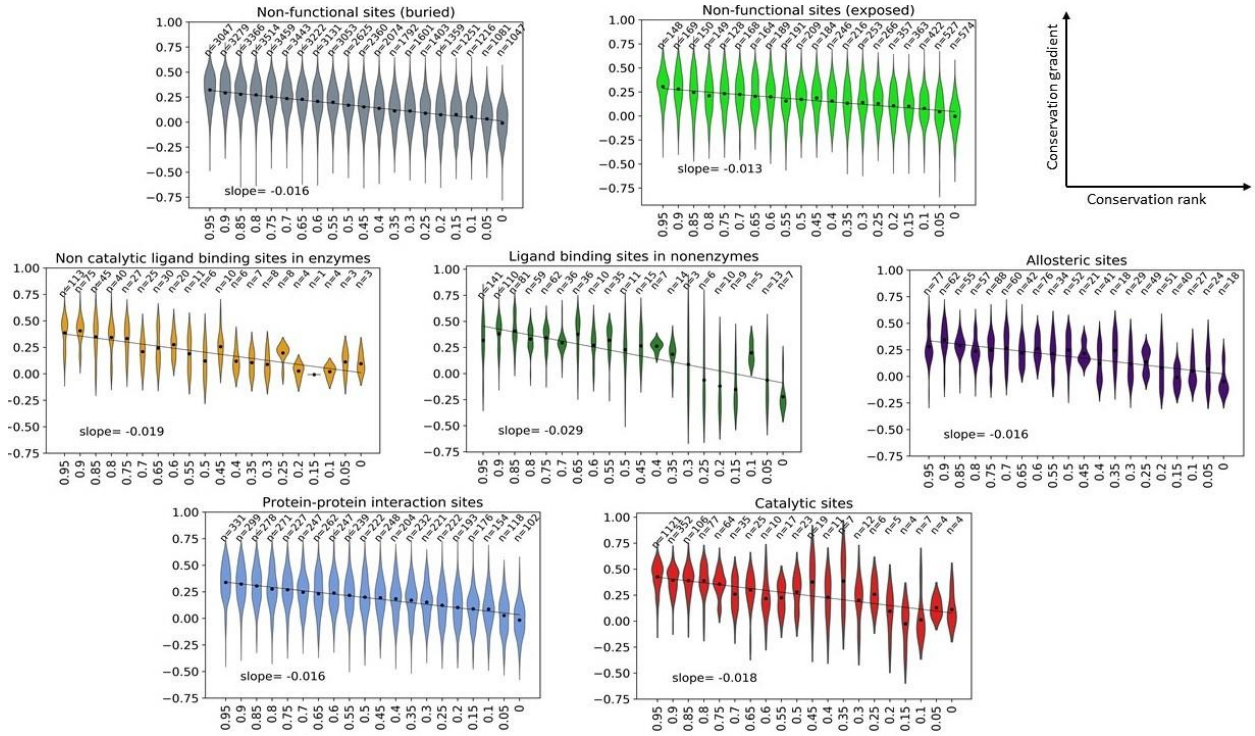


Figure 2. Residues in functional and structural sites exhibit linear correlation between conservation and conservation gradient. Violin plots of conservation gradient of residues as a function of their conservation rank within a protein for residues in different types of structural and functional sites.

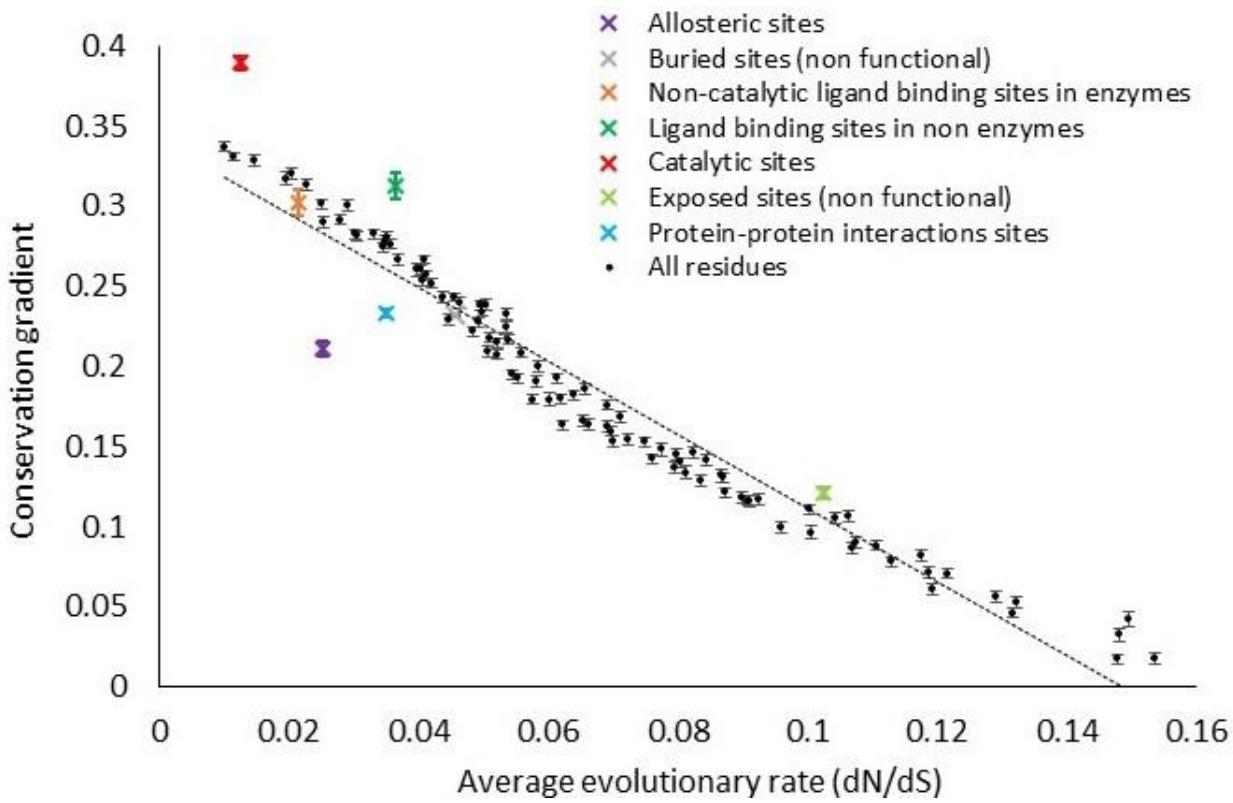


Fig 3. **Conservation gradient induced from residues is linearly (negatively) correlated with their evolutionary rate (dN/dS), with catalytic site residues inducing stronger conservation gradient than expected by the linear trend**. Average conservation gradient (calculated as the average Pearson correlation between conservation of residues and their distance from a site) as a function of the average evolutionary rate (dN/dS) for all yeast protein residues binned according to their annotated conservation rank into 100 equally spaced bins (black) as well as the average conservation gradients of different types of functional sites.

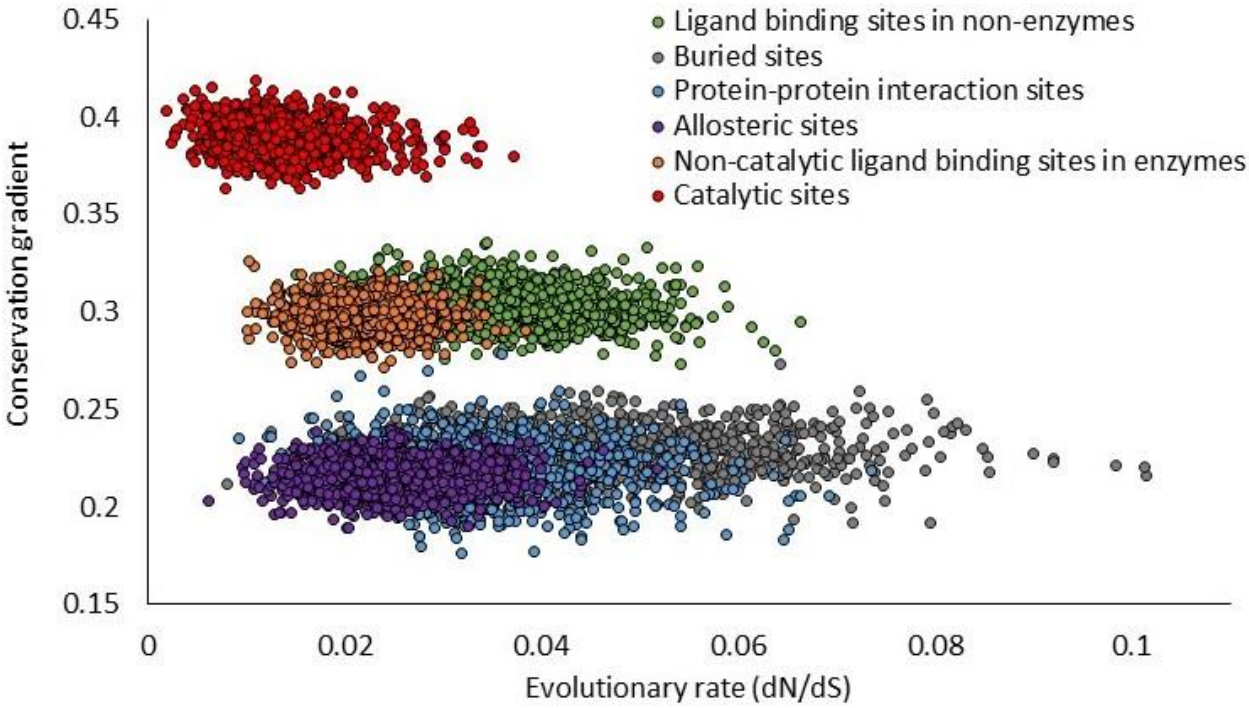


Figure 4. **Catalytic site residues induce stronger conservation gradients than non-catalytic functional site residues with similar evolutionary rates.** Average conservation gradients of subsets of functional residues. Each circle represents a subset of residues, colored by different types of functional sites.

