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Reply to comments

CRISPR–Cas adaptive immunity and developments in CRISPR–Cas applications Reply to comments on "Diversity, evolution, and therapeutic applications of small RNAs in prokaryotic and eukaryotic immune systems"

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We appreciate the commentaries received from Stern [1], Pinti and Cossarizza [2], Plagens and Randau [3], Koonin [4], Wanner et al. [5], and Severinov [6], and their observations and insights, which we will now discuss. As is mentioned in many of the commentaries received, the emergence of knowledge about CRISPR–Cas systems marks an important shift in the mindset of immunology. For a long time, eukaryotic immune systems were viewed through the paradigm of self/not-self. This paradigm was rooted in clonal selection and the dual innate and adaptive model [7–9]. With the advent of the danger hypothesis, it became apparent that the self/not-self pattern was not impervious to upheaval [7,10]. Similarly, small RNA systems have shifted the perspective of immunology. When RNA systems were first discovered, they were seen as an exception to the suite of well-known protein-recognition adaptive immune defenses. However, in the words of Pinti and Cossarizza, "it is now clear even to us, human immunologists, that RNA-based defense mechanisms are the rule, and not the exception, and that the modern, adaptive immune system has been built on an ancient basement made by RNA" [2].

Stern similarly remarks that the wealth of RNA systems reveals the "general solutions to the problem of parasites" [1]. The recognition of not-self motifs, infected cell death, and retained memory of past exposures are all mechanisms of immune defense that are observed across the tree of life [1]. Koonin likens small RNA defense mechanisms to an idea that evolution found " 'too good' to be abandoned" [4]. We agree that the pervasiveness of small RNA systems in prokaryotes and eukaryotes demonstrates the importance of these systems to life throughout the phylogenetic tree, and speaks to the power of these types of systems. As Koonin states, "there seems to be underlying logic in their evolution that is both universal and simple" [4].

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As for the evolution of CRISPR–Cas and other analogous RNA systems, Pinti and Cossarizza express uncertainty as to whether these systems could be homologous, and not simply analogous [2]. Koonin proposes that CRISPR–Cas system elements have not been conserved because Cas2, an mRNA interferase, is likely to possess toxic properties. Because of this, CRISPR–Cas was likely eliminated through purifying selection when eukaryotes evolved, in the same way as toxin–antitoxin and restriction–modification systems [4]. However, Plagens and Randau suggest that the structure of RNA and DNA-interfering elements in CRISPR–Cas have a common ancestor, and also have some structural similarity to the Argonaute proteins of RNAi. We agree that further research into the connection between RNAi and CRISPR–Cas systems in individual organisms is necessary to elucidate their relationship, since functional Argonaute homologs have been found in prokaryotes [3].

Koonin supports our assertion that CRISPR–Cas is an example of a Lamarckian system. Additionally, "piRNA clusters in the genomes of animal germline cells incorporate transposon sequences" in another example of Lamarckian inheritance [4]. However, Severinov feels that CRISPR–Cas represents a pseudo-Lamarckian inheritance system. Severinov commented that the Lamarckian inheritance of spacers is problematic, given that lytic phages have the potential to kill prokaryotes before a helpful spacer could be passed on [6]. While this is certainly a possibility, it does not change the fact that should the prokaryote succeed in integrating a spacer and subsequently reproducing, the genetic adaptations passed to the next generation would be in response to, and address, the environment of the prokaryote. We maintain that while CRISPR–Cas does undergo Darwinian selection, it is also a strong example of Lamarckian inheritance.

Severinov also questions how CRISPR–Cas could be an efficient immune system in *E. Coli*, given that the system lacks the delineation between self/not-self DNA seen in restriction–modification systems [6]. However, the *E. Coli* Type I–E CRISPR–Cas system does differentiate between target and non-target DNA. While Type I–E systems do not rely on protospacer-flanking sequences for self/not-self distinction, target recognition is dependent on PAM recognition by Cse-1. According to Westra et al., self DNA is not targeted in Type I–E because there are no PAM sequences in the CRISPR array, which serves as a "robust mechanism" of self/not-self discrimination [11].

We do agree with Severinov in that it is important to note that while CRISPR–Cas can provide immunity, this is likely not its sole function [6]. For example, it has been suggested that CRISPR–Cas systems may contribute to the regulation of endogenous genes during the interaction of *Francisella novicida* (Type II CRISPR–Cas system) with eukaryotic hosts [12,13]. Additionally, Cas9 is required for *Neisseria meningitidis* and *Campylobacter jejuni* to attach to, invade, and replicate in epithelial cells [13]. Like Severinov, we worry that now that CRISPR–Cas has been shown to have practical applications, the examination of its biological functions will be abandoned [6]. However, the demonstration of CRISPR–Cas' relevance to bioengineering may also spur forward research into its native role in prokaryotes and its analogs such as piRNA, as we seek out a deeper understanding of the systems we wish to harness. Plagens and Randau mentioned errors in Sections 3.1 and 6.4 of our review [3], which we would like to acknowledge and clarify: Cas1 and Cas9 both cleave DNA. Additionally, further studies into the genetic engineering potential of CRISPR–Cas elements have emerged since the conception of our review [3]. As noted by Wanner et al., "PubMed now has more than 600 CRISPR citations, nearly half of which are from 2013, documenting uses of CRISPR–Cas systems from bacteria to human cells" [5].

For example, as cited by Stern [1], Maeder et al. demonstrated that guide RNAs can direct catalytically inactive Cas9 to repress endogenous genes in humans [14]. In a study by Cheