

Systematic genetic characterization of the human PKR kinase domain highlights its functional malleability to escape a poxvirus substrate mimic

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Abstract

Evolutionary arms races can arise at the contact surfaces between host and viral proteins, producing dynamic spaces in which genetic variants are continually pursued. However, the sampling of genetic variation must be balanced with the need to maintain protein function. A striking case is given by protein kinase R (PKR), a member of the mammalian innate immune system. PKR detects viral replication within the host cell and halts protein synthesis to prevent viral replication by phosphorylating eIF2 α , a component of the translation initiation machinery. PKR is targeted by many viral antagonists, including poxvirus pseudosubstrate antagonists that mimic the natural substrate, eIF2 α , and inhibit PKR activity. Remarkably, PKR has several rapidly evolving residues at this interface, suggesting it is engaging in an evolutionary arms race, despite the surface's critical role in phosphorylating eIF2 α . To systematically explore the evolutionary opportunities available at this dynamic interface, we generated and characterized a library of 426 SNP-accessible nonsynonymous variants of human PKR for their ability to escape inhibition by the model pseudosubstrate inhibitor K3, encoded by the vaccinia virus gene *K3L*. We identified key sites in the PKR kinase domain that harbor K3-resistant variants, as well as critical sites where variation leads to loss of function. We find K3-resistant variants are readily available throughout the interface and are enriched at sites under positive selection. Moreover, variants beneficial against K3 were also beneficial against an enhanced variant of K3, indicating resilience to viral adaptation. Overall, we find that the eIF2 α -binding surface of PKR is highly malleable, potentiating its evolutionary ability to combat viral inhibition.

eLife Assessment

This **important** revised report describes the control of the activity of the RNA-activated protein kinase, PKR, by the Vaccinia virus K3 protein. A strength of the manuscript is the powerful combination of a classic yeast-based assay with high-throughput sequencing and its **convincing** experimental use to characterize large numbers of PKR variants, now with improved controls for potential biases. A minor current limitation that the authors may address in the future is the scope of the screen in terms of the segments of PKR included.

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Introduction

Molecular interfaces between interacting host and pathogen proteins are atomic battlegrounds that give rise to evolutionary arms races (1). Successful infection typically involves critical interactions between host and pathogen proteins, such as binding to cell-surface receptors or inhibition of cellular defense mechanisms. In such scenarios, the host benefits from genetic mutations that disrupt the interaction while the pathogen benefits from mutations that maintain the interaction. The pursuit of genetic mutations by the host and pathogen can develop into an evolutionary arms race where the accumulation of mutations leads to abnormally high sequence diversity, which is used to identify sites experiencing positive selection.

A key member of the antiviral innate immune repertoire in vertebrates is protein kinase R (PKR) (2–4). PKR contains two N-terminal double-stranded RNA (dsRNA) binding domains and a C-terminal kinase domain, separated by a long linker (5). The presence of dsRNA in the cytoplasm is a hallmark of viral infection (6). PKR binds dsRNA, initiating PKR homodimerization and autophosphorylation, which activates the kinase (7–10). Active PKR binds and phosphorylates eIF2 α on Ser51 (Figure 1A), a member of the translation initiation machinery. Phosphorylation of eIF2 α leads to a halt in protein synthesis, thereby preventing viral replication (8,11,12).

Vertebrate PKR homologs have many sites under positive selection (13), including residues at its binding surface with eIF2 α (Figure 1B). This is remarkable given the critical nature of this interface and that eIF2 α is an unvarying protein under purifying selection (14). Many viruses encode PKR inhibitors to evade its antiviral activity, some of which are known to interact with PKR at its eIF2 α -binding interface (15–17). One example is vaccinia virus, whose well-characterized pseudosubstrate inhibitor protein K3 is encoded by the *K3L* gene (Figure 1C) (18–20). Vaccinia K3 mimics the structure and sequence of eIF2 α to inhibit PKR and prevent the halt of protein synthesis (19,21–23). Thus, despite evolutionary pressure on PKR to recognize the conserved eIF2 α substrate, variation at the same interface would be evolutionarily beneficial if it allowed evasion of viral pseudosubstrate antagonists. Indeed, previous studies have identified some single-residue variants at sites under positive selection in the PKR kinase domain that maintain recognition of eIF2 α yet resist vaccinia K3 (13,14,24). However, without comprehensively exploring the genetic landscape at this interface, it is unclear how readily PKR can mutate to evade viral antagonists while preserving its kinase function.

Here we use a prospective, systematic, and unbiased approach to map SNP-accessible PKR variants in the kinase domain that are resistant or susceptible to the vaccinia wild-type K3, nonfunctional K3, and the enhanced mutant allele K3-H47R. We found many novel K3-resistant variants,

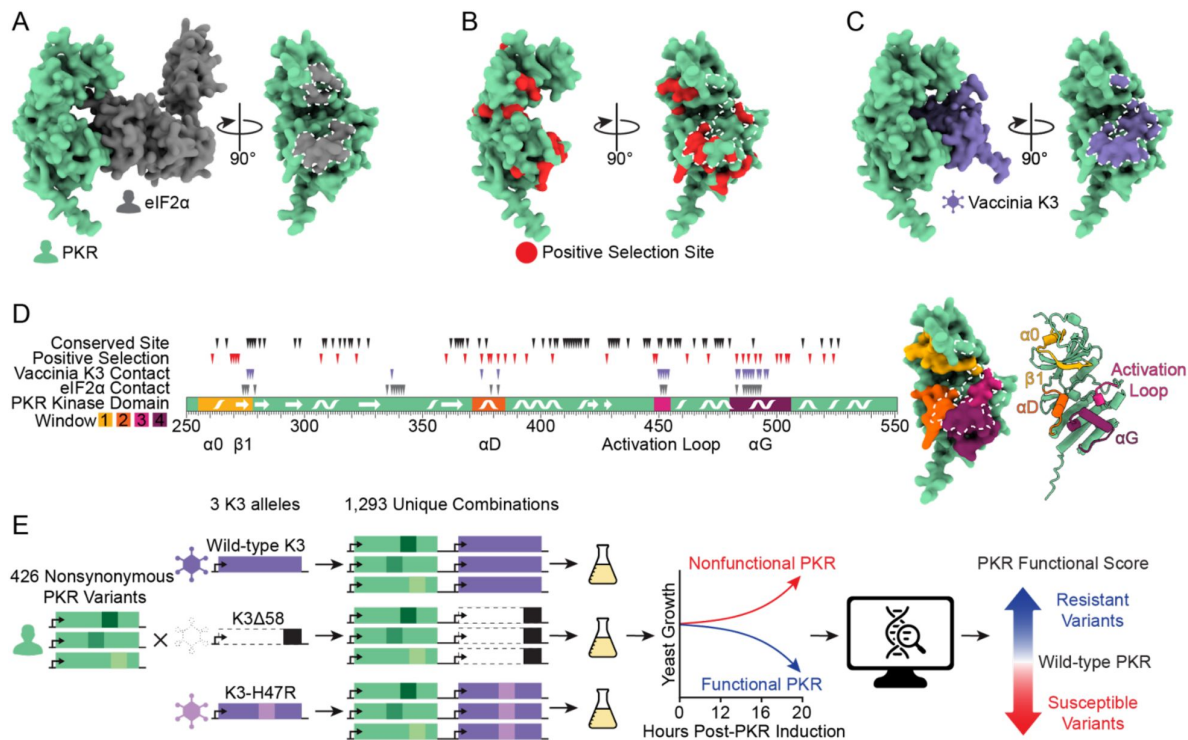


Figure 1.

Exploring genetic variants of human PKR against the pseudosubstrate antagonist vaccinia K3.

(A) (Left) AlphaFold2 model of the PKR kinase domain (green) bound to its target, eIF2α (gray). (Right) Rotated 90°, the eIF2α binding surface of PKR is shaded gray and outlined in white dashed line. (B) PKR kinase domain with vertebrate positive selection sites highlighted in red (13), with the K3 binding surface delineated with white dashed line. (C) AlphaFold2 model of the PKR kinase domain bound to vaccinia K3 (purple). Rotated 90°, the K3 binding surface of PKR is shaded purple and outlined in white dashed line. (D) (Left) The PKR kinase domain with four windows highlighted in which we designed 426 nonsynonymous variants. Secondary structures occurring in these windows (alpha helices α0, αD, and αG, beta strand β1, and the kinase activation loop) are marked (5). The positions of conserved sites and sites under positive selection (13) are denoted above with black and red triangles, respectively, and the positions of sites within 5 angstroms of either K3 or eIF2α in an AlphaFold2 binding model are denoted with purple and gray triangles, respectively. Note that the modeled interaction between PKR and eIF2α resembles the pre-phosphorylation state in which the phosphoacceptor loop of eIF2α has not entered the PKR active site, and thus there are likely additional sites in PKR that contact eIF2α. (Right) Mutated windows are highlighted on the surface of the PKR kinase domain, with the K3 binding surface delineated with a white dashed line, and in cartoon form with secondary structures marked. (E) Methodological approach to explore the effects of variants of human PKR in the presence of different K3 alleles. 426 nonsynonymous variants of PKR were generated and paired with wild-type K3, K3Δ58, and K3-H47R. Variant effects were characterized using a high-throughput yeast growth assay and massively parallel sequencing.

especially at sites under positive selection, and note a strong correlation between variants that resist wild-type K3 and the enhanced allele. We also identified sites in the PKR kinase domain where variants increased susceptibility to vaccinia K3. Essential PKR residues that did not tolerate variation were infrequent despite the critical nature of this interface; they were primarily found surrounding the ATP-binding site. These results paint a portrait of PKR balancing the tension between conserved function and evasion of viral inhibitors through mutational resilience in its substrate interface.

Results

Generation of nonsynonymous PKR variant library

We systematically made genetic variants in four windows of interest in the kinase domain of PKR (**Figure 1D** [↗](#)). The four windows were selected based on having sites within five angstroms of eIF2α or K3 and sites under positive selection across vertebrate PKR homologs as identified by Rothenburg et al. ([13](#) [↗](#)). Our windows of interest include helix αD and αG, as well as the glycine-rich loop and activation loop surrounding the ATP-binding site. In these windows, we focused on testing 426 variants that could be acquired with a single nucleotide change from the predominant human PKR allele (Genbank M85294.1) (**Supplemental Figure 1A** [↗](#)), as those are the most likely to be evolutionarily sampled.

Overexpression of PKR in the budding yeast *Saccharomyces cerevisiae* is toxic, as PKR auto-activates and shuts down protein synthesis by phosphorylating yeast eIF2α, which is highly conserved between yeast and humans ([25](#) [↗](#), [26](#) [↗](#)). This toxicity can be partially counteracted by co-expression of vaccinia K3 ([23](#) [↗](#)). Thus, yeast provides a useful experimental system to test the functionality of PKR variants: variants that maintain the ability to phosphorylate eIF2α while escaping K3 inhibition will further arrest growth, while variants that lose eIF2α kinase activity or become more susceptible to K3 will improve growth.

We sought to leverage this assay in a high-throughput manner to determine the functionality of PKR variants by tracking their abundances in a pool over time. We generated the 426 single-residue variants of PKR on a single-copy plasmid with a galactose-inducible promoter in a plasmid pool (**Figure 1E** [↗](#)). Each unique plasmid in the pool carried a DNA barcode randomly generated during cloning. We used long-read circular consensus sequencing to pair DNA barcodes with PKR variants, identifying an average of 43 barcodes per variant (**Supplemental Figure 1B** [↗](#)). We then created three libraries by combining the PKR variant library with wild-type K3, nonfunctional K3, or the enhanced mutant allele K3-H47R ([23](#) [↗](#), [27](#) [↗](#)). The K3 alleles were expressed from a constitutive *TDH3* promoter. We transformed the three PKR-K3 paired libraries in duplicate into yeast grown in glucose media, such that PKR expression was repressed. We then inoculated the libraries in liquid galactose media to induce PKR expression, and sampled cells at 0, 12, 16, and 20 hours post-inoculation. We quantified the abundance of the PKR variants at each timepoint using high-throughput short-read sequencing of the DNA barcodes. For each PKR variant we calculated a functional score against each K3: PKR variants that evade K3 inhibition and reduce yeast growth have high functional scores, while PKR variants that are non-functional or are inhibited by vaccinia K3, and thus are associated with good yeast growth, have low functional scores (**Supplemental Figure 2**). The correlation of functional scores between replicates 1 and 2 was very strong (Pearson correlation coefficient > 0.98 for each K3 allele, **Supplemental Figure 3** [↗](#)), so we combined the reads from replicates 1 and 2 to generate PKR functional scores for subsequent analyses.

Many PKR variants evade K3 while maintaining kinase function

We first examined how each PKR variant performed against wild-type K3. We were encouraged to find that variants previously characterized as resistant to K3-H47R (24) were resistant to wild-type K3 in our screen (Figure 2A). We found many additional K3-resistant variants across all four tested windows and validated select PKR variants using serial dilution to measure yeast growth (Supplemental Figure 4). Notably, every variant made at Glu375 increased evasion of K3, with the exception of Glu375Asp. In the AlphaFold2-predicted complex, Glu375 forms an attractive electrostatic interaction with Lys45 of K3 (Figure 2B).

Consistent with our findings, this predicted interaction would be disrupted by all tested variants at site 375 aside from the negatively charged aspartic acid. Interestingly, Glu375 is the only site under positive selection in vertebrates that was proximal to K3 but not eIF2 α in the AlphaFold2 models, making it a prime candidate for disrupting K3 inhibition without affecting eIF2 α phosphorylation.

We found a large number of K3-resistant variants in the vicinity of PKR's helix α G (Figure 2C,D). Compared to other protein kinase structures, eIF2 α kinases have a noncanonical helix α G that is extended by one full turn and rotated 40° counterclockwise with a 5 angstrom translation relative to its C-terminus, owing to a reduced α F- α G linker (5). The noncanonical orientation of helix α G contributes to both eIF2 α binding specificity and Ser51 presentation to PKR (5). It has been proposed that mutations causing movement in helix α G might be especially capable of disrupting K3 binding due to the rigid structure of K3 between its contact points on helix α G and the PKR active site. In contrast, PKR mutations that move helix α G could be tolerated in the context of eIF2 α binding because eIF2 α contacts PKR's active site using a flexible loop (5). We observe a gradient of PKR functional scores along the helix α G region, starting with highly resistant or susceptible variants (positions 480-492) and shifting to neutral wild-type-like scores (493-500) (Figure 2A). We identified four sites within or near the N-terminus of helix α G for which many variants evade K3: Glu480, Val484, Glu490, and Thr491 (Figure 2C).

We looked at what features of PKR were associated with sites of K3-resistant variants and were excited to find that sites under positive selection in vertebrates were strongly enriched for K3-resistant variants relative to sites conserved across vertebrate PKRs (Figure 2E). Comparing K3 contact and non-contact sites, we find significantly lower functional scores at K3 contact sites (Figure 2F). However, functional scores at K3 contact sites form a bimodal distribution (Hartigan's dip test statistic = 0.105, $p < 0.0001$). Considering only functional variants (PKR functional score > -2) we find that variants at K3 contact sites have significantly higher functional scores than those at non-contact sites (Supplemental Figure 5). Thus, variants at K3 contact sites tend towards large effects either improving or diminishing PKR function versus K3.

Sampled sites in the PKR kinase domain are largely resilient to genetic variation

To understand the constraint placed on the PKR kinase domain, we examined variants that failed to maintain PKR function. We screened our PKR variant library against a nonfunctional K3 (hereafter referred to as K3 Δ 58) in which the first 173 bases were deleted, removing the first 58 residues, including the initial methionine. When paired with K3 Δ 58, most PKR variants were comparable to wild-type PKR (Figure 3A), suggesting the tested regions were not highly functionally constrained for kinase activity despite their proximity to eIF2 α during its phosphorylation. The few highly constrained sites include Gly276 and Gly277, which compose the glycine-rich loop above the ATP-binding site; Gly450 and Thr451 in the activation loop; and Thr487 in helix α G, which stabilizes helix α G (5) and induces the conformational change in eIF2 α required for its phosphorylation (Figure 3B) (28). We also found proline variants to generally disrupt PKR function (Figure 3C). We found nonfunctional variants of varying degrees in all

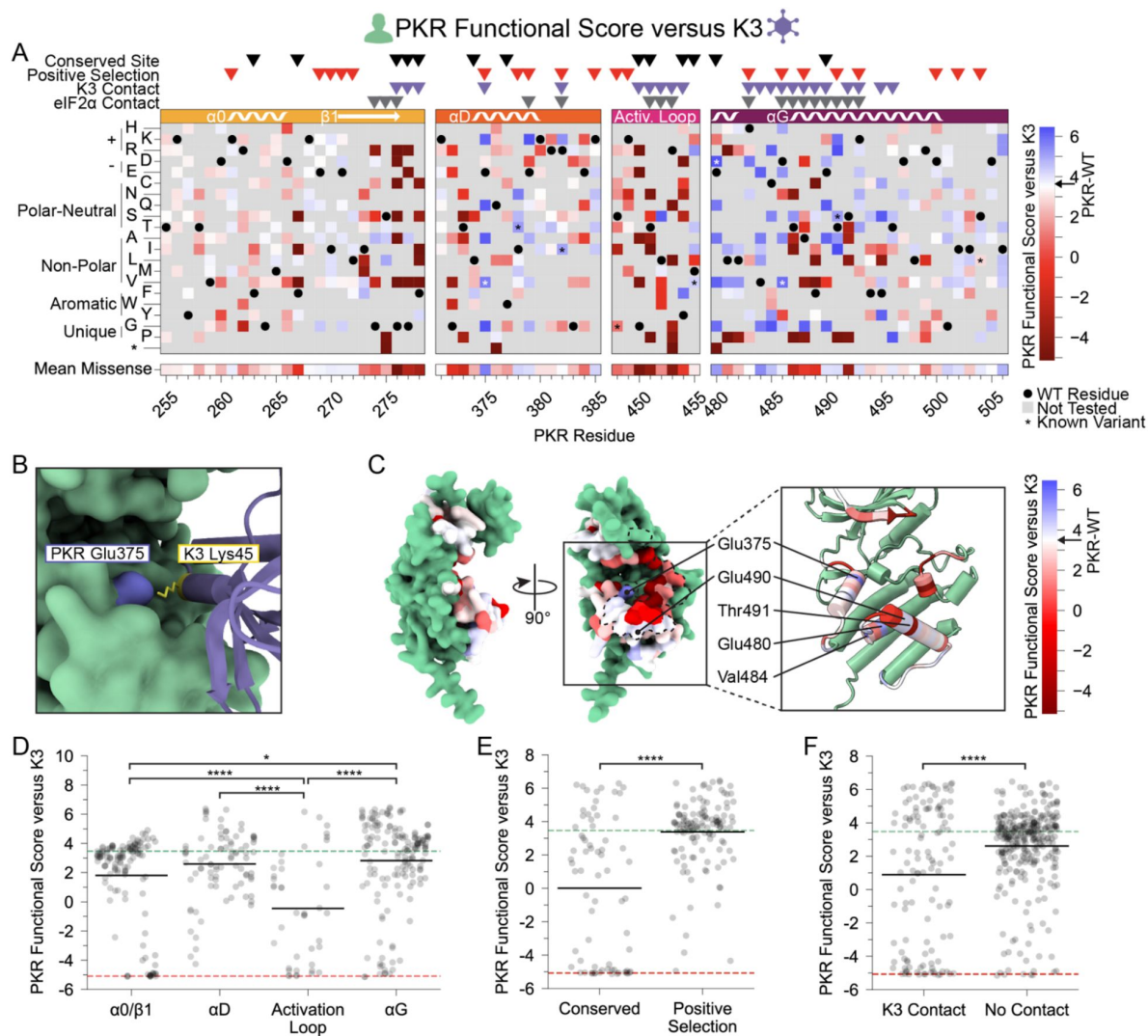


Figure 2.

PKR variants that evade K3 and maintain kinase function are enriched at positive selection sites and helices α D and α G.

(A-B) PKR functional scores versus K3 are colored ranging from susceptible (red) to WT-like (white) to resistant (blue). (A) Heatmap of PKR variants with cells colored by the PKR functional score versus K3 for SNP-accessible variants. Wild-type PKR residues and untested variants are denoted with black circles and gray squares, respectively. Previously characterized K3-H47R-resistant variants are noted with an asterisk (24). (B) Attractive electrostatic interaction between PKR-Glu375 (blue) and K3-Lys45 (yellow) in the AlphaFold2 model of PKR bound to K3. (C) Surface structure of the PKR kinase domain with sites colored by the mean PKR functional score versus K3 for missense variants. The K3 binding surface is delineated with black dashed line. (Inset) Location of K3-resistant sites cited in the text. (D-F) Strip plots of PKR functional score versus K3 for variants, with green and red dashed lines representing mean scores for WT PKR and nonsense variants, respectively. Variants are partitioned by nearest secondary structural element (D), level of conservation in vertebrates (E), or predicted contact with K3 (F). Points in D are ordered left to right by their position along the kinase domain, whereas they are randomly jittered along the x-axis in E and F. (* $p < .05$, **** $p < .0001$, Tukey's HSD (D) and two-sample t-test (E, F).)

four windows of interest, but with very few in helix α D and the C-terminus of helix α G (**Figure 3C**), consistent with these regions having pliable secondary structures in which variants can evade K3 while not disrupting eIF2 α recognition.

More PKR variants had low functional scores in the presence of functional K3 (**Figure 2A**) than in its absence (**Figure 3C**). We plotted the functional scores against each other (**Figure 4A**), and found that variants with low PKR functional scores versus K3 separated into two classes: some switched to high functional scores in the absence of K3, indicating they caused enhanced susceptibility to K3 but otherwise retained PKR function, while others had low functional scores in both conditions, indicating they had diminished eIF2 α kinase activity irrespective of K3 antagonism. We quantified this effect by calculating residuals from the line connecting PKR nonsense variants to PKR-WT, which highlights variants whose functional scores diverged between the K3 conditions. We find that the activation loop and the start of helix α G, which were less tolerant of mutation when paired with wild-type K3 (**Figure 2D**), contain a mix of variants that lose function outright or that increase susceptibility to K3 (**Figures 3B-C**, **4B-C**). Variants at five sites were especially susceptible to K3 inhibition: Phe278, Thr373, Arg453, Phe489, and Ser492 (**Figure 4B**). None appear under positive selection across vertebrates, and Phe278 is conserved across vertebrate PKR homologs. Interestingly, Phe278 is predicted to form a pi interaction with His47 of K3, which aligns to Arg52 of eIF2 α (**Supplemental Figure 6**) (23). Mutation of K3-H47 to arginine, matching eIF2 α , is known to improve K3's antagonism of PKR (23,27), suggesting PKR can discriminate against K3 based on its difference from eIF2 α at this position. This is consistent with our observation of the importance of Phe278 specifically in the presence of K3.

K3-resistant variants were largely resistant to enhanced K3-H47R

We also characterized PKR variants in the presence of an enhanced mutant allele of K3, K3-H47R (**Figure 5**). This mutant allele was first identified in a genetic screen of K3 mutants as an enhanced antagonist of PKR (23) and independently identified in a directed evolution screen

(27). We sought to understand the resilience of improved PKR variants to alternative K3 alleles by examining the concordance between beneficial PKR variants against wild-type K3 and K3-H47R. We found strong correlation between PKR functional scores versus K3 and K3-H47R (Spearman correlation = 0.832, **Figure 5A**). Indeed, many of the K3-resistant variants are also resistant to K3-H47R (**Figure 5B-D**), suggesting resilience of PKR to allelic variation in K3. A general change is the struggle for PKR to overcome the enhanced inhibition of K3-H47R, compressing the functional score range between wild-type-like and nonfunctional scores. This nonlinear relationship between the functional scores in the two K3 conditions (**Supplemental Figure 7**) led to some differences in the patterns of resistance to the two K3 alleles. For instance, positively selected PKR sites did not have significantly higher PKR functional scores versus K3-H47R than conserved sites (**Figure 5E**), a sharp contrast to the PKR functional scores versus wild-type K3 (**Figure 2E**). This result appears to be caused by the many wild-type-like scores at positively selected sites now being similar to the many nonsense-like scores at conserved sites. We also noted that variants at sites proximal to K3 had slightly higher functional scores than those at non-contact sites (**Figure 5F**), the opposite of what we observed against wild-type K3 (**Figure 2F**). This could result from wild-type-like PKR variants being enriched at non-contact sites. Overall, the strong correlation of functional scores versus K3 and K3-H47R suggests K3-resistant variants would often be resilient against alternate K3 alleles.

Previous work identified twelve PKR variants with enhanced resistance to K3-H47R (24), nine of which were included in our experiment (**Supplemental Figure 8**). We examined the other nonsynonymous variants introduced at these sites and found that most sites contained additional novel resistant variants, indicating the previously uncovered individual mutants reflect sites of general opportunity for pseudosubstrate evasion.

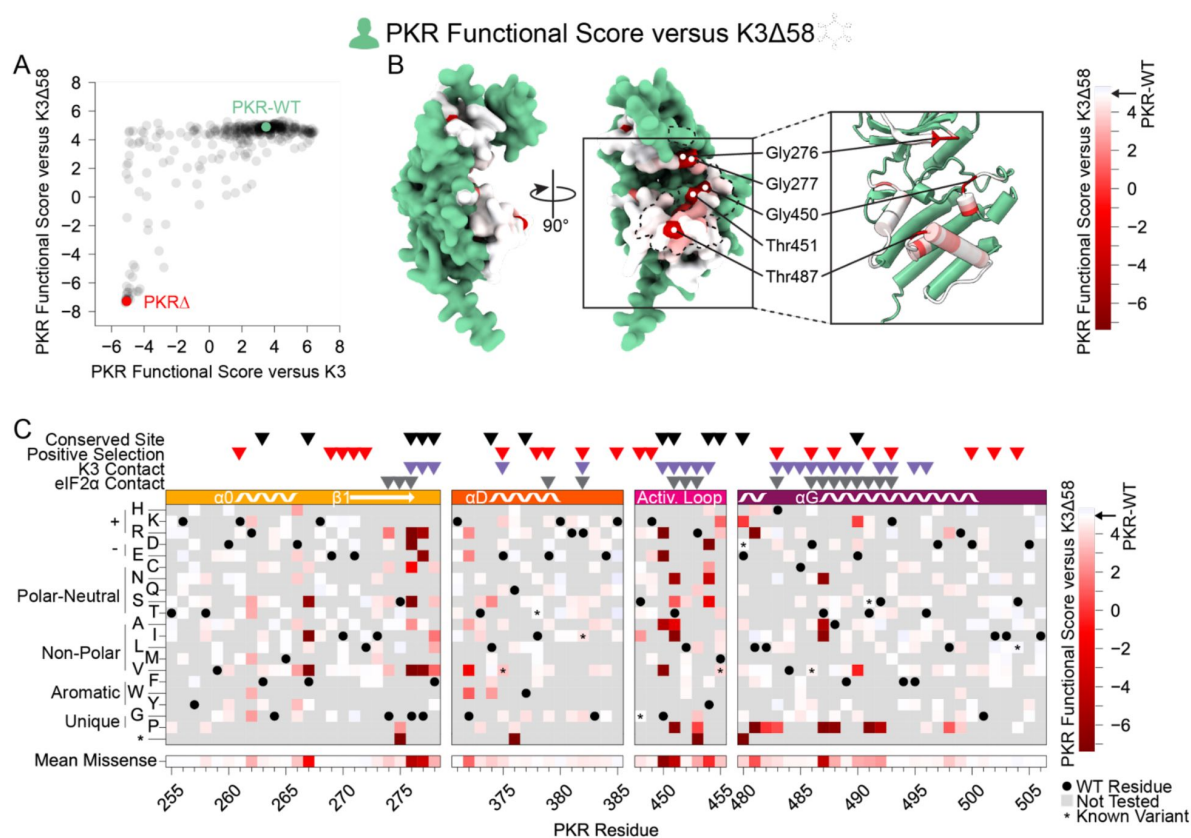


Figure 3.

Few nonfunctional PKR variants identified in the absence of K3 inhibition.

(A) Scatter plot showing each variant's PKR functional score versus wild-type K3 plotted against its PKR functional score versus K3Δ58. The data point for PKR-WT is colored green and a data point representing the average of the four nonsense variants (PKRΔ) is colored red. (B-C) PKR functional scores versus K3Δ58 are colored ranging from nonfunctional (red) to WT-like (white). (B) Surface structure of the PKR kinase domain with sites colored by the mean PKR functional score versus K3Δ58 for missense variants. The K3 binding surface is delineated with a black dashed line. (Inset) Location of highly constrained sites cited in the text. (C) Heatmap of PKR variants with cells colored by the PKR functional score versus K3Δ58 for each variant.

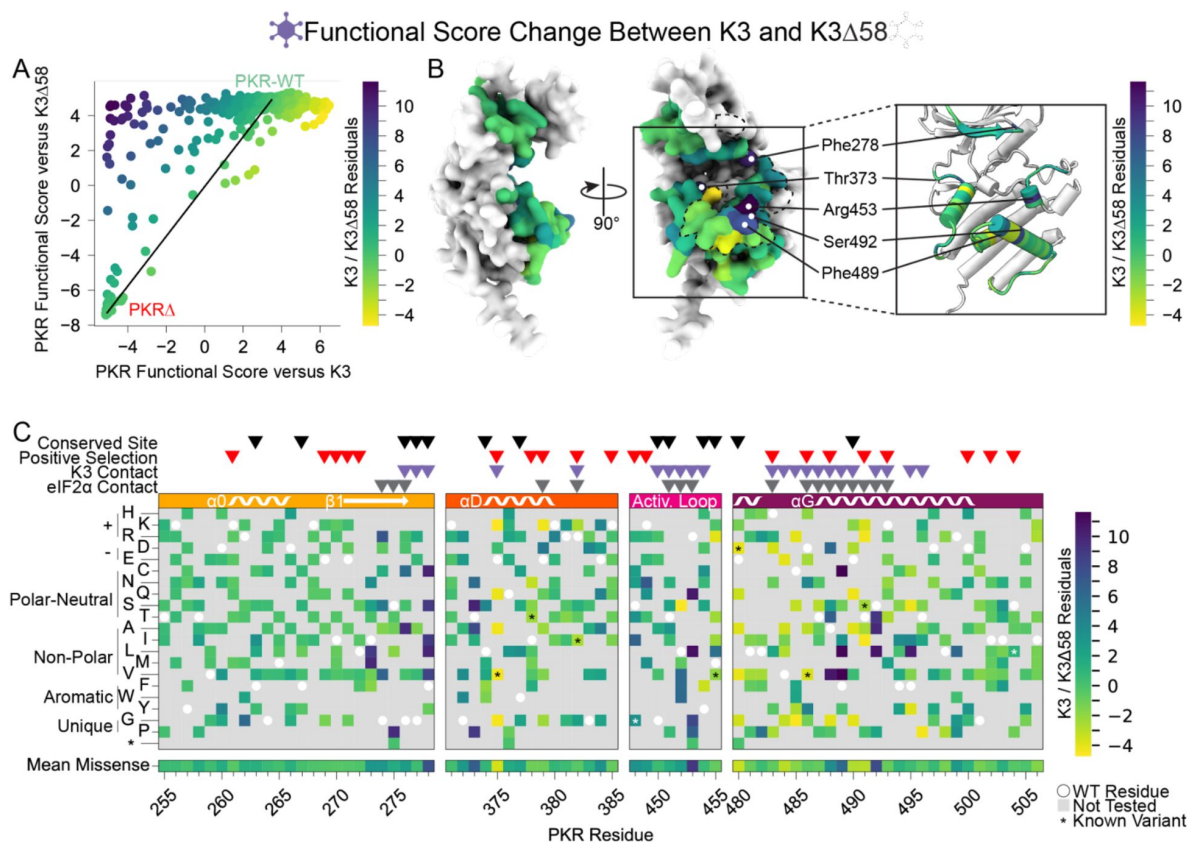


Figure 4.

Identification of PKR sites highly susceptible to K3 inhibition.

(A) A line was drawn connecting the data point for PKR-WT to the position of the average data point for the four nonsense variants (PKRΔ) from the data in the scatter plot from **Figure 3A**, with all other data points colored by their residual from that line (K3 / K3Δ58 Residuals), ranging from K3-susceptible (purple) to K3-indifferent (green) to K3-resistant (yellow). This color scheme is used in panels B and C. (B) Surface structure of the PKR kinase domain with sites colored by the mean K3 / K3Δ58 residuals for missense variants. The K3 contact site is delineated with a black dashed line. (Inset) Location of K3-susceptible sites cited in the text. (C) Heatmap of PKR variants with cells colored by the K3 / K3Δ58 residuals for each variant.

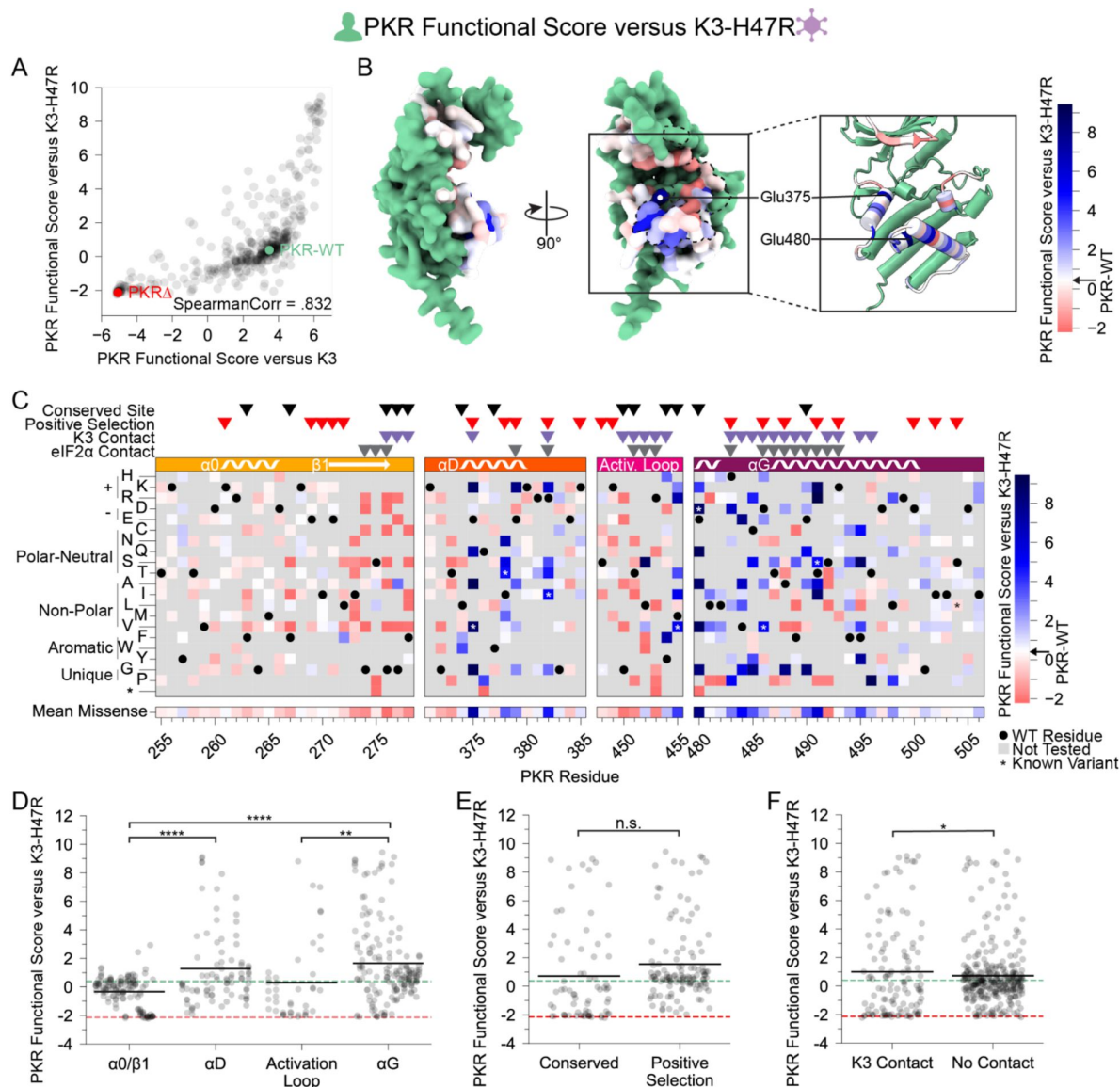


Figure 5.

PKR variants that are K3-resistant are also largely K3-H47R-resistant.

(A) Scatter plot of PKR functional scores versus wild-type K3 plotted against PKR functional scores versus K3-H47R. The data point for PKR-WT is colored green and a datapoint representing the average of the four nonsense variants (PKR Δ) is colored red. (B-C) PKR functional score versus K3-H47R ranging from susceptible (red) to WT-like (white) to resistant (blue). (B) Surface structure of the PKR kinase domain with sites colored by the mean PKR functional score versus K3-H47R for missense variants. The K3 binding surface is delineated with a black dashed line. (Inset) Location of K3-H47R resistant sites cited in the text. (C) Heatmap of PKR variants with cells colored by the PKR functional score versus K3-H47R for each variant. (D-F) Strip plots of PKR functional scores versus K3-H47R for variants, as plotted in [Figure 2D-F](#). Variants are partitioned by nearest secondary structural element (D), level of conservation in vertebrates (E), or predicted contact with K3 (F). Points in D are ordered left to right by their position along the kinase domain, whereas they are randomly jittered along the x-axis in E and F. (* $p < .05$, ** $p < .01$, **** $p < .0001$, Tukey's HSD (D) and two-sample t-test (E, F).)

Discussion

Here we characterize the local evolutionary space available to the human innate immunity protein PKR to maintain functionality while evading the well-studied vaccinia virus antagonist, K3. One strategy viruses leverage against PKR is pseudosubstrate inhibition, in which proteins like K3 bind PKR by structurally mimicking its natural substrate eIF2 α , inhibiting PKR kinase activity. Interestingly, this binding interface contains many residues under positive selection. However, positively selected genetic variants across the binding interface would need to maintain binding with PKR's natural substrate while evading viral pseudosubstrate antagonists. In this study, we quantified the abundance of such “tightrope-walking” genetic variants relative to the abundance of functionally deleterious variants, which highlighted particular sites of opportunity and constraint within the kinase domain.

We identified many sites containing variants that evade vaccinia K3 without losing eIF2 α targeting. Resistant variants clustered around helices α D and α G, secondary structures with sites under positive selection and where resistant variants have been previously characterized (13, 24). Overall, we found few genetic variants that impair the kinase function of PKR (Figure 3), underscoring the genetic resilience of PKR, with functionally constrained sites mostly localized to the vicinity of the ATP-binding site. Finally, we found a strong correlation between PKR variants that are resistant against both K3 and the enhanced antagonist K3-H47R, suggesting the K3-resistant variants are resilient to K3 allelic variation.

We identified sites across the PKR kinase domain that were K3-resistant, K3-susceptible, or essential to eIF2 α phosphorylation (Figure 6A). Sites enriched with genetic variants that improve evasion of K3 were classified as K3-resistant sites, such as Glu375 and Glu480. We find clusters of these sites located across helices α D and α G, at the binding surface of K3 and eIF2 α . Positive selection sites are enriched for K3-resistant variants. There were sites under positive selection where we did not observe much K3 resistance, such as sites 269-272, which may suggest this region is antagonized by a completely different viral antagonist. Still, we found these sites to be generally able to adopt variants that maintain PKR function (Figure 3), making them prime “tightrope-walking” sites for thwarting any alternate viral inhibitors that interact at those sites. We also identified some K3-resistant sites that are not under positive selection, such as Glu480, which is in fact conserved across vertebrate PKR homologs and the four human eIF2 α kinases (9, 13). Perhaps it has an important role in a function not tested in our assay, such as avoiding off-target phosphorylation of other human proteins.

Our analysis does not account for variation in PKR expression that may result from the introduced variation. Previous work demonstrated that K3-resistant PKR variants were not enhanced due to increased expression (13). We note that we did not observe much variation in PKR functional scores in the absence of K3 inhibition (Figure 3A), which suggests PKR variants did not often substantially modulate inherent properties of PKR, like its expression.

We found PKR variants that were not tolerated, though perhaps fewer than we would expect given the kinase domain is responsible for carrying out its critical function. These came in two flavors: variants that increased susceptibility to K3, and variants that were deleterious for PKR function. We were surprised to find sites enriched for K3-susceptible variants, such as Phe278, Arg453, and Ser492. These could be sites where the wild-type residues help discriminate between eIF2 α and K3. These sites do not cluster together; however, when projected onto the surface of PKR they appear to be intermingled with essential residues (Figure 6A). This could suggest that while they are not needed to recognize eIF2 α in normal conditions, they are well-positioned to discriminate between eIF2 α and a pseudosubstrate inhibitor. Finally, the correlation of PKR functional scores between K3 and K3-H47R suggests resistant variants are resilient. This agrees with prior work

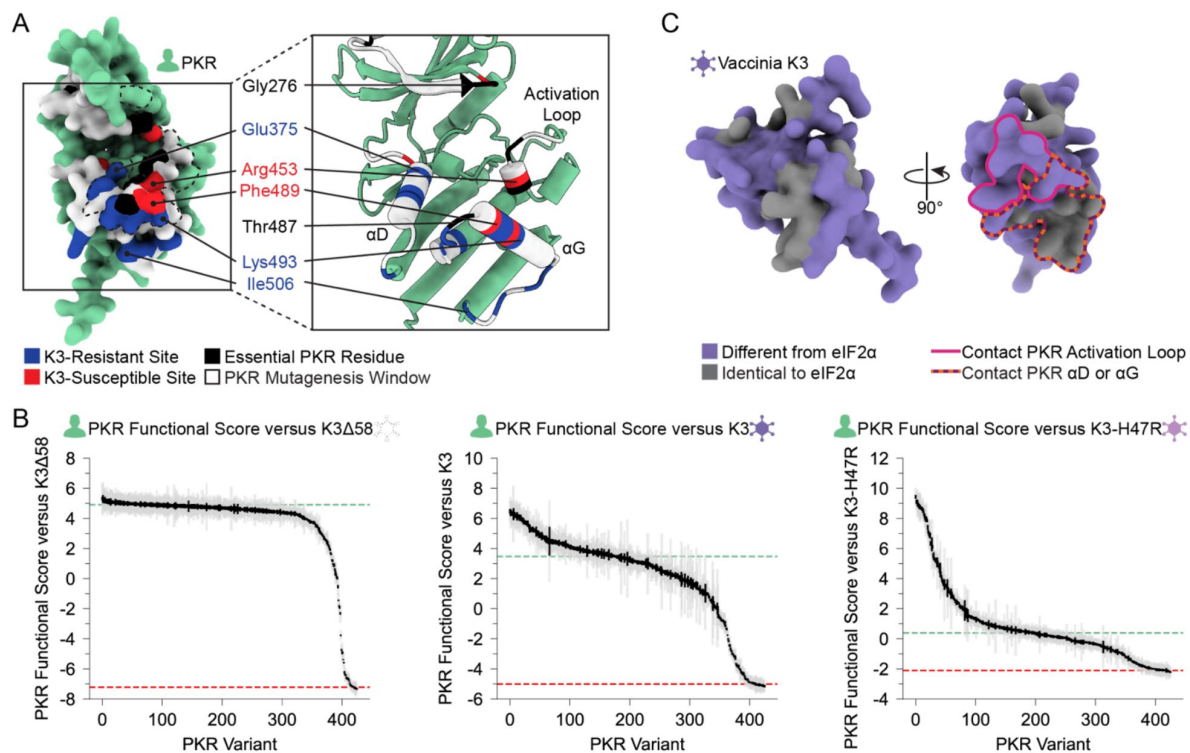


Figure 6.

Unified spatial view of highlighted PKR sites across the K3-binding interface.

Positions of sites classified as essential residues (black), K3-resistant (blue), and K3-susceptible (red) based upon the above analyses. All other tested sites are marked white. (Left) The vaccinia K3 binding surface of PKR is outlined with a black dashed line. (B) PKR functional scores versus K3 Δ 58 (Left), K3 (Center), and K3-H47R (Right), with each vertical line representing a PKR variant. Variants are sorted by functional score from high (left) to low (right). Gray and black lines indicate the standard deviation and standard error, respectively, in PKR functional scores across barcodes associated with the given variant. (C) (Left) AlphaFold2 model of Vaccinia K3 with surface residues colored as different from eIF2 α (purple) or identical to eIF2 α (gray) based on sequence alignment (**Supplemental Figure 6**). (Right) Rotated 90°, K3 residues that contact the PKR activation loop or helix α D or α G are outlined in pink and dashed orange and burgundy, respectively.

screening a few PKR variants against vaccinia K3-H47R and the K3 homolog from variola virus (13 [13](#)). We would be curious to know if this pattern holds for more highly diverged K3 orthologs (29 [29](#)) and other pseudosubstrate inhibitors, such as Ranavirus vIF2 α (30 [30](#)).

The genetic pliability observed in the PKR kinase domain is reminiscent of that seen with another innate immunity factor, TRIM5 α , which is locked in an evolutionary arms race with retroviral capsids (31 [31](#), 32 [32](#)). Both PKR and TRIM5 α have access to “rolling hills” of resistant variants (**Figure 6B** [32](#)), in opposition to sharp cliffs that are highly optimized (32 [32](#)). Notably, unlike PKR, TRIM5 α does not have to balance pathogen conflict with enzymatic function. We also note the structural context in which we identified K3-resistant sites, with many being found within secondary structures (e.g. Glu375, Il378, and Glu379 in helix α D, and Glu490, Lys493, and Thr491 in helix α G), whereas in TRIM5 α , as well as the immune proteins MxA and NLRP1 (33 [33](#), 34 [34](#)), positively selected sites and improved variants have been described in structurally flexible loops and linkers. We also considered contrasts between PKR and ACE2, a host enzyme with sites under positive selection likely due to being targeted by coronaviruses for cell entry (35 [35](#)–37 [37](#)). However, the ACE2 sites under positive selection are not located near the catalytic domain of the enzyme, in contrast to PKR (38 [38](#)–40 [40](#)). Ultimately, it appears that the resolution of this conundrum is that PKR maintains pliability in this region despite its criticality.

This pliability is likely made possible by the flexibility of eIF2 α ’s Ser51 loop in which the phosphorylation site resides; indeed, the loop is unstructured in the co-crystal of PKR and eIF2 α (5 [5](#)). As such, while we still don’t have a clear picture of what phosphorylation of eIF2 α looks like, we can appreciate the flexible nature of eIF2 α during its interaction with PKR (5 [5](#), 14 [14](#), 24 [24](#), 28 [28](#)). Further biochemical and structural investigation will be necessary to interrogate the ability of variants in PKR to disrupt interactions with K3 while maintaining eIF2 α targeting. We note that while we were able to identify many sites of interest across the PKR kinase domain, variation at many other sites in PKR remains to be explored and may harbor allosteric effects on pseudosubstrate resistance.

Barring possible variation in under-studied populations, there is little allelic diversity of PKR in the human population, with all nonsynonymous variation listed as rare in gnomAD (41 [41](#)). PKR does exhibit sequence diversity in comparisons across species, including primates (14 [14](#), 42 [42](#)).

We expect viruses could exert selective pressure on rare heterozygous variation in PKR, as we would expect beneficial variants in PKR that evade pseudosubstrate inhibitors to be dominant, given that a small amount of eIF2 α phosphorylation is capable of halting protein synthesis (43 [43](#)).

We found an enrichment for vaccinia K3-resistant variants at sites previously found to be under positive selection (13 [13](#), 14 [14](#)). In effect, these are two independent approaches that both pinpoint these sites as genetically pliable: positive selection analysis retrospectively considers existing variation in nature across multiple homologs, whereas deep mutational scanning prospectively tests novel variants in a single homolog. We and others (32 [32](#), 33 [33](#)) view deep mutational scanning experiments as a complementary approach to positive selection analysis. One strength of deep mutational scanning is its potential to illuminate sites of opportunity against pathogens that have not exerted historical evolutionary pressure. It can also allow for the testing of insertions or deletions (44 [44](#)) which are generally discarded in positive selection analysis. On the other hand, a limitation of the deep mutational scanning approach is that it only tests variant function in a limited set of conditions, when the evolutionary selection could be a symphonic signal made up of a myriad of unknown pressures. Deep mutational scanning also requires the function of the protein to be testable in a selectable assay. While proteins with cell-extrinsic or whole-organism phenotypes would be harder to test, recent advances in high-throughput genetic experimentation may allow deep mutational scans of those proteins as well (45 [45](#), 46 [46](#)).

Ideas and speculation

One might naively expect a PKR pseudosubstrate inhibitor to be a closer mimic of the natural PKR substrate, eIF2 α , which would make it more difficult for PKR to distinguish it from eIF2 α . One possibility is that K3 pseudosubstrate divergence from eIF2 α has been selected to allow the inhibitor to bind PKR with even higher affinity than eIF2 α does. But mutations that improve binding to one species' PKR homolog might decrease binding to the divergent PKR homologs from other species (47–49). Thus, pseudosubstrate inhibitors of generalist poxviruses may need to evolutionarily balance improving PKR binding for one host against maintaining PKR binding of multiple hosts. Interestingly, the structural component most diverged between K3 and eIF2 α , a rigid helix in K3 in place of eIF2 α 's flexible phospho-acceptor site, primarily interacts with the residues around the activation loop of PKR (Figure 6C), which we found to be highly functionally constrained. In contrast, amino acids 72–83 of K3 are highly identical to eIF2 α and interact primarily with PKR's divergent helix α G (Supplemental Figure 6). Thus, vaccinia K3 may well have been evolutionarily constrained to avoid limiting host range. While the host range of vaccinia is not fully understood (50), current evidence suggests vaccinia is a generalist (29, 48). Poxviruses as a whole infect a very wide range of species, which suggests the ancestral K3 needed to be compatible with a wide host range.

Despite its pliability, PKR helix α G is believed to play a critical role in eIF2 α binding and phosphorylation, as it induces a conformational shift in eIF2 α that moves the Ser51 phospho-acceptor site into PKR's ATP-binding site. Based on our findings and positive selection analyses, this conformational shift is apparently amenable to variation in the PKR kinase domain. Perhaps the resilience of the binding surface between PKR and eIF2 α could be informative for the budding field of de novo protein design, as resilient design is a critical consideration for vaccines and therapeutics faced with rapidly evolving infectious agents.

Materials and methods

PKR and K3 plasmid construction

We generated the plasmid MSp508_MCS as the base plasmid for all experiments by inserting a multiple-cloning site (MCS) into the single-copy plasmid YCp50, a gift from Mark Rose (51). The MCS contained seven unique restriction enzyme cut sites: AgeI, BsiWI, BstEII, NotI, MluI, PvuII, and SacI. YCp50 was digested using restriction enzymes EcoRI-HF (NEB Cat#R3101S) and BspDI (NEB Cat#R0557S) with rCutSmart buffer, followed by purification and size selection on a 1% agarose gel with 0.6 μ g/mL ethidium bromide (BioRad Cat#1610433), selecting for the 7,961 bp band. The extracted band was purified using a QIAquick Gel Extraction kit (Qiagen Cat#28706) and eluted with 30 μ L of water. For Gibson Assembly the purified band was combined with a single-stranded DNA oligonucleotide containing the MSC and homology arms spanning the digest site, named Oligo 1 (Supplemental File), in a 1:5 molar ratio in a total volume of 5 μ L, along with an additional 5 μ L 2x Gibson Assembly Master Mix (NEB Cat#E2611L). The reaction mixture was incubated at 50°C for 1 hour. One microliter of the Gibson Assembly reaction was transformed into 10 μ L 5-alpha competent *E. coli* (NEB Cat#C2987I) and plated onto 10-cm LB-AMP plates (IPM Scientific Cat#11006-016) and incubated overnight at 37°C. Colonies were selected for overnight outgrowth in 3 mL LB-AMP (IPM Scientific Cat#11006-004), followed by a QIAprep spin miniprep (Qiagen Cat#27106) and Sanger sequencing to validate the plasmid sequence using the primer Oligo 2. This produced the plasmid MSp508_MCS.