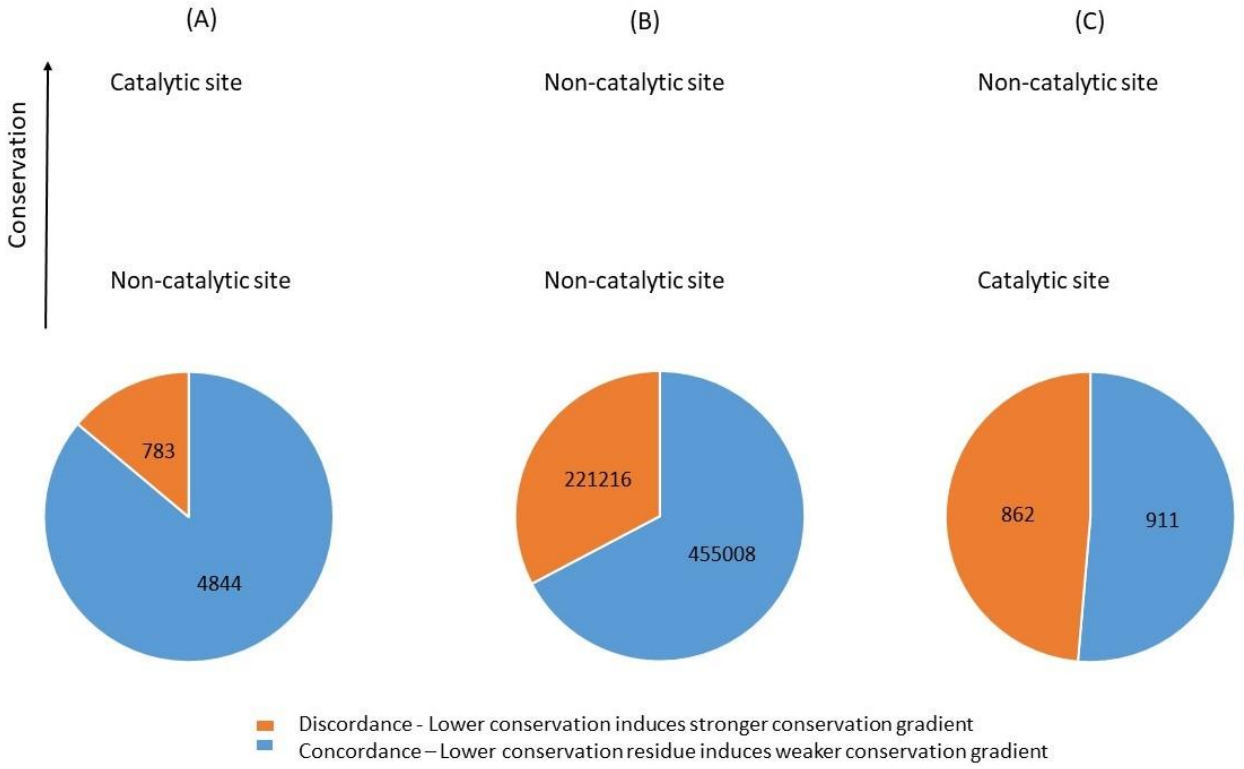
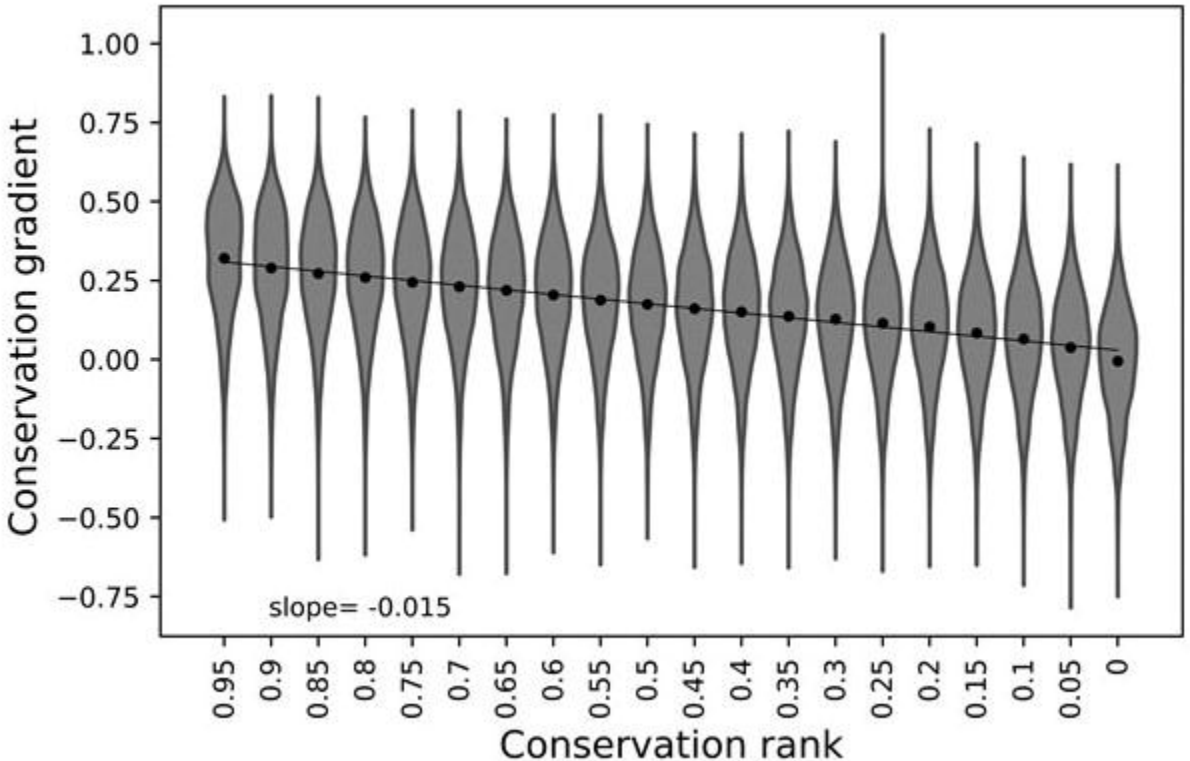
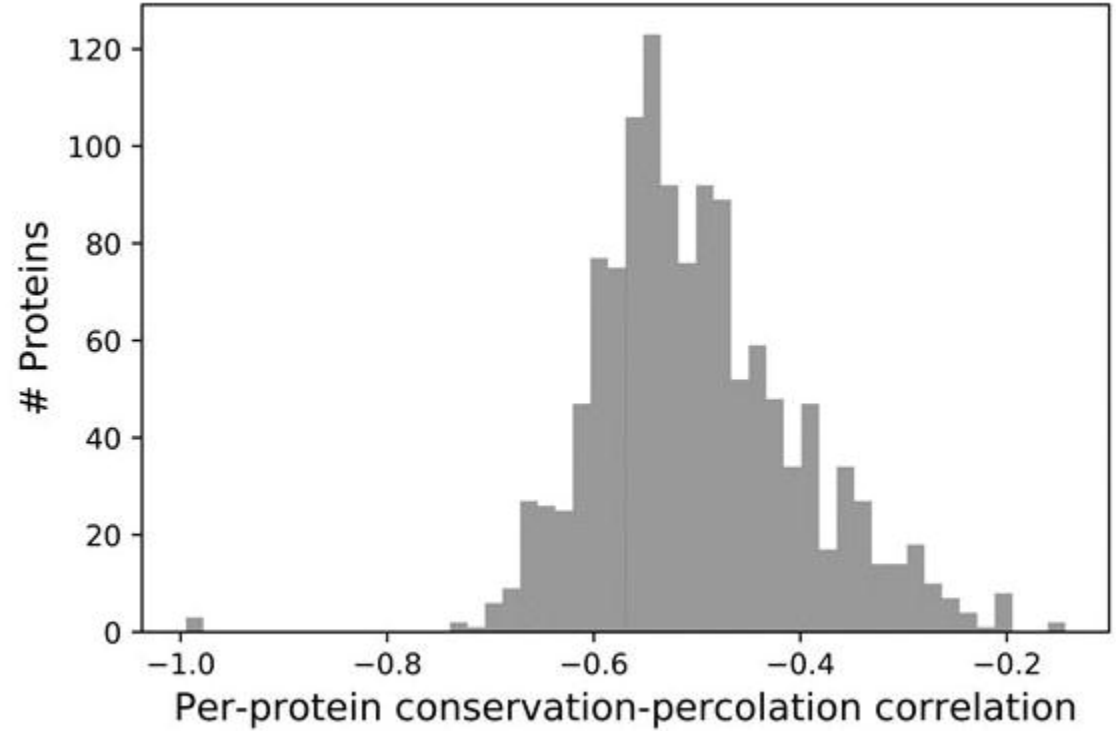
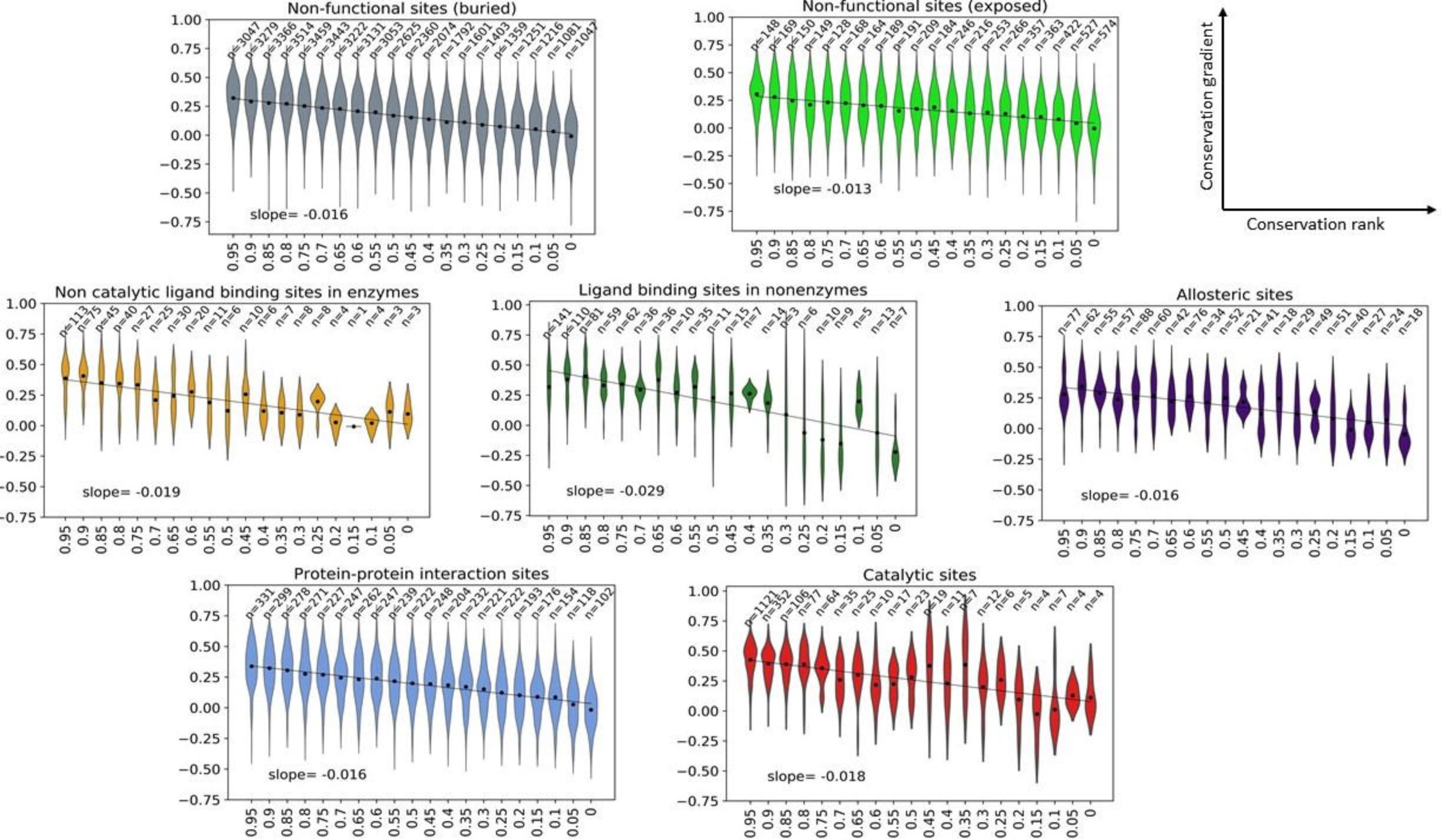
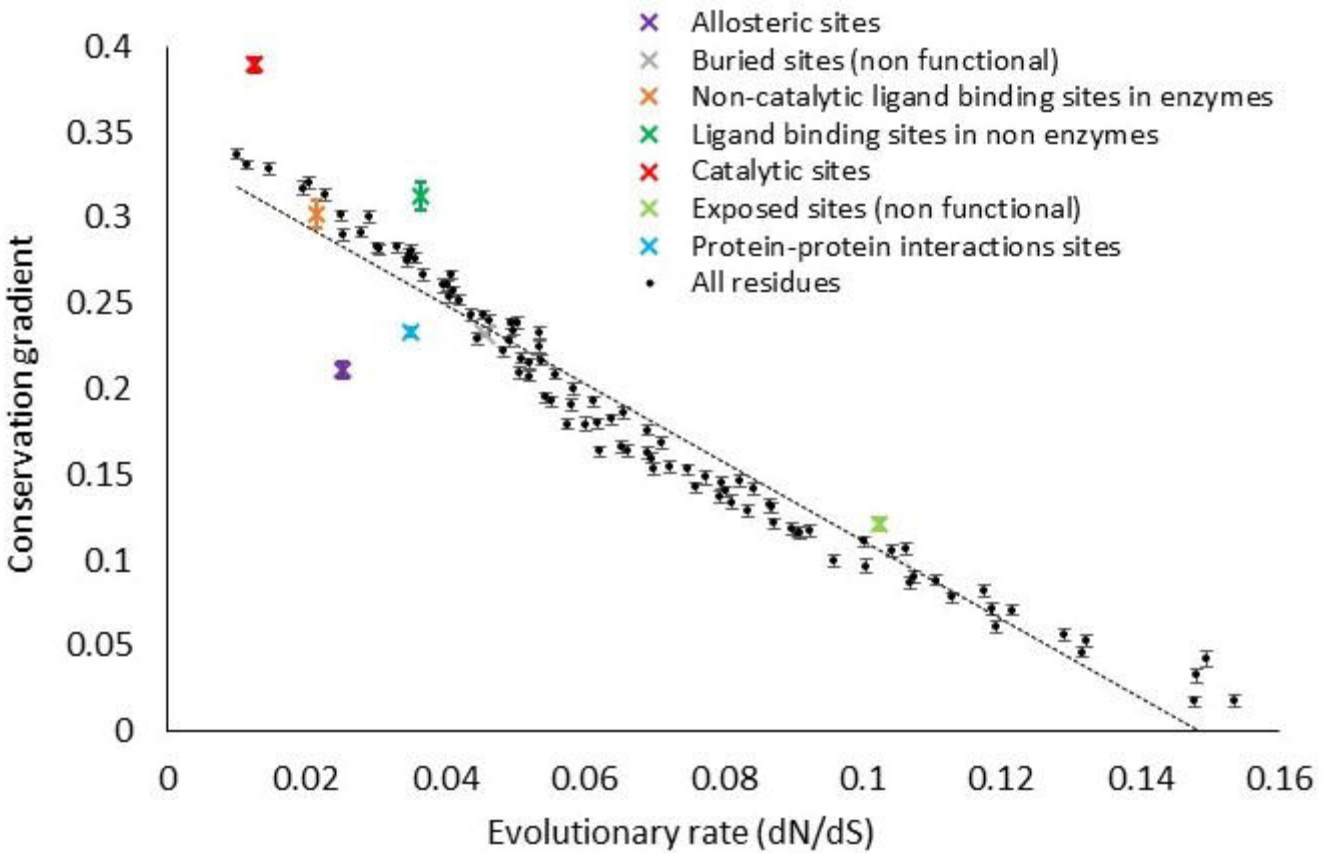


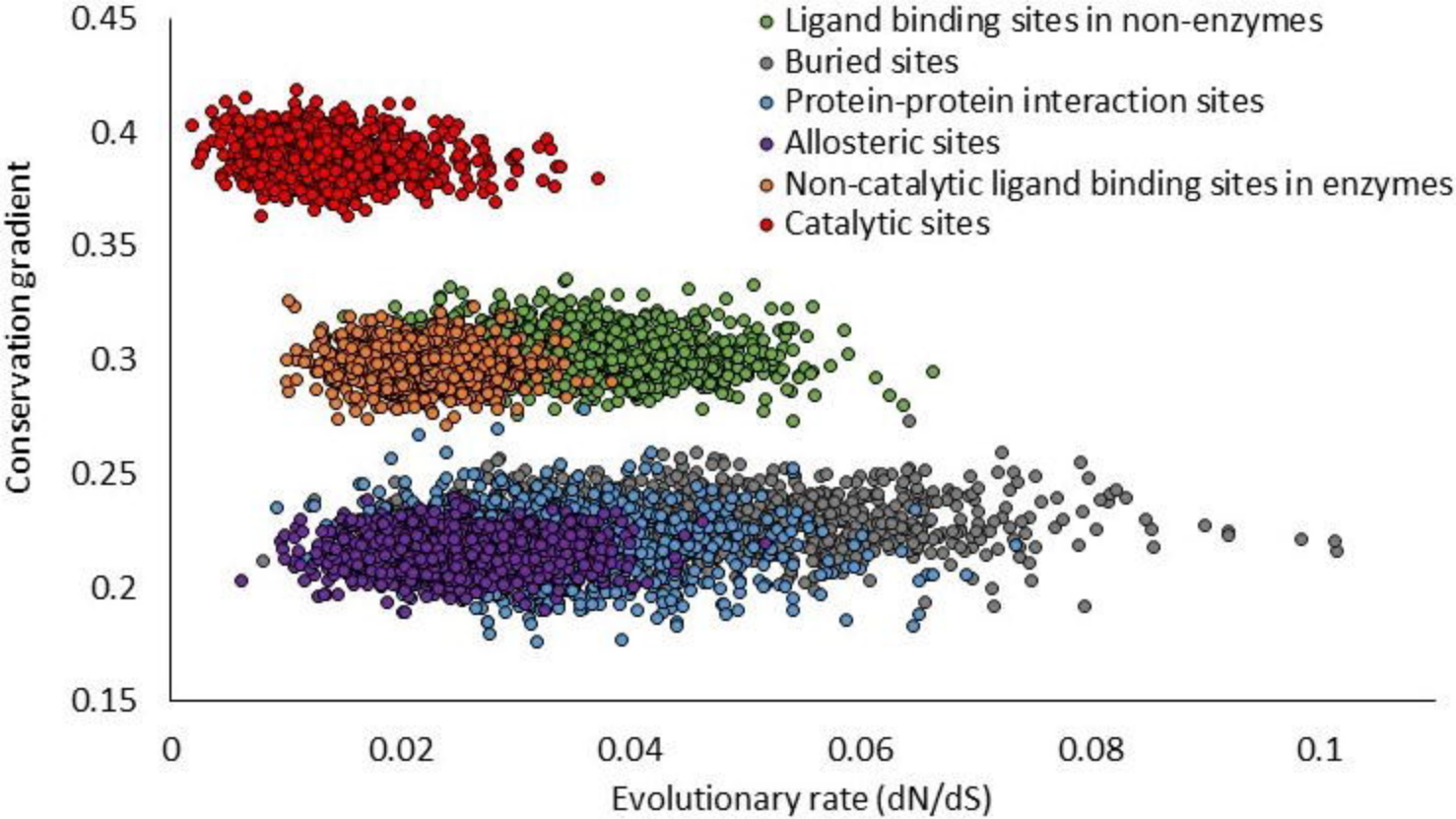
Fig 5. Catalytic site residues often induce stronger conservation gradients than more conserved non-catalytic functional site residues within the same protein. Within the same protein, (A) more conserved catalytic site residues tend to induce stronger conservation gradient than less conserved non-catalytic site residues (binomial test, P<<0.001); (B) more conserved non-catalytic site residues tend to induce stronger conservation gradient than less conserved non-catalytic site residues (binomial test, P <<0.001); (C) less conserved catalytic site residues often induce stronger conservation gradient than more conserved non-catalytic site residues (discordance for half of the cases, binomial test, P=0.13). Functional site residue pairs for which the ordering of residue conservation agrees with the ordering of induced conservation gradient (concordance) are marked in blue. Functional site residue pairs for which the ordering of residue conservation disagrees with the ordering of induced conservation gradient (discordance) are marked in orange.