We cloned wild-type PKR, wild-type K3, and K3-H47R into the MCS of MSp508_MCS to produce the MSp509_PKR-WT, MSp510_K3-WT, and MSp511_K3-H47R plasmids, respectively. The plasmid p1419 (23) contains the *PKR* gene (standard name *EIF2AK2*) under the control of the galactose-inducible pGAL10/CYC1 promoter for yeast expression. We PCR-amplified the PKR expression

construct using primers (Oligos 3 and 4) to place pGAL10/CYC1-*PKR* on MSp508_MCS between AgeI and BstEII. Oligo 4 incorporated a synthetic terminator for *PKR*, Tsynth1 (52), a 28-bp spacer sequence, and a 26-bp barcode (CGCTTAATATGCAATGAAATTGCTTA); the primers added 20-bp homology arms for cloning onto the MSp508_MCS plasmid. PCR was performed using the polymerase PfuUltraII (Agilent Technologies Cat#600674) for 30 cycles with an annealing temperature of 55°C, followed by gel purification. MSp508_MCS was digested with restriction enzymes AgeI-HF (NEB Cat#R3552S) and BstEII-HF (NEB Cat#R3162S), followed by gel purification. The digested MSp508_MCS and pGAL10/CYC1-*PKR* fragments were joined via Gibson Assembly in a 1:5 molar ratio, followed by transformation into 5-alpha competent *E. coli* and plating onto LB-AMP. A resultant clone, validated by Sanger sequencing, was designated MSp509_PKR-WT.

To produce MSp510_K3-WT, the *K3L* coding sequence was amplified from the plasmid pC140 (23) using primers Oligo 5 and Oligo 6. Similarly, to produce MSp511_K3-H47R, the *K3L-H47R* coding sequence was amplified from the plasmid pC407 using primers Oligo 6 and Oligo 7. pC140 and pC407 were gifts from Thomas Dever. We note that the *K3L* alleles on these plasmids carry a Val2Leu mutation relative to the VACV-WR *K3L* (Genbank AAO89313.1), which was introduced during the cloning of *K3L* on the original plasmid pTM1 (19). Oligos 5 and 7 incorporated a synthetic terminator (T_{synth8}) for the K3 allele (52), a 28-bp nucleotide spacer sequence, a 26-bp barcode (ATCGTAATAGGTTTCCGGCTTGTTTCG and ACAGGAAATGCTTTCGGGGTTGTATT, respectively), and a 20-bp homology arm to MSp508_MCS. The pTDH3 promoter, also known as pGPD, was amplified from genomic DNA of the yeast strain BY4742 (53) using primers Oligo 8 and Oligo 9. K3-WT, K3-H47R, and pTDH3 were amplified using PfuUltraII, followed by gel purification. MSp508_MCS was digested with restriction enzymes NotI-HF (NEB Cat#R3189S) and PvuII-HF (NEB Cat#R3151S). Each K3 allele (K3-WT and K3-H47R) was joined with a pTDH3 promoter onto the digested MSp508_MCS via Gibson Assembly in a 1:5:5 molar ratio, followed by transformation into 5-alpha competent *E. coli* and plating onto LB-AMP. Resultant clones, validated by Sanger sequencing, were designated as MSp510_K3-WT and MSp511_K3-H47R.

MSp512_K3Δ58 was made by removing nucleotides 1-173 of the *K3L* sequence of YCp50_K3, leaving codons 59-88 to generate a nonfunctional *K3L* allele (denoted K3Δ58) under the pTDH3 promoter. The K3Δ58 sequence was amplified from the MSp510_K3-WT plasmid using primers Oligo 10 and Oligo 11. MSp510_K3-WT was digested with restriction enzymes NotI-HF and MluI-HF (NEB Cat#R3198S), followed by gel purification and size selection for the 8,684 bp band. The K3Δ58 allele was joined with the MSp508_MCS digested fragment via Gibson Assembly in a 1:5 molar ratio, followed by transformation into 5-alpha competent *E. coli* and plating onto LB-AMP. A resultant clone was validated by Sanger sequencing and designated as MSp512_K3Δ58.

Generation of the *PKR* variant library

We made the *PKR* variant library by 15 Gibson Assembly reactions using pairs of PCR amplicons targeting 4 windows of *PKR* protein sequence: 255-278 (5 pairs of reactions), 371-385 (3 pairs of reactions), 448-455 (2 pairs of reactions), and 480-506 (5 pairs of reactions). In each pair, one of the PCR reactions, PCR-1, utilized a pool of forward primers, termed a variant primer tile set, containing single-base deviations from the *PKR* sequence to generate all SNP-accessible variants in a stretch of X to Y consecutive codons (Supplemental Figure 9). Each forward primer in a tile set was composed of a 20-bp homology arm, the variant region, and a priming region with a melting temperature of 50°C. The reverse primer of all PCR-1 reactions had a constant 20-bp homology arm, a barcode region with 20 fully mixed bases, and a constant PCR-priming region homologous to the end of *PKR* with a melting temperature of 55°C (Supplemental Figure 10). Mixed bases in the barcodes were limited to 5-nucleotide stretches to prevent the unintended generation of restriction sites, spacing these stretches with “AA” or “TT” sequences (54). The forward PCR-1 primer tile sets are listed in the Supplemental File as Oligos 12-26, and the reverse PCR-1 barcode primer is listed as Oligo 27. The paired PCR-2 reaction was an inverse PCR that generated the remainder of the *PKR* gene and plasmid backbone, with overlaps to the homology arms of PCR-1; the forward primer for PCR-2 reactions was Oligo 28, and the reverse primers were

Oligos 29-43. Thus, Gibson Assembly of these PCR products would generate sets of *PKR* variants in defined segments of *PKR* along with random barcodes at the end of the gene (**Supplemental Figure 11** [↗](#)).

We used custom Python (v3.8.18) scripts to design each variant-introducing primer, utilizing IUPAC degenerate base symbols (e.g. “D” represents a mix of “A”, “G”, and “T”) ([55](#) [↗](#), [56](#) [↗](#)). The introduced variant amino acids were all within a single base change from the canonical human *PKR* coding sequence (Genbank M85294.1:31-1686); we note the *PKR* sequence encoded on MSp509_PKR-WT differs somewhat from the canonical sequence through synonymous substitutions. Primers were synthesized by Integrated DNA Technologies, then were pooled manually into variant primer tiles sets.

PCR-1 reactions were amplified from the MSp509_PKR-WT plasmid using PfuUltraII polymerase with an annealing temperature of 45°C. Each reaction generated a pool of *PKR* gene fragments containing a single nonsynonymous variant paired with a unique barcode sequence. In parallel, PCR-2 reactions amplified vector fragments from MSp509_PKR-WT plasmid using Herculase II polymerase (Agilent Technologies Cat#600677) using cycling conditions for vector targets >10 kb. PCR-1 and PCR-2 amplicons were digested with DpnI (NEB Cat#R0176S) for 2 hours at 37°C to remove the MSp509_PKR-WT template DNA, followed by gel purification. Each PCR-1 amplicon was paired with its corresponding PCR-2 amplicon for Gibson Assembly in a 1:5 molar ratio.

Two μL of each Gibson Assembly was transformed into 50 μL of 5-alpha competent *E. coli* and all cells were plated on LB-AMP and incubated overnight at 37°C. Colonies were counted on each plate and plates were bottlenecked at approximately 30 colonies per selected nonsynonymous variant (i.e. each nonsynonymous variant would be linked to approximately 30 unique barcode sequences). Plates were washed with 15 mL LB-AMP, cell densities were measured as the optical density at 600 nm (OD_{600}), and an equal number of cells (approximately 5×10^9 cells) from each reaction were pooled to form a single *PKR* variant library. A 200 mL LB-AMP outgrowth was grown to an OD_{600} measurement of approximately 3, followed by a Qiagen MAXI plasmid prep (Qiagen Cat#12963).

PacBio sequencing of barcoded variant libraries

The *PKR* variant library was sequenced using the PacBio Sequel II instrument to identify the barcodes that were linked to each nonsynonymous variant. Sixteen micrograms of the *PKR* variant library were digested with restriction enzymes AgeI-HF and NotI-HF to create linear fragments of approximately 2,100 bp for sequencing. The digest was incubated for 2 hours at 37°C then purified using a QiaQuick PCR purification kit (Qiagen Cat#28106). Fragments of 2,100 bp were size-selected using a SageELF instrument (Sage Science) before PacBio circular consensus sequencing (CCS) HiFi sequencing on a Sequel II instrument (Pacific Biosciences). From the CCS reads we generated a table of *PKR* barcodes paired with genetic variants using alignparse v0.2.6 and custom Python scripts ([57](#) [↗](#)).

Combining *PKR* variant library with K3 alleles

We next cloned the three K3 alleles (K3, K3-H47R, and K3 Δ 58) onto the *PKR* variant library plasmids. The *PKR* variant library was digested with NotI-HF and PvuII-HF to generate a 10,270 bp receiver DNA fragment, while the K3 alleles were separately digested with BstEII-HF and SacI-HF (NEB Cat#R3156S) to generate a 1,130 bp insert DNA fragment with 20-bp homology arms with homology to the receiver fragment for Gibson Assembly. For each of the reactions, 10 μg of plasmid DNA was digested with 5 μL of each restriction enzyme in a 50 μL reaction to generate a linear fragment, followed by gel purification. The *PKR* and K3 fragments were combined in three separate Gibson Assembly reactions in a 1:5 molar ratio with 100 ng of the *PKR* vector fragment in

a reaction volume of 20 μ L and incubated at 50°C for 1 hour. A standard ethanol precipitation was performed on each reaction, adding 100 μ L of 100% ETOH and incubating overnight at -20°C before resuspending the pellets in 2 μ L of water (58 [↗](#)).

We transformed each of the three concentrated reactions into *E. coli* 10G SUPREME electrocompetent cells (Lucigen Cat#60080-2) using a MicroPulser Electroporator (BioRad), with 2 μ L of the concentrated reaction combined with 25 μ L of competent cells. Following the electroporation and recovery, cells were plated onto two 15-cm LB-AMP plates. A 1:1000 dilution was plated with 100 μ L of water on a 10-cm plate to estimate the number of transformed colonies across the two 15-cm plates, which produced an estimate of approximately 2 million colonies per electroporation. Plates were washed with 25 mL LB-AMP, combining the two plates for each of the reactions. Each library was expanded in 200 mL LB-AMP to an OD₆₀₀ measurement of approximately 3, followed by a Qiagen MAXI plasmid prep.

Screening the PKR-K3 variant pairs in a yeast growth assay

All yeast growth was at 30°C in CSM-Ura (Sunrise Science Products Cat#1004-010), and liquid cultures were shaken at 200 revolutions per minute. We transformed each of the three paired libraries (PKR + K3, PKR + K3Δ58, and PKR + K3-H47R) into yeast in duplicate. The paired plasmid libraries were transformed into the yeast strain BY4742 (*MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0*; strain name MSY2) (53 [↗](#)) using a standard large-scale high efficiency lithium acetate transformation protocol (59 [↗](#)) and plated onto 15-cm plates with 2% dextrose, using 1:1,000 and 1:10,000 dilutions on 10-cm plates to estimate colony counts, which produced approximately 100,000 colonies. Colonies were washed off plates with 25 mL media with 2% dextrose.

To start the yeast growth assay, six cultures were seeded at low density (OD₆₀₀ measurement of 0.01) in 40 mL media with 2% dextrose and grown overnight for 16 hours. The following morning cultures were moved from 30°C to 4°C to pause growth, then restarted at an OD₆₀₀ of 0.25 in 40 mL media with 2% dextrose 3 hours prior to GAL induction. After 3 hours, once all cultures were in a log growth phase, approximately 1×10^8 cells were taken from each culture as the starting timepoint sample (designated as “0 hours”). All timepoint samples were spun down in a 1.5 mL Eppendorf tube at 5,000 rpm for 1 minute, after which supernatant was removed and the pellet was frozen at -80°C. Approximately 2×10^8 cells from the remaining cultures were pelleted in 50 mL conical tubes at 3,000 rpm, supernatant was removed, and cells were resuspended in 80 mL CSM-Ura media with 2% galactose (OD₆₀₀ measurement of 0.125 OD₆₀₀) to induce PKR expression. Cultures were incubated overnight at 30°C, 200 rpm.

Additional samples were harvested at 12, 16, and 20 hours post-PKR induction, with approximately 1×10^8 cells taken at each timepoint. The culture density was monitored and back diluted to maintain a log growth phase (OD₆₀₀ measurement less than 1). With three K3 allele conditions (K3, K3Δ58, and K3-H47R), four timepoints (0, 12, 16, and 20 hours) and two replicates, a total of 24 samples were taken across the yeast growth assay.

Plasmid extraction and barcode amplification

To quantify changes in barcode abundance between timepoints in the yeast growth assay, we harvested plasmids from the four timepoints for each K3 allele, then amplified and sequenced the adjacent PKR and K3 barcodes in each plasmid. Plasmids were harvested from the yeast samples using a modified QIAprep spin miniprep protocol (60 [↗](#)). Sampled cell pellets were first thawed at room temperature, followed by the addition of 250 μ L QIAprep P1 buffer and 2 μ L zymolyase (2.5 units per μ L), and incubation at 37°C for 30 minutes, followed by adding 250 μ L P2 buffer and following the manufacturer instructions for the remainder of the protocol.

To identify and quantify the abundance of each PKR variant paired with each of the three K3 alleles we performed Illumina sequencing of the PKR barcodes from the plasmids. We used a two-step PCR protocol, the first to amplify the PKR barcode from the plasmids and the second to attach Illumina adapters and Nextera indices for downstream sequencing and sample demultiplexing. For the first PCR reaction, we designed forward and reverse primers as a pool of five primers each, that had 0 to 4 “N” bases between the Nextera transposase adapter sequence and the priming sequence to stagger the base signal per cycle and maintain sequence diversity across the flow cell (61 [↗](#)). The forward primers (Oligos 44-48) with a melting temperature of 60°C were pooled together and used for all PCR-1 reactions, annealing immediately upstream of the PKR barcode locus. Five reverse primers were designed and pooled for amplification from each of the three K3 alleles that annealed within the three distinct K3 barcode sequences (Oligos 49-53 for K3, Oligos 54-58 for K3Δ58, and Oligos 59-63 for K3-H47R). One microliter of each primer pool was combined with 50 ng of sample plasmid DNA and 25 μL Kapa HiFi Hotstart ReadyMix (Kapa Biosystems Cat# KK2602), and topped off to a total volume of 50 μL with water. PCR cycling was performed as follows: (1 [↗](#)) 95°C for 3 minutes, (2 [↗](#)) 98°C for 20 seconds, (3 [↗](#)) 65°C for 15 seconds, (4 [↗](#)) 72°C for 1 minute, repeat steps 2-4 for a total of 18 cycles, (5 [↗](#)) 72°C for 1 minute, (6 [↗](#)) 12°C hold. PCR products were purified using a MinElute PCR purification kit (Qiagen Cat#28006) using 11 μL water for the final elution, then quantified using a Qubit dsDNA High Sensitivity (HS) kit (ThermoFisher Scientific Cat#Q32851).

For the second PCR reaction, 10 ng of amplicon DNA from the first PCR reaction was combined with 10 μL of Nextera adapter index primers (Illumina Cat#20027213) and 25 μL Kapa HiFi Hotstart ReadyMix, and topped off to a total volume of 50 μL with water. PCR cycling was performed as follows: (1 [↗](#)) 100°C for 45 seconds, (2 [↗](#)) 100°C for 15 seconds, (3 [↗](#)) 60°C for 30 seconds, (4 [↗](#)) 72°C for 30 seconds, repeat steps 2-4 for a total of 8 cycles, (5 [↗](#)) 72°C for 1 minute, (6 [↗](#)) 12°C hold. PCR products were purified using a MinElute PCR purification kit using 11 μL of water for the final elution, then quantified using a Qubit dsDNA Broad Range (BR) kit (ThermoFisher Scientific Cat#Q32850). 500 ng of the second PCR reaction amplicons were gel purified on a Size Select II E-Gel (ThermoFisher Scientific Cat#G661012) for approximately 13 minutes to extract the approximately 250-bp amplicon band, followed by quantification with a Qubit dsDNA HS kit. Amplicon samples were diluted to 4 nM before being pooled together, followed by manufacture denature and dilution protocols (Illumina Document#15039740v10) before sequencing on an Illumina NextSeq 2000 instrument.

PKR functional scores and screening analysis

Next, we extracted PKR barcode sequences from the Illumina reads and mapped the barcodes back to their corresponding select nonsynonymous variants using the table of PKR barcodes paired with genetic variants generated from the PacBio CCS HiFi reads. Paired reads were assembled into contiguous sequences using PEAR v0.9.11 (62 [↗](#)), followed by Bartender v1.1 (63 [↗](#)) to extract and cluster PKR barcodes using the barcode search pattern "CAAGG[25-27]GGTGA".

We wrote Python v3.8.18 scripts to tally the PKR barcodes and map them back to genetic variants using the table generated from the PacBio CCS HiFi reads. Barcode counts were normalized to the total read count for each of the four timepoints across each of the K3 alleles, followed by a fold change calculation for each PKR barcode across timepoints 0, 12, 16, and 20 hours:

$$FC_{TP} = -\log_2 \left(\frac{\text{Normalized Reads}_{TP}}{\text{Normalized Reads}_0} \right)$$

where FC_{TP} is the fold change in normalized reads for a PKR barcode from 0 hours to a subsequent timepoint (TP), $\text{Normalized Reads}_{TP}$ is the read count for a given barcode divided by the total number of reads at the given timepoint, and $\text{Normalized Reads}_0$ is the read count for a given barcode divided by the total number of reads at the 0-hour timepoint. Of note, the $\log_2$ of the

fold change is inverted such that functional PKR variants that inhibit yeast growth and decreased in abundance were assigned positive fold change value. We then calculated PKR functional scores for each PKR barcode in each of the K3 alleles by calculating the area under the curve using the composite trapezoidal rule. We calculated the PKR functional score for each variant for each K3 allele by averaging the PKR functional score across all representative barcodes for a given variant. As we found a strong correlation between replicates (Pearson correlation coefficient > 0.98 for each K3 allele, **Supplemental Figure 3**), we proceeded by combining all reads from the two replicate experiments for each K3 allele and recalculating the fold changes and PKR functional scores for each barcode as described above.

Predicted PKR complexes and substrate contacts

All molecular graphics and analyses were performed using UCSF ChimeraX v1.5 and PyMol v2.5.4. To define PKR residues contacting K3 and eIF2 α we used the AlphaFold2 structure prediction tool (ColabFold v1.5.5) in UCSF ChimeraX v1.5 (64,65) (**Supplemental Figure 12A**). We aligned these predictions to existing crystal structures (PDB 2A1A and 1LUZ) and found the AlphaFold2 predictions to largely represent the crystal structures (RMSD < 1 angstrom for both alignments, **Supplemental Figure 12B-D**) (5,22). We note the low confidence score (pLDDT < 50) of the eIF2 α flexible loop containing Ser51, which adopts a compact helical conformation in the predicted model and closely matches the conformation observed in the crystal structure of unbound yeast eIF2 α (PDB 1Q46) (66). As Ser51 of eIF2 α would need to travel approximately 17 Å to reach PKR's active site (5), this predicted structure is likely reflective of a pre-phosphorylation complex, and thus would not capture contacts between PKR and eIF2 α made during phosphorylation. The corresponding loop is unresolved in the crystal structure of human PKR in complex with eIF2 α (PDB 2A1A). Note that we opted not to use the existing structure for defining contact residues, both for consistency with the K3 contact definitions, as there is no PKR-K3 co-crystal, as well as for the potential to capture interactions mediated by residues unresolved in the crystal structure (PDB 2A1A, PKR Asp338-Asn350 and eIF2 α Glu49-Lys60).

To identify potential PKR contact sites from the predicted complex structures, we used PyMol v2.5.4 to select all PKR residue branches within 5 Å of K3 or eIF2 α using the command: “select contacts, br. /{pdb file name}//A within 5 of /{pdb file name}//B” with chain “A” being PKR and chain “B” being either K3 or eIF2 α . Predicted PKR sites that contact K3 were: 275, 276, 278, 304, 339, 343, 345, 375, 379, 382, 414, 416, 435, 448, 449, 450, 451, 452, 453, 455, 460, 485, 486, 487, 488, 489, 490, 492, 493, and 496. Predicted PKR sites that contact eIF2 α were: 274, 275, 276, 279, 335, 337, 338, 339, 340, 341, 342, 379, 382, 451, 452, 453, 483, 486, 487, 488, 489, 490, 491, 492, and 493.

PKR sites in the kinase domain under positive selection across vertebrate species were identified by Rothenburg et al. (13): 261, 269-272, 307, 314, 322, 360, 368, 375, 378, 379, 382, 385, 389, 394, 405, 428, 448, 449, 462, 471, 483, 486, 488, 491, 493, 500, 502, 504, 505, 514, 520, and 524. Sites conserved across vertebrate PKR homologs were also identified by Rothenburg et al. (13) which were used for supplementary analysis: 263, 267, 276-279, 281, 283, 296, 298, 308, 309, 312, 315, 317, 319, 320, 323, 327, 362, 364-367, 369, 374, 377, 397, 401, 404, 406, 407, 410-417, 419, 420, 429-433, 437, 444-446, 450, 451, 454, 455, 457-459, 465, 469, 470, 474-477, 480, 490, 511, 519, 523, and 526.

Data access

Supplemental data and code are available under the DOI: 10.5281/zenodo.13788100

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Author contributions

Designed research/conceptualization: M.J.C. and M.J.S. designed research;

Performed research: M.J.C. and S.B.S. performed research;

Analyzed data: M.J.C., S.B.S., and M.J.S. analyzed data;

Wrote paper: M.J.C. and M.J.S. wrote the paper.

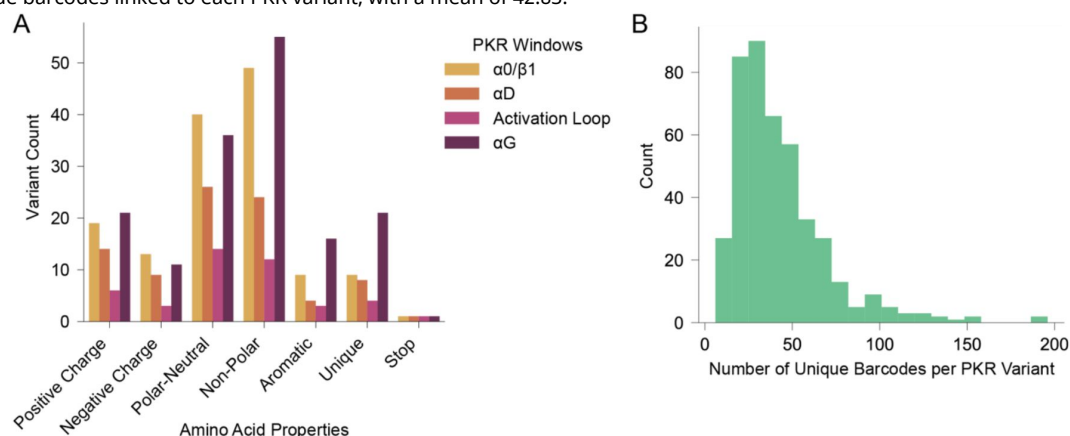
All authors read and revised the paper.

Supplemental figures

Supplemental Figure 1.

Composition of the PKR variant library.

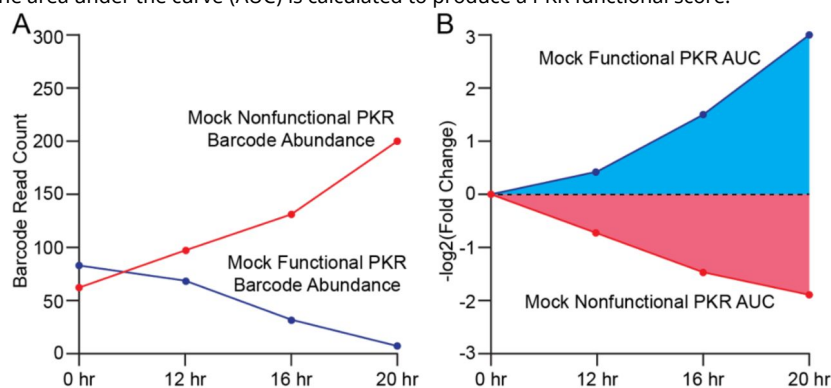
(A) Barchart depicts the number of variants made at each of the four PKR windows categorized by amino acid properties: Positive Charge = H, K, R; Negative Charge = D, E; Polar-Neutral = C, N, Q, S, T; Non-Polar = A, I, L, M, V; Aromatic = F, W, Y; Unique = G, P; Stop = *. A total of 140 variants were made in $\alpha 0/\beta 1$, 86 in αD , 43 in the Activation Loop, and 161 in αG for a total of 430 variants, which includes the 4 nonsense variants made in each window. (B) Histogram depicts the number of unique barcodes linked to each PKR variant, with a mean of 42.83.

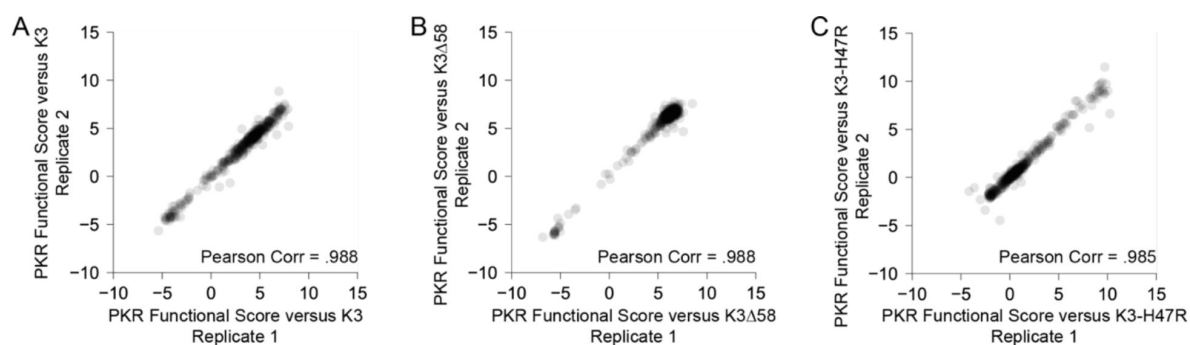


Supplemental Figure 2.

Calculation of PKR functional scores from yeast growth assay.

(A) Line plot of mock barcode read count data over time for cells expressing nonfunctional (red) or functional (blue) PKR across four sampled timepoints. As PKR activity is toxic to yeast, the number of cells in the pool expressing the functional PKR will decrease over time, and thus the associated barcode read count will also decrease, while the read count for the nonfunctional PKR will increase. (B) Line plot depicting the fold changes from timepoint 0 of barcode abundance derived from Panel A, with a $-\log_2$ transformation to assign positive values to functional PKR and negative values to nonfunctional PKR, from which the area under the curve (AUC) is calculated to produce a PKR functional score.

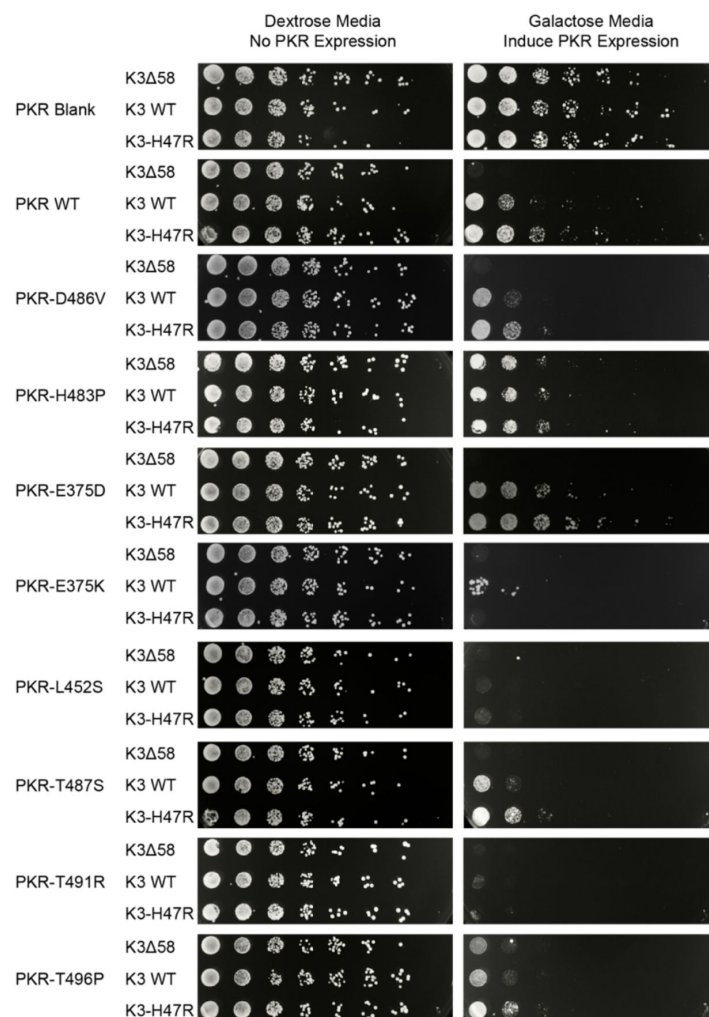




Supplemental Figure 3.

Replication of PKR variants paired with K3 alleles.

(A) Scatter plot of PKR functional scores for variants paired with K3-WT for two biological replicate experiments. Functional scores were calculated across four timepoints as the area under the curve (see Materials and Methods). Thus, variants with increased evasion of K3 would have higher PKR functional scores, while those with increased susceptibility to K3 or loss of eIF2 α kinase activity would have lower scores.



Supplemental Figure 4.

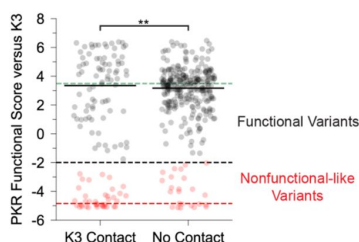
Experimental validation of the K3 resistance phenotypes of PKR variants.

Select PKR variants were generated and screened against K3Δ58, WT, and H47R alleles using a yeast growth assay visualized through serial dilution (23). PKR is under the inducible pGAL10/CYC1 hybrid promoter, and thus is repressed in dextrose media (left) and expressed in galactose media (right). We selected one variant that exhibited loss of function in the high-throughput screen (H483P, **Figure 3C**), one variant whose phenotype was similar to PKR WT (E375D, **Figures 2A**, 3C, and 5C), and five variants whose K3 resistance was improved (E375K, L452S, T487S, T491R, and T496P, **Figures 2A** and 5C).

Supplemental Figure 5.

Bimodal distribution of PKR functional scores at K3 contact sites.

Strip plot of PKR functional scores versus K3 partitioned by predicted contact with K3. Black dashed line denotes the threshold at which variants were separated as nonfunctional-like or functional. Green and red dashed lines represent mean scores for WT PKR and nonsense variants. (** $p < 0.01$, two-sample t-test, comparing just the variants classified as functional in the two classes.)



Supplemental Figure 6.

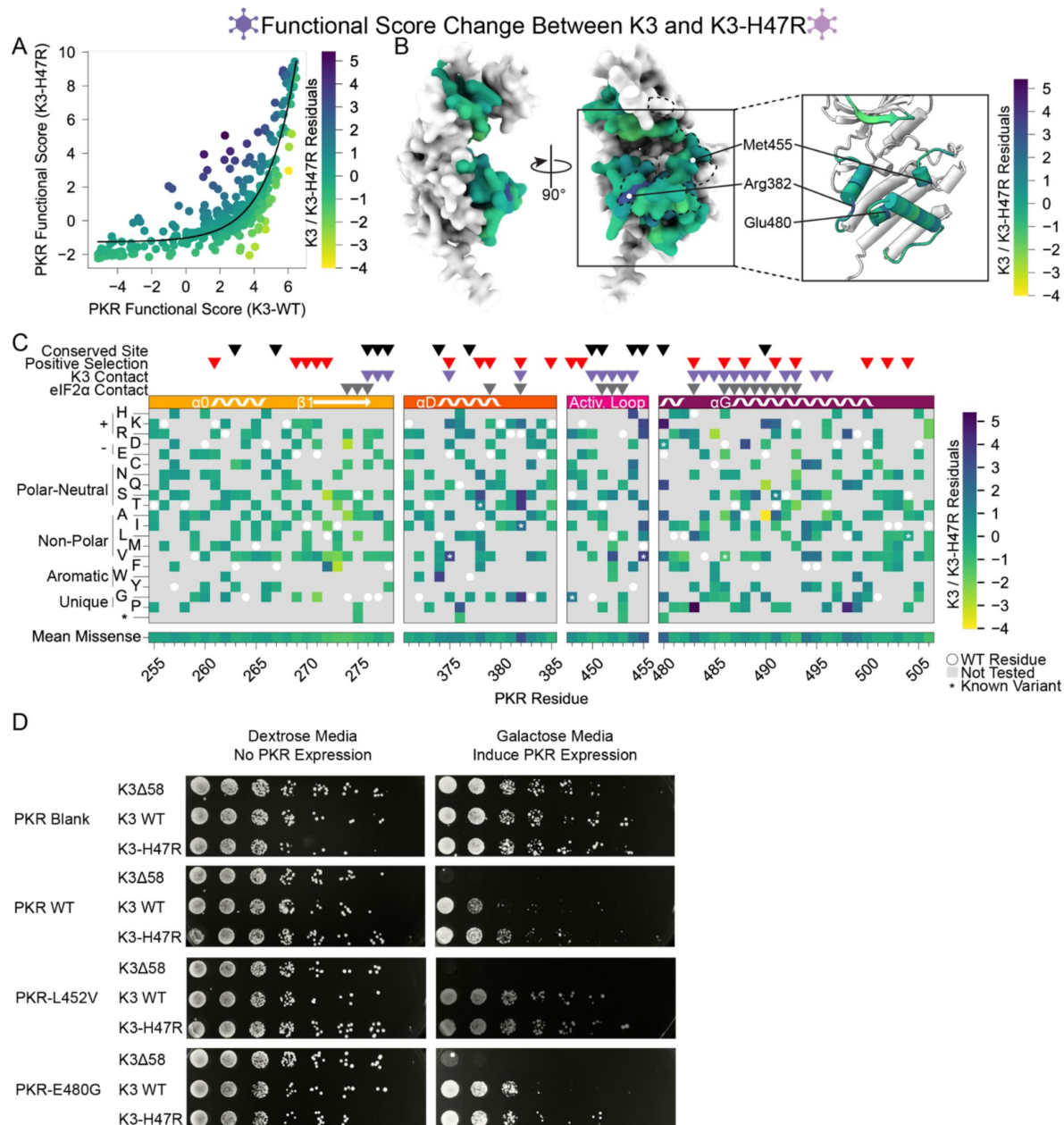
Sequence similarity between human eIF2 α and vaccinia K3.