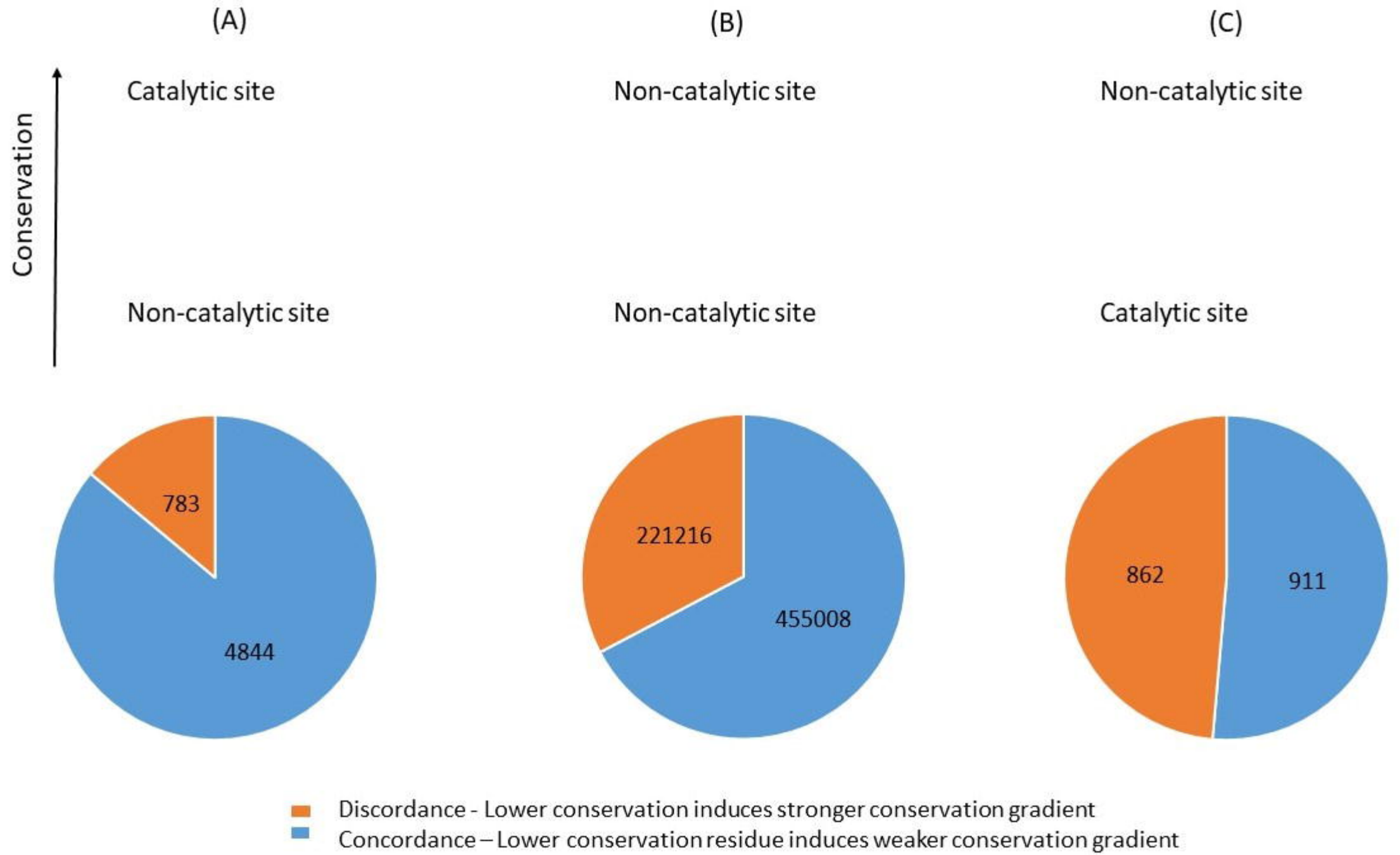

Conservation gradient
1.00
0.75
0.50
0.25
0.00
−0.25
−0.50
−0.75
slope= -0.015
0.95
0.9
0.85
0.8
0.75
0.7
0.65
0.6
0.55
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Conservation rank


Proteins
120
100
80
60
40
20
0
−1.0
−0.8
−0.6
−0.4
−0.2
Per-protein conservation-percolation correlation


Non-functional sites (buried)
slope= -0.016
Non-functional sites (exposed)
slope= -0.013
Conservation gradient
Conservation rank
Non catalytic ligand binding sites in enzymes
slope= -0.019
Ligand binding sites in nonenzymes
slope= -0.029
Allosteric sites
slope= -0.016
Protein-protein interaction sites
slope= -0.016
Catalytic sites
slope= -0.018


Allosteric sites
Buried sites (non functional)
Non-catalytic ligand binding sites in enzymes
Ligand binding sites in non enzymes
Catalytic sites
Exposed sites (non functional)
Protein-protein interactions sites
All residues
Conservation gradient
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
0.16
Evolutionary rate (dN/dS)


Ligand binding sites in non-enzymes
Buried sites
Protein-protein interaction sites
Allosteric sites
Non-catalytic ligand binding sites in enzymes
Catalytic sites
Conservation gradient
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0
0.02
0.04
0.06
0.08
0.1
Evolutionary rate (dN/dS)


(A)
(B)
(C)
Conservation
Catalytic site
Non-catalytic site
Non-catalytic site
Non-catalytic site
Non-catalytic site
Catalytic site
783
4844
221216
455008
862
911
Discordance - Lower conservation induces stronger conservation gradient
Concordance – Lower conservation residue induces weaker conservation gradient