Alignment of human eIF2 α (RefSeq Accession NP_004085.1, residues 0-117) to vaccinia K3 (RefSeq Accession YP_232916.1). The Ser51 site of phosphorylation in eIF2 α is indicated by #. Sequences were aligned using Muscle 3.8 and displayed in Clustal format, * = fully conserved, : = strong group conservation, . = weak group conservation. Sites 41-58 of K3 correspond to the rigid helix insert, which is proximal to PKR's ATP-binding site in the AlphaFold2-predicted complex, while sites 72-83 are proximal to PKR's helix α G.

```

#
0      10      20      30      40      50
Human eIF2α  MPGLSCRFYQHKFPEVEDVVMNVRSIAEMGAYVSLLEYNNIEGMILLSER--RRIRS
              1      10      20      30      40      50
Vaccinia K3  -----MLAFCYSLPNAGDVIKGRVYE--KDYALYIYLFDPYHPFEAILAESVKMHMDRYVEY
              : :.:. :. **: . * . : . * : :.: * : * :
              60      70      80      90      100     110
Human eIF2α  INKLIRIGRNECVVIRVDKEKGYIDLSKRRVSPEEAIKCEDKFTKSKTVYSILRHVAEV
              55      60      70      80
Vaccinia K3  RDKL--VGKTVKVKVIRVDYTKGYIDVNYKRMCRHQ-----
              : **: : * . : * * * * * : * : :

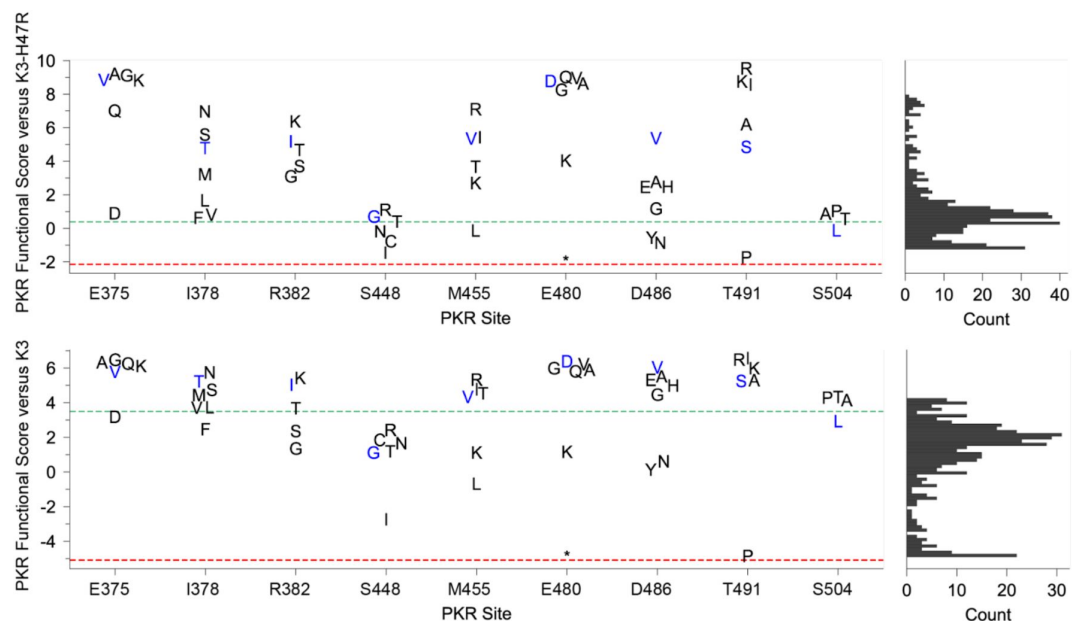
```



Supplemental Figure 7.

Differing patterns of resistance between wild-type K3 and K3-H47R.

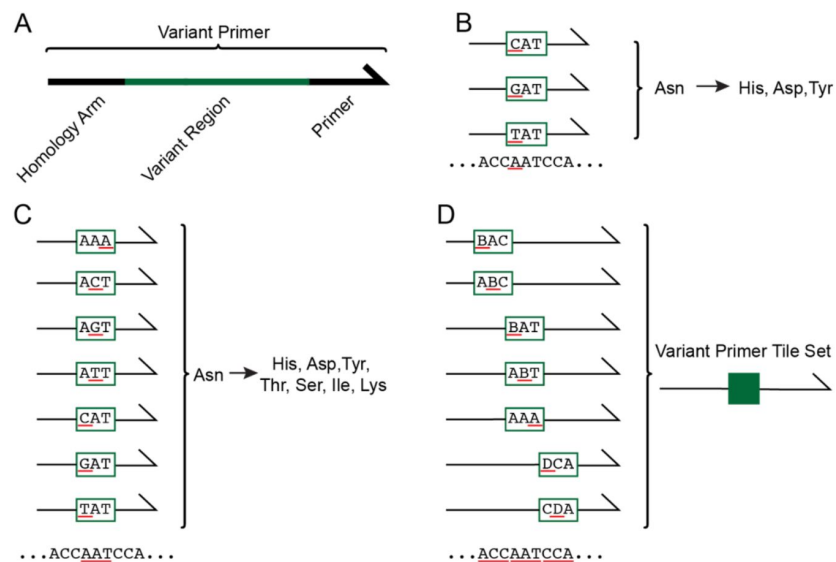
(A) A nonlinear exponential curve (black line) was fitted to the data in the scatter plot from [Figure 5A](#). Points are colored by their residuals from that curve (K3 / K3-H47R Residuals), ranging from enhanced (purple) to decreased (yellow) resistance to K3-H47R relative to the expectation from K3 resistance. This color scheme is used in panels B and C. (B) Surface structure of PKR kinase domain with sites colored by the mean K3 / K3-H47R residuals for missense variants. The K3 contact site is delineated with a black dashed line. (Inset) Location of K3-H47R-resistant sites where variants deviated most strongly between the wild-type K3 and K3-H47R conditions. (C) Heatmap of PKR variants with cells colored by the K3 / K3-H47R residuals for each variant. (D) Two PKR variants that did not follow the general trend of enhanced susceptibility to K3-H47R relative to K3 WT were selected for validation. The variants were generated and screened against K3Δ58, WT, and H47R alleles using a yeast growth assay visualized through serial dilution as in Supplemental Figure 4.



Supplemental Figure 8.

Additional variants at the sites of previously identified K3-H47R- resistant variants often also conferred resistance to K3.

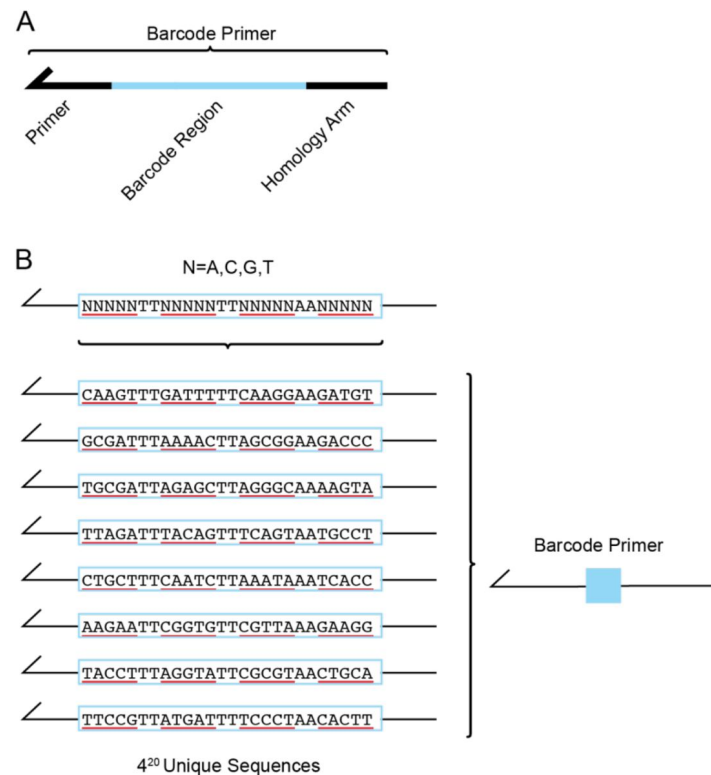
Strip plots of PKR functional scores of variants paired with K3-H47R (top) and K3-WT (bottom). Each plot highlights PKR variants made at sites where an improved PKR variant was previously identified (24). Blue markers denote previously identified variants; black markers are additional variants made at the same site. Green and red dashed lines represent mean scores for WT PKR and nonsense variants, respectively. Histograms (right) show PKR functional scores versus K3-H47R (top) and K3 (bottom) for all variants. * = stop codon.



Supplemental Figure 9.

Systematic generation of PKR variants using mixed-base primer tile sets.

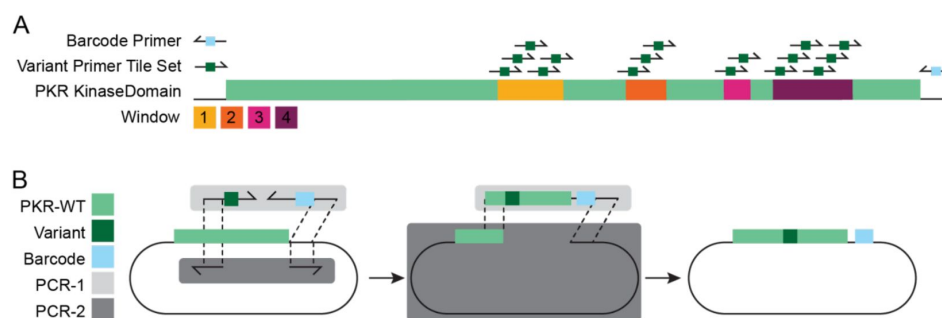
(A) Each variant primer is composed of a homology arm, variant region, and priming region. (B) Nonsynonymous SNP-accessible variants are generated by altering the codon in the variant region of the primer. This example depicts the codon "AAT" encoding Asn. The first nucleotide in the codon, "A", is underlined in red, with three codons above having changes to "C", "G", and "T" underlined in red, which generate the nonsynonymous variants His, Asp, and Tyr. (C) Variant primers were designed across all three nucleotides in each codon, as underlined in red. (D) Variant primer tile sets, represented in dark green, were made by pooling variant primers that modify adjacent codons. Primers included in a given variant primer tile sets have differing variant regions but share homology arms and priming region sequences.



Supplemental Figure 10.

Multiple unique barcode sequences were attached to each PKR variant.

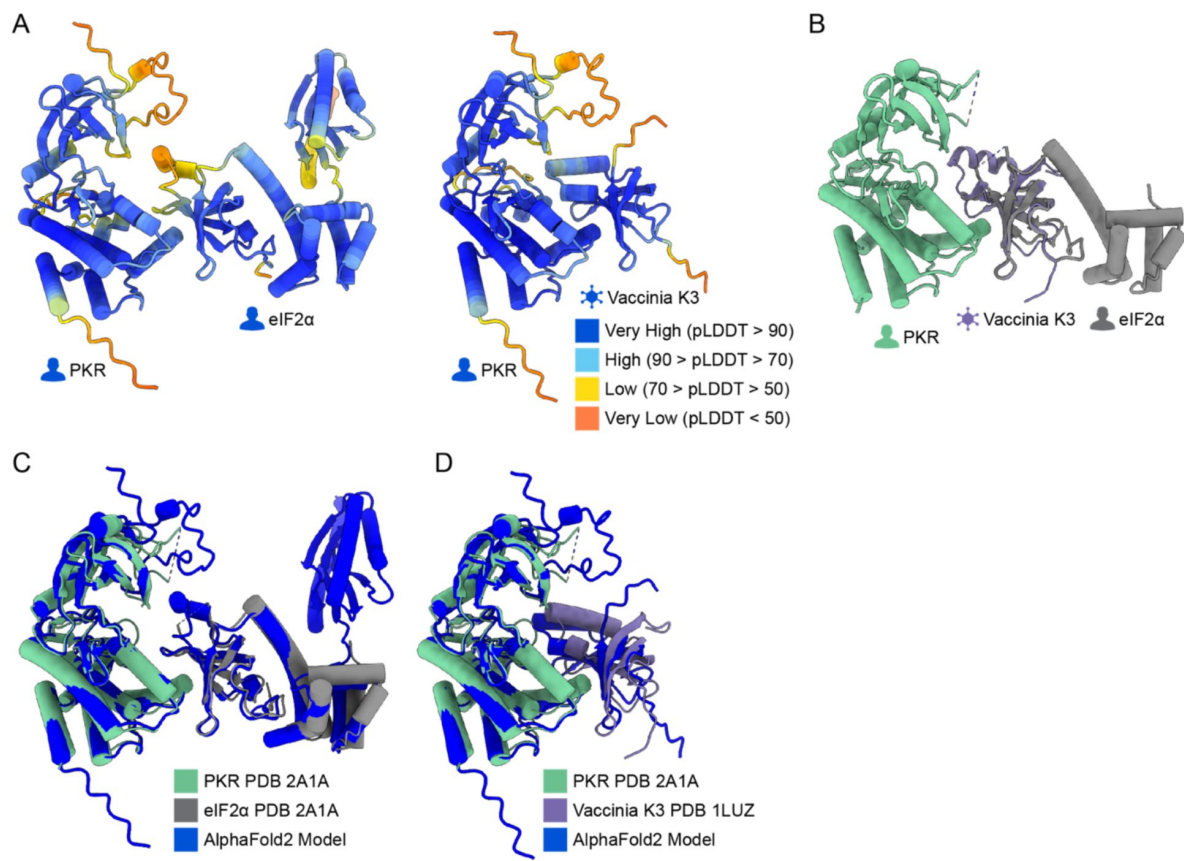
(A) The barcode primer is composed of a homology arm, barcode region, and priming region. The barcode primer is used as the reverse primer in the variant-generating PCR reactions to attach a unique nucleotide sequence after the PKR variant sequence. (B) The barcode region of the primer is composed of 20 "N", representing an equal mix of the nucleotides "A", "C", "G", and "T". A barcode primer with 20 random nucleotides can take on 4^{20} unique nucleotide sequences. Dinucleotide sequences "TT" and "AA" are interspersed throughout the barcode region to avoid making unintended restriction enzyme cut sites.



Supplemental Figure 11.

Assembly of PKR variant library using variant tile sets and barcode primers.

(A) 15 variant primer tile sets were designed to generate variants across four windows of interest in PKR. The full-length PKR sequence is denoted in green, with Windows 1- 4 overlaid in yellow, orange, magenta, and burgundy, respectively. Variant primer tile sets were used to generate 426 PKR missense variants, for a total of 15 PCR-1 reactions. (B) Two separate PCR reactions were used to generate complementary insert and vector fragments. PCR-1 primers (light gray box) include a single variant primer tile set (dark green, see Supplemental Figure 9) and a single doped barcode primer (light blue, see Supplemental Figure 10) that amplified from WT PKR (green) and made the PCR-1 insert fragment containing a select nonsynonymous variants (dark green) and a unique barcodes (blue). PCR-2 primers included a single forward and reverse primer that amplified from WT PKR and made a larger vector fragment with 20-bp homology arms that complement the homology arms of the PCR-1 insert fragment. The two fragments were combined via Gibson Assembly to form a pool of complete vectors, each vector containing a single, nonsynonymous variant with a unique barcode.



Supplemental Figure 12.

AlphaFold2 Multimer predictions used to identify PKR sites proximal to eIF2α and K3.

(A) AlphaFold2 Multimer prediction of PKR in complex with eIF2α (Left) and K3 (Right). Residues are colored by AlphaFold2 pLDDT confidence scores per residue. In both predicted models, PKR is on the left and its binding partner is on the right, as in **Figure 1A,B**. (B) Crystal structure of PKR (green, PDB 2A1A) in complex with eIF2α (gray, PDB 2A1A) with the crystal structure of vaccinia K3 (purple, PDB 1LUZ) aligned to eIF2α (root-mean-square deviation (RMSD) = 0.819 Å). (C) Overlay of the PKR-eIF2α from PDB 2A1A (green and gray) and AlphaFold2 (blue), RMSD = 0.970 Å. (D) Overlay of the PKR-Vaccinia K3 complex from PDB 2A1A and 1LUZ (green and purple) to AlphaFold2 (blue), RMSD = 0.944 Å.

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Reviewer #1 (Public review):

Summary:

The report examines the control of the antiviral RNA-activated protein kinase, PKR, by the Vaccinia virus K3 protein. K3 binds to PKR, hindering its ability to control protein translation by blocking its phosphorylation of the eukaryotic initiation factor EIF2 α . Kinase function is probed by saturation mutation of the K3/EIF2 α -binding surface on PKR, guided by models of their interaction. The findings identify specific residues at the predicted interface that asymmetrically influence repression by K3 and the phosphorylation of EIF2 α . This recognises the potential of PKR alleles to resist control by the viral virulence factor.

Strengths:

The experimentation is diligent, generating and screening many point mutants to identify residues at the interface between PKR and EIF2 α or K3 that distinguishes PKR's phosphor control of its substrate from the antithetical interaction with the viral virulence factor.

Weaknesses:

The protein interaction between PKR and K3 has already been well-explored through phylogenetic and functional analyses and molecular dynamics studies, as well as with more limited site-directed mutational studies using the same experimental assays. Accordingly, the findings are not pioneering but reinforce and extend what had previously been established.

The authors responded to this comment by pointing out that their more comprehensive screen better defined the extent of the plasticity of the K3/EIF2 α -binding surface on PKR.

Also in their response, the authors added the caveat that the equivalent expression of the different PKR mutants has not been verified, added information clarifying the states of the

model proteins compared to their determined molecular structures, and provided clarifications or responses to all other questions.

I question eLife's assessment that the development of the yeast-based assay is a key advancement of this report, as this assay has been used for over 30 years.

<https://doi.org/10.7554/eLife.99575.2.sa2>

Reviewer #2 (Public review):

Chambers et al. (2024) present a systematic and unbiased approach to explore the evolutionary potential of the kinase domain of the human antiviral protein kinase R (PKR) to evade inhibition by a poxviral antagonist while maintaining one of its essential functions.

The authors generated a library of 426 single-nucleotide polymorphism (SNP)-accessible non-synonymous variants of PKR kinase domain and used a yeast-based heterologous virus-host system to assess PKR variants' ability to escape antagonism by the vaccinia virus pseudo-substrate inhibitor K3. The study identified determinant sites in the PKR kinase domain that harbor K3-resistant variants, as well as sites where variation leads to PKR loss of function. The authors found that multiple K3-resistant variants are readily available throughout the domain interface and are enriched at sites under positive selection. They further found some evidence of PKR resilience to viral antagonist diversification. These findings highlight the remarkable adaptability of PKR in response to viral antagonism by mimicry.

Significance of the findings: The findings are important with implications to various fields, including evolutionary biology, virus-host interfaces, genetic conflicts, antiviral immunity.

Strength of the evidence: Convincing methodology using state-of-the-art mutational scanning approach in an elegant and simple setup to address important challenges in virus-host molecular conflicts and protein adaptations.

Strengths

Systematic and Unbiased Approach: The study's comprehensive approach to generating and characterizing a large library of PKR variants provides valuable insights into the evolutionary landscape of PKR kinase domain. By focusing on SNP-accessible variants, the authors ensure the relevance of their findings to naturally occurring mutations.

Identification of Key Sites: The identification of specific sites in the PKR kinase domain that confer resistance or susceptibility to a poxvirus pseudosubstrate inhibition is a significant contribution.

Evolutionary Implications: The authors performed meticulous comparative analyses throughout the study between the functional variants from their mutagenesis screen ("prospective") and the evolutionarily-relevant past adaptations ("retrospective").

Experimental Design: The use of a yeast-based assay to simultaneously assess PKR capacity to induce cell growth arrest and susceptibility/resistance to various VACV K3 alleles is an efficient approach. The combination of this assay with high-throughput sequencing allows for the rapid characterization of a large number of PKR variants.

Areas of improvement

Validation of the screen: In the revised version, the authors now provide the results of two independent experiments in a complete yeast growth assay on a handful of candidates to control the screen's results. This strengthens the direct findings from the screen. It would strengthen the study to complement this validation by another method to assess PKR functions; for example, in human PKR-KO cells, because results between yeast and human cells can differ. These limitations are now acknowledged in the revised version.

Evolutionary Data: Beyond residues under positive selection, the screen allows the authors to also perform a comparative analysis with PKR residues under purifying selection. Because they are assessing one of the most conserved ancestral functions of PKR (i.e. cell translation arrest), it may also be of interest to discuss these highly conserved sites. The authors now discuss the implications for the conserved residues.

Mechanistic insights and viral diversity: While the study identifies key sites and residues involved in vaccinia K3 resistance, it could benefit from further investigation into the underlying molecular mechanisms and the diversity of viral antagonists. The authors have now acknowledged these limitations in the Discussion and updated the manuscript to be more specific. These exciting research avenues will be the objectives of a next study.

Overall Assessment

The systematic approach, identification of key sites, and evolutionary implications are all notable strengths. While there is room for a stronger validation of the functions and further investigation into the mechanistic details and broader viral diversity, the findings are robust and already provide important advancements. The manuscript is well-written and clear, and the revised figures are informative and improved.

<https://doi.org/10.7554/eLife.99575.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Reviewer #1 (Public Review):

Summary:

The report describes the control of the activity of the RNA-activated protein kinase, PKR, by the Vaccinia virus K3 protein. Repressive binding of K3 to the kinase prevents phosphorylation of its recognised substrate, EIF2 α (the α subunit of the Eukaryotic Initiation Factor 2). The interaction of K3 is probed by saturation mutation within four regions of PKR chosen by modelling the molecules' interaction. They identify K3-resistant PKR variants that recognise that the K3/EIF2 α -binding surface of the kinase is malleable. This is reasonably interpreted as indicating the potential adaptability of this antiviral protein to combat viral virulence factors.

Strengths:

This is a well-conducted study that probes the versatility of the antiviral response to escape a viral inhibitor. The experimentation is very diligent, generating and screening a large number of variants to recognise the malleability of residues at the interface between PKR and K3.

Weaknesses:

(1) These are minor. The protein interaction between PKR and K3 has been previously well-explored through phylogenetic and functional analyses and molecular dynamics studies, as well as with more limited site-directed mutational studies using the same experimental assays.

Accordingly, these findings largely reinforce what had been established rather than making major discoveries.

First, thank you for your thoughtful feedback. We agree that our results are concordant with previous findings and recognize the importance of emphasizing what we find novel in our results. We have revised the introduction (lines 65-74 of the revised_manuscript.pdf) to emphasize three findings of interest: (1) the PKR kinase domain is largely pliable across its substrate-binding interface, a remarkable quality that is most fully revealed through a comprehensive screen, (2) we were able to differentiate variants that render PKR nonfunctional from those that are susceptible to Vaccinia K3, and (3) we observe a strong correlation between PKR variants that are resistant to K3 WT and K3-H47R.

There are some presumptions:

(2) It isn't established that the different PKR constructs are expressed equivalently so there is the contingency that this could account for some of the functional differences.

This is an excellent point. We have revised the manuscript to raise this caveat in the discussion (lines 247-251). One indirect reason to suppose that expression differences among our PKR variants are not a dominant source of variation is that we did not observe much variation in kinase activity in the absence of K3.

(3) Details about the confirmation of PKR used to model the interaction aren't given so it isn't clear how accurately the model captures the active kinase state. This is important for the interaction with K3/eIF2 α .

We have expanded on Supplemental Figure 12 and our description of the AlphaFold2 models in the Materials and Methods section (lines 573-590). We clarify that these models may not accurately capture the phosphoacceptor loop of eIF2 α (residues Glu49-Lys60) and the PKR β 4-5 linker (Asp338-Asn350) as these are highly flexible regions that are absent in the existing crystal structure complex (PDB 2A1A) and have low AlphaFold2 confidence scores (pLDDT < 50). We also noted, in the Materials and Methods section and in the caption of Figure 1, that the modeled eIF2 α closely resembles the crystal structure of standalone yeast eIF2 α , which places the Ser51 phosphoacceptor site far from the PKR active site. Thus, we expect there are additional undetermined PKR residues that contact eIF2 α .

(4) Not all regions identified to form the interface between PKR and K3 were assessed in the experimentation. It isn't clear why residues between positions 332-358 weren't examined, particularly as this would have made this report more complete than preceding studies of this protein interaction.

Great questions. We designed and generated the PKR variant library based on the vaccinia K3 crystal structure (PDB 1LUZ) aligned to eIF2 α in complex with PKR (PDB 2A1A), in which PKR residues 338-350 are absent. After the genesis of the project, we generated the AlphaFold2-predicted complex of PKR and vaccinia K3, and have become very interested in the β 4- β 5 linker, a highly diverse region across PKR homologs which includes residues 332-358. However, this region remains unexamined in this manuscript.

Reviewer #2 (Public Review):

Chambers et al. (2024) present a systematic and unbiased approach to explore the evolutionary potential of the human antiviral protein kinase R (PKR) to evade inhibition by a poxviral antagonist while maintaining one of its essential functions.

The authors generated a library of 426 single-nucleotide polymorphism (SNP)-accessible non-synonymous variants of PKR kinase domain and used a yeast-based heterologous virus-host system to assess PKR variants' ability to escape antagonism by the vaccinia

virus pseudo-substrate inhibitor K3. The study identified determinant sites in the PKR kinase domain that harbor K3-resistant variants, as well as sites where variation leads to PKR loss of function. The authors found that multiple K3-resistant variants are readily available throughout the domain interface and are enriched at sites under positive selection. They further found some evidence of PKR resilience to viral antagonist diversification. These findings highlight the remarkable adaptability of PKR in response to viral antagonism by mimicry.

Significance of the findings:

The findings are important with implications for various fields, including evolutionary biology, virus-host interfaces, genetic conflicts, and antiviral immunity.

Strength of the evidence:

Convincing methodology using state-of-the-art mutational scanning approach in an elegant and simple setup to address important challenges in virus-host molecular conflicts and protein adaptations.

Strengths:

Systematic and Unbiased Approach:

The study's comprehensive approach to generating and characterizing a large library of PKR variants provides valuable insights into the evolutionary landscape of the PKR kinase domain. By focusing on SNP-accessible variants, the authors ensure the relevance of their findings to naturally occurring mutations.

Identification of Key Sites:

The identification of specific sites in the PKR kinase domain that confer resistance or susceptibility to a poxvirus pseudosubstrate inhibition is a significant contribution.

Evolutionary Implications:

The authors performed meticulous comparative analyses throughout the study between the functional variants from their mutagenesis screen ("prospective") and the evolutionarily-relevant past adaptations ("retrospective").

Experimental Design:

The use of a yeast-based assay to simultaneously assess PKR capacity to induce cell growth arrest and susceptibility/resistance to various VACV K3 alleles is an efficient approach. The combination of this assay with high-throughput sequencing allows for the rapid characterization of a large number of PKR variants.

Areas for Improvement:

(5) Validation of the screen: The results would be strengthened by validating results from the screen on a handful of candidate PKR variants, either using a similar yeast heterologous assay, or - even more powerfully - in another experimental system assaying for similar function (cell translation arrest) or protein-protein interaction.

Thank you for your thoughtful feedback. We agree that additional data to validate our findings would strengthen the manuscript. We have individually screened a handful of PKR variants in duplicate using serial dilution to measure yeast growth, and found that the results generally support our original findings. We have revised the manuscript to include these validation experiments (lines 117-119 of the revised_manuscript.pdf, Supplemental Figure 4).

(6) Evolutionary Data: Beyond residues under positive selection, the screen would allow the authors to also perform a comparative analysis with PKR residues under purifying selection. Because they are assessing one of the most conserved ancestral functions of PKR (i.e. cell translation arrest), it may also be of interest to discuss these highly conserved sites.

This is a great point. We do find that there are regions of the PKR kinase domain that are not amenable to genetic perturbation, namely in the glycine rich loop and active site. We contrast the PKR functional scores at conserved residues under purifying selection with those under positive selection in Figure 2E (lines 141-143).

(7) Mechanistic Insights: While the study identifies key sites and residues involved in vaccinia K3 resistance, it could benefit from further investigation into the underlying molecular mechanisms. The study's reliance on a single experimental approach, deep mutational scanning, may introduce biases and limit the scope of the findings. The authors may acknowledge these limitations in the Discussion.

We agree that further investigation into the underlying molecular mechanisms is warranted and we have revised the manuscript to acknowledge this point in the discussion (lines 284-288).

(8) Viral Diversity: The study focuses on the viral inhibitor K3 from vaccinia. Expanding the analysis to include other viral inhibitors, or exploring the effects of PKR variants on a range of viruses would strengthen and expand the study's conclusions. Would the identified VACV K3-resistant variants also be effective against other viral inhibitors (from pox or other viruses)? or in the context of infection with different viruses? Without such evidence, the authors may check the manuscript is specific about the conclusions.

This is a fantastic question that we are interested in exploring in our future studies. In the manuscript we note a strong correlation between PKR variants that evade vaccinia wild-type K3 and the K3-H47R enhanced allele, but we are curious to know if this holds when tested against other K3 orthologs such as variola virus C3. That said, we have revised the manuscript to clarify this limitation to our findings and specify vaccinia K3 where appropriate.

Reviewer #3 (Public Review):

Summary:

- This study investigated how genetic variation in the human protein PKR can enable sensitivity or resistance to a viral inhibitor from the vaccinia virus called K3.
- The authors generated a collection of PKR mutants and characterized their activity in a high-throughput yeast assay to identify 1) which mutations alter PKR's intrinsic biochemical activity, 2) which mutations allow for PKR to escape from viral K3, and 3) which mutations allow for escape from a mutant version of K3 that was previously known to inhibit PKR more efficiently.
- As a result of this work, the authors generated a detailed map of residues at the PKR-K3 binding surface and the functional impacts of single mutation changes at these sites.

Strengths:

- Experiments assessed each PKR variant against three different alleles of the K3 antagonist, allowing for a combinatorial view of how each PKR mutant performs in

different settings.

- Nice development of a useful, high-throughput yeast assay to assess PKR activity, with highly detailed methods to facilitate open science and reproducibility.
- The authors generated a very clean, high-quality, and well-replicated dataset.

Weaknesses:

(9) The authors chose to focus solely on testing residues in or near the PKR-K3 predicted binding interface. As a result, there was only a moderately complex library of PKR mutants tested. The residues selected for investigation were logical, but this limited the potential for observing allosteric interactions or other less-expected results.

First, we greatly appreciate all your feedback on the manuscript, as well as raising this particular point. We agree that this is a moderately complex library of PKR variants, from which we begin to uncover a highly pliable domain with a few specific sites that cannot be altered. We have revised the manuscript to raise this limitation (lines 284-288 of the revised_manuscript.pdf) and encourage additional exploration of the PKR kinase domain.

(10) For residues of interest, some kind of independent validation assay would have been useful to demonstrate that this yeast fitness-based assay is a reliable and quantitative readout of PKR activity.

We agree that additional data to validate our findings would strengthen the manuscript. We have individually screened a handful of PKR variants in duplicate using serial dilution to measure yeast growth, and generally found that the results support our original findings. We have revised the manuscript to include this validation experiment (lines 117-119, Supplemental Figure 4).

(11) As written, the current version of the manuscript could use more context to help a general reader understand 1) what was previously known about these PKR and K3 variants, 2) what was known about how other genes involved in arms races evolve, or 3) what predictions or goals the authors had at the beginning of their experiment. As a result, this paper mostly provides a detailed catalog of variants and their effects. This will be a useful reference for those carrying out detailed, biochemical studies of PKR or K3, but any broader lessons are limited.

Thank you for bringing this to our attention. We have revised the introduction of the manuscript to provide more context regarding previous work demonstrating an evolutionary arms race between PKR and K3 and how single residue changes alter K3 resistance (lines 51-64).

(12) I felt there was a missed opportunity to connect the study's findings to outside evolutionary genetic information, beyond asking if there was overlap with PKR sites that a single previous study had identified as positively selected. For example, are there any signals of balancing selection for PKR? How much allelic diversity is there within humans, and are people typically heterozygous for PKR variants? Relatedly, although PKR variants were tested in isolation here, would the authors expect their functional impacts to be recessive or dominant, and would this alter their interpretations? On the viral diversity side, how much variation is there among K3 sequences? Is there an elevated evolutionary rate, for example, in K3 at residues that contact PKR sites that can confer resistance? None of these additions are essential, but some kind of discussion or analysis like this would help to connect the yeast-based PKR phenotypic assay presented here back to the real-world context for these genes.

We appreciate this suggestion to extend our findings to a broader evolutionary context. There is little allelic diversity of PKR in humans, with all nonsynonymous variation listed in gnomAD being rare. (PKR shows sequence diversity in comparisons across species, including across primates.) Thus, barring the possibility of variation being present in under-studied populations, there is unlikely to be balancing selection on PKR in humans. Our expectation is that beneficial mutations in PKR for evading a pseudosubstrate inhibitor would be dominant, as a small amount of eIF2 α phosphorylation is capable of halting translation (Siekierka, PNAS, 1984). There is a recent report citing PKR missense variants associated with dystonia that can be dominantly or recessively inherited (Emy et al. 2020 PMID 33236446). Elde et al. 2009 (PMID 19043403) notes that poxvirus K3 homologs are under positive selection but no specific residues have been cited to be under positive selection. The lack of allelic diversity in PKR in humans notwithstanding, PKR could experience future selection in the human population as evidenced by its rapid evolution in primates, so we fully agree that a connection to the real-world context is useful. We have noted these topics in the discussion section (lines 289-294).

Reviewer #1 (Recommendations For The Authors):

I have no major criticisms but ask for some clarifications and make some comments about the perceived weaknesses.

(13) If the authors disagree with my summation that the findings largely replicate what was known, could they detail how the findings differ from what was known about this protein interaction and the major new insights stemming from the study? Currently, the abstract is a little philosophical rather than listing the explicit discoveries of the study.

Thank you again for raising the need for us to clearly convey the novelty of our findings. We have revised the final paragraph in our introduction as described in comment #1.

(14) As the experimental approach is well reported it is unnecessary to confirm the proposed activity by, for instance, measures of Sui2 phosphorylation. However, previous reports have recognised that point mutants of PKR can be differentially expressed. The impact of this potential effect is unknown in the current experimentation as there are no measures of the expression of the different mutant PKR constructs. The large number of constructs used makes this verification onerous. The potential impact could be ameliorated by redundant replacing each residue (hoping different residues have different effects on expression). Still, this limitation of the study should be acknowledged in the text.

We greatly appreciate this comment and agree that this should be made clear in the text, which we have added to the discussion of the manuscript (lines 247-251).

(15) Preceding findings and the modeling in this report recognise an involvement in the kinase insert region (residues 332 to 358) in PKR's interaction with K3 but this region is excluded from the analysis. These residues have been largely disregarded in the preceding analysis (it is absent from the molecular structure of the kinase) so its inclusion here might have lent a more novel aspect or delivered a more complete investigation. Is there a justification for excluding this flexible loop?

The PKR variant library was designed based on the crystal structure of K3 (PDB 1LUZ) aligned to eIF2 α in complex with PKR (PDB 2A1A). After the library was designed and made we attained complete predicted structures of PKR in complex with eIF2 α and K3, which largely agrees with the predicted crystal structures but contain the additional flexible loops that

were not captured in the crystal structures. Though the library studied here does not explore variation in the kinase insert region, we are very interested in doing so in our future studies.

(16) Could the explanation of the 'PKR functional score' be clarified? The description given within the legend of SF1 was helpful, so could this be replicated earlier in the main body of the text when introducing these experiments? e.g. As PKR activity is toxic to yeast, the number of cells in the pool expressing the functional PKR will decrease over time. Thus the associated barcode read count will also decrease, while the read count for the nonfunctional PKR will increase. This is termed the PKR function score, which will be relatively lower for cells transformed with less active PKR than those with more active PKR.

Thank you for suggesting this clarification, we have revised the manuscript to clarify our definition of the PKR functional score (lines 106-109).

(17) Another suggestion to clarify this term is to modify the figures. Currently, the intent of the first simulated graph in Fig 1E is clear but the inversion of the response (shown by the transposition of the colours) in the next graph (to the right) is less immediately obvious. Accordingly, the orientation of the 'PKR functional score' is uncertain. Could the authors add text to the rightmost graphic in Figure 1E by, for instance, indicating the PKR activity in the vertical column with text such as 'less active' (at the bottom), 'WT' (in the centre), and 'more activity' (at the top)? Also, the position of the inactive K296R mutant might be added to Figure 2A complementing the positioning of the active WT kinase in the first data graph of this kind.

We appreciate your specific feedback to improve the figures of the manuscript, we have made adjustments to Figure 1E to clarify how we derive the PKR functional scores.

(18) The authors don't use existing structures of PKR in their modelling. However, there is no information about the state of the PKR molecule used for modelling. Specific elements of the kinase domain affect its interaction with K3 so it would be informative to know the orientation of these elements in the model. Could the authors detail the state of pivotal kinase elements in their models? This could involve the alignment of the N- and C-lobes, the orientation of kinase spines (C- and R-spines), and the phosphorylation status of residues in the activation loop, or at least the position of this loop in relationship to that adopted in the active dimeric kinase (e.g. PDB-2A1A, 3UIU or 6D3L). Alternatively, crystallographic structures of active inactive PKR could be overlayed with the theoretical structure used for modelling (as supplementary information).

We have revised the manuscript to describe the alignment of the predicted PKR-K3 complex with active and inactive PKR, and we have extended Supplemental Figure 12 with an overlay of the predicted structures with existing structures. We have also added a supplemental data file containing the RMSD values of PKR (from the predicted PKR-K3 complex) aligned to active (PDB 2A1A) and inactive (PDB 3UIU) or unphosphorylated (PDB 6D3L) PKR (5_Structure-Alignment-RMSD-Values.xlsx). We have also provided the AlphaFold2 best model predictions for the PKR-eIF2 α complex (6_AF2_PKR-KD-eIF2a.pdb) and PKR-K3 complex (7_AF2_PKR-KD_VACV-K3.pdb). Looking across the RMSD values, the AlphaFold2 model of PKR most closely resembles unphosphorylated PKR (PDB 6D3L) though we note the activation loop is absent from PDB 6D3L and 3UIU. We also aligned the Ser51 phosphoacceptor loop of AlphaFold2 eIF2 α model to PDB 1Q46 and we see that the model reflects the pre-phosphorylation state. This loop is expected to interact with the PKR active site, which is not captured in our model and we state this explicitly in the caption of Figure 1 (lines 665-668).

(19) Could some specific residue in Figure 7 be labelled (numbered) to orient the findings? Also, the key in this figure doesn't title the residues coloured white (RE red/black/blue). The white also isn't distinguished from the green (outside the regions targeted for mutagenesis).

Excellent suggestion, we have revised this figure to include labels for the sites to orient the reader and clarify our categorization of PKR residues in the kinase domain.

(20) Regarding the discussion, the authors adopt the convention of describing K3 as a pseudosubstrate. Although I realize it is common to refer to K3 as a pseudosubstrate, it isn't phosphorylated and binds slightly differently to PKR so alternative descriptors, such as 'a competitive binder', would more accurately present the protein's function. Possibly for this reason, the authors declared an expectation that evolution pressures should shift K3 to precisely mimic EIF2 α . However, closer molecular mimicry shouldn't be expected for two reasons. The first is a risk of disrupting other interactions, such as the EIF2 complex. Secondly, equivalent binding to PKR would demote K3 to merely a stoichiometric competitor of EIF2 α . In this instance, effective inhibition would require very high levels of K3 to compete with equivalent binding by EIF2 α . This would be demanding particularly upon induction of PKR during the interferon response. To be an effective inhibitor K3 has to bind more avidly than EIF2 α and merely requires a sufficient overlap with the EIF2 α interface on PKR to disrupt this alternative association. This interpretation predicts that K3 is under pressure to bind PKR by a different mechanism than EIF2 α .

We appreciate your thoughtful point about the usage of the term pseudosubstrate. Ultimately, we've decided to continue using the term due to its historical usage in the field. The question of the optimal extent of mimicry in K3 is a fascinating one, and we greatly appreciate your thoughts. We wholly agree that the possibility of K3 having superior PKR binding relative to eIF2 α would be preferable to perfect mimicry. In our Ideas and Speculation section, we propose that benefits towards increasing PKR affinity may need to be balanced against potential loss of host range resulting from overfitting to a given host's PKR. However, the possibility that reduced mimicry could be selected to avoid disruption of eIF2 function had not occurred to us; thank you for pointing it out!

(21) The discussion of the 'positive selection' of sites is also interesting in this context. To what extent has the proposed positive selection been quantified? My understanding is that all of the EIF2 α kinases are conserved and so demonstrate lower levels of residue change that might be expected by random mutagenesis i.e. variance is under negative selection. The relatively higher rate of variance in PKR orthologs compared to other EIF2 α kinases could reflect some relaxation of these constraints, rather than positive selection. Greater tolerance of change may stem from PKR's more sporadic function in the immune response (infrequent and intermittent presence of its activating stimuli) rather than the ceaseless control of homeostasis by the other EIF2 α kinases. Also, induction of PKR during the immune response might compensate for mutations that reduce its activity. I believe that the entire clade of extant poxviruses is young relative to the divergence between their hosts. Accordingly, genetic variance in PKR predates these viruses. Although a change in PKR may become fixed if it affords an advantage during infection, such an advantage to the host would be countered by the much higher mutation rates of the virus. This would appear to diminish the opportunity for a specific mutation to dominate a host population and, thereby, to differentiate host species. Rather, pressure to elude control by a rapidly evolving viral factor would favour variation at sites where K3 binds. This speculation offers an alternative perspective to the current discussion that the variance in PKR orthologs stems from positive selection driven by viral infection.

We appreciate this stimulating feedback for discussion. Three of the four eIF2 α kinases (HIR, PERK, and GCN2) appear to be under purifying selection (Elde et al. 2009, PMID 19043403), which stand in contrast to PKR. Residues under positive selection have been found throughout PKR, including the dsRNA binding domains, linker region, and the kinase domain. Importantly, the selection analysis from Elde et al. and Rothenburg et al. concluded that positive selection at these sites is more likely than relaxed selection. We agree that poxviruses are young, though we would guess that viral pseudosubstrate inhibition of PKR is ancient. Many viral proteins have been reported to directly interact with PKR, including herpes virus US11, influenza A virus NS1A, hepatitis C virus NS5A, and human immunodeficiency virus Tat. The PKR kinase domain does contain residues under purifying selection that are conserved among all four eIF2 α kinases, but it also contains residues under positive selection that interface with the natural substrate eIF2 α . Our work suggests that PKR is genetically pliable across several sites in the kinase domain, and we are curious to know if this pliability would hold at the same sites across the other three eIF2 α kinases.

(22) The manuscript is very well written but has a small number of typos; e.g. an aberrant 'e' in 7 of the introduction, capitalise the R in ranavirus on the last line of the fourth paragraph of the discussion, and eIF2 α (EIF2 α ?) is occasionally written as eIF α in the materials&methods.

Thank you for bringing these typos to our attention! We've deleted the aberrant 'e' in the introduction, capitalized 'Ranavirus' in the discussion (line 265), and corrected 'eIF α ' to 'eIF2 α ' throughout the manuscript.

Reviewer #2 (Recommendations For The Authors):

Additional minor edits or revisions:

(23) Paragraph 3 of the Introduction gives the impression that most of the previous work on the PKR-virus arms race is speculative. However, it is one of the best-described and most convincing examples of virus-host arms races. Can the authors edit the paragraph accordingly?

Thank you for bringing this to our attention. We have revised the third paragraph and strengthened the description of the evolutionary arms race between PKR and viral pseudosubstrate antagonists.

(24) Introduction: PKR has "two" double-stranded RNA binding domains. Can the authors update the text accordingly?

We have updated the manuscript to clarify PKR has two dsRNA binding domains (lines 44-45).

(25) The authors test here for one of the key functions of PKR: cell growth/translation arrest. Because of PKR pleiotropy, the manuscript may be edited accordingly: For example, statements such as "We found few genetic variants render the PKR kinase domain nonfunctional" are too speculative as they may retain other (not tested here) functions.

This is a great suggestion, we have revised the manuscript to specify our definition of nonfunction in the context of our experimental screen (lines 86-92 and 106-109) and acknowledge this limitation in our experimental screen (lines 304-307).

(26) The authors should specify "vaccinia" K3 whenever appropriate.

We appreciate this comment and have revised the manuscript to specify vaccinia K3 where appropriate (e.g. lines 62,66, 70, 80, 108, and 226).

| (27) *Ref for ACE2 diversification may include Frank et al 2022 PMID: 35892217.*

Thank you for pointing us to this paper, we have included it as a reference in the manuscript (line 277).

| (28) *Positive selection of PKR as referred to by the authors corresponds to analyses performed in primates. As shown by several studies, the sites under positive selection may vary according to host orders. Can the authors specify this ("primate") in their manuscript? And/or shortly discuss this aspect.*

Thank you for raising this point. In the manuscript we performed our analysis using vertebrate sites under positive selection as identified in Rothenburg et al. 2009 PMID 19043413 (lines 51 and figure legends). We performed the same analysis using sites under positive selection in primates (as identified by Elde et al. 2009 PMID 19043403) and again found a significant difference in PKR functional scores versus K3. We have revised the manuscript to clarify our use of vertebrate sites under positive selection (line 80-81).

| (29) *We view deep mutational scanning experiments as a complementary approach to positive selection": The authors should edit this and acknowledge previous and similar work of other antiviral factors, in particular one of the first studies of this kind on MxA (Colon-Thillet et al 2019 PMID: 31574080), and TRIM5 (Tenthorey et al 2020 PMID: 32930662).*

Thank you for raising up these two papers, which we acknowledge in the revised manuscript (line 299).

| (30) *We believe Figure S7 brings important results and should be placed in the Main.*

We appreciate this suggestion, and have moved the contents of the former supplementary Figure 7 to the main text, in Figure 6.

| (31) *The title may specify "poxvirus".*

Thank you for the suggestion to specify the nature of our experiment, we have adjusted the title to: Systematic genetic characterization of the human PKR kinase domain highlights its functional malleability to escape a poxvirus substrate mimic (line 3).

| **Reviewer #3 (Recommendations For The Authors):**

| (32) *No line numbers or page numbers are provided, which makes it difficult to comment.*

We sincerely apologize for this oversight and have included line numbers in our revised manuscript as well as the tracked changes document.

| (33) *In the introduction, I recommend defining evolutionary arms races more clearly for a broad audience.*

Thank you for this suggestion. We have revised the manuscript in the first and third paragraphs to more clearly introduce readers to the concept of an evolutionary arms race.

(34) The introduction could use a clearer statement of the question being considered and the gap in knowledge this paper is trying to address. Currently, the third paragraph includes many facts about PKR and the fourth paragraph jumps straight into the approach and results. Some elaboration here would convey the significance of the study more clearly. As is, the introduction reads a bit like "We wanted to do deep mutational scanning. PKR seemed like an ok protein to look at", rather than conveying a scientific question.

This is a great suggestion to improve the introduction section. We have heavily revised the third and fourth paragraphs of the introduction to clarify the motivation, approach, and significance of our work.

(35) Relatedly, did the authors have any hypotheses at the start of the experiment about what kinds of results they expected? e.g. What parts of PKR would be most likely to generate escape mutants? Would resistant mutants be rare or common? etc? This would help the reader to understand which results are expected vs. surprising.

These are all great questions. We have revised the introduction of the manuscript to point out that previous studies have characterized a handful of PKR variants that evade vaccinia K3, and these variants were made at sites found to be under positive selection (lines 60-64).

(36) A description of the different K3 variants and information about why they were chosen for study should also be added to the Introduction. It was not until Figure 5 that the reader was told that K3-H47R was the same as the 'enhanced' K3 allele you are testing.

Thank you for bringing this to our attention, we have revised the introduction to clarify the experimental conditions (lines 65-67) and specify K3-H47R as the enhanced allele earlier in the manuscript (line 100).

(37) Does every PKR include just a single point mutation? It would be nice to see data about the number and types of mutations in each PKR window added to Supplemental Figure 1.

Thank you for the suggestion to improve this figure. Every PKR variant that we track has a single point mutation that generates a nonsynonymous mutation. In our PacBio sequencing of the PKR variant library we identified a few off-target variants or sequences with multiple variants, but we identified the barcodes linked to those constructs and discarded those variants in our analysis. We have revised Supplemental Figure 1 to include the number and types of mutations made at each PKR window.

(38) In terms of the paper's logical flow, personally, I would expect to begin by testing which variants break PKR's function (Figure 3) and then proceeding to see which variants allow for K3 escape (Figure 2). Consider swapping the order of these sections.

Thank you for this suggestion, and we can appreciate how the flow of the manuscript may be improved by swapping Figures 2 and 3. We have decided to maintain the current order of the figures because we use Figure 3 to emphasize the distinction of PKR sites that are nonfunctional versus susceptible to vaccinia K3.

(39) Figure 3A seems like a less-informative version of Figure 4A, recommend combining these two. Same comment with Figure 5A and Figure 6A.

We appreciate this specific feedback for the figures. Though there are similarities between figure panels (e.g. 3A and 4A) we use them to emphasize different points in each figure. For example, in Figure 3 we emphasize the general lack of variants that impair PKR kinase activity, and in Figure 4 we distinguish kinase-impaired variants from K3-susceptible variants. For this reason, and given space constraints, we have chosen to maintain the figures separately. We did decide to move the former Figure 6 to the supplement.

(40) In general, it felt like there was a lot of repetition/re-graphing of the same data in Figures 3-6. I recommend condensing some of this, and/or moving some of the panels to supplemental figures.

Thank you for your suggestion, we have revised the manuscript and have moved Figure 6 to Supplemental Figure 7.

(41) In contrast, Supplemental Figure 7 is helpful for understanding the distribution of the data. Recommend moving to the main text.

This is a great recommendation, and we have moved Supplemental Figure 7 into Figure 6.

(42) How do the authors interpret an enrichment of positively selected sites in K3-resistant variants, but not K3-H74R-resistant variants? This seems important. Please explain.

Thank you for this suggestion to improve the manuscript; we agree that this observation warranted further exploration. We found a strong correlation in PKR functional scores between K3 WT and K3-H47R, and with that we find sites under positive selection that are resistant to K3 WT are also resistant to K3-H47R. The lack of enrichment at positively selected sites appears to be caused by collapsed dynamic range between PKR wild-type-like and nonfunctional variants in the K3-H47R screen. We have revised the manuscript to clarify this point (line 202-204).

(43) Discussion: The authors compare and contrast between PKR and ACE2, but it would be worth mentioning other examples of genes involved in antiviral arms races wherein flexible, unstructured loops are functionally important and are hotspots of positive selection (e.g. MxA, NLRP1, etc).

We greatly appreciate this suggestion to improve the discussion. We note this contrast between the PKR kinase domain and the flexible linkers of MxA and NLRP1 in the revised manuscript (lines 273-274).

(44) Speculation section: What is the host range of the vaccinia virus? Is it likely to be a generalist amongst many species' PKRs (and if so, how variable are those PKRs)? Would be worth mentioning for context if you want to discuss this topic.

Thank you for raising this question. Vaccinia virus is the most well studied of the poxviruses, having been used as a vaccine to eradicate smallpox, and serves as a model poxvirus. Vaccinia virus has a broad host range, and though the name vaccinia derives from the Latin word “vacca” for cow the viruses origin remains uncertain (Smith 2007 https://doi.org/10.1007/978-3-7643-7557-7_1). has been used to eradicate smallpox as a vaccine and serves as a model poxvirus. Though the natural host is unknown, it appears to be a general inhibitor of vertebrate PKRs The natural host of vaccinia virus is unknown, though there is some evidence to suggest it may be native to rabbits and does appear to be generalist.

(45) Many papers in this field discuss interactions between PKR and K3L, rather than K3. I understand that this is a gene vs. protein nomenclature issue, but consider matching the K3L literature to make this paper easier to find.

Thank you for bringing this to our attention. We have revised the manuscript to specify that vaccinia K3 is expressed from the K3L gene in both the abstract (line 26) and the introduction (line 56) to help make this paper easier to find when searching for “K3L” literature.

(46) Which PKR sequence was used as the wild-type background?

This is a great question. We used the predominant allele circulating in the human population represented by Genbank m85294.1:31-1686. We cite this sequence in the Methods (line 421) and have added it to the results section as well (lines 84).

(47) Figure 1C: the black dashed line is difficult to see. Recommend changing the colors in 1A-1C.

Thank you for this suggestion, we have changed the dashed lines from black to white to make them more distinguishable.

(48) Figure 1D: Part of the point of this figure is to convey overlaps between sites under selection, K3 contact sites, and eIF2alpha contact sites, but at this scale, many of the triangles overlap. It is therefore impossible to tell if the same sites are contacted vs. nearby sites. Perhaps the zoomed-in panels showing each of the four windows in the subsequent figures are sufficient?

Thank you for bringing this to our attention. We have scaled the triangles down to reduce their overlap in Figure 1D and list all sites of interest (predicted eIF2α and vaccinia contacts, conserved sites, and positive selection sites) in the Materials and Methods section “Predicted PKR complexes and substrate contacts”.

(49) Figure 1E: under "1,293 Unique Combinations", there is a line between the PKR and K3 variants, which makes it look like they are expressed as a fusion protein. I believe these proteins were expressed from the same plasmid, but not as a fusion, so I recommend re-drawing. Then in the graph, the y-axis says "PKR abundance", but from the figure, it is not clear that this refers to relative abundance in a yeast pool. Perhaps "yeast growth" or similar would be clearer?

Thank you for the specific feedback to improve Figure 1. We have made the suggested edits to clarify that PKR and vaccinia K3 are not fused but each is expressed from their own promoter. We have also changed the y-axis from “PKR Abundance” to “Yeast Growth”.

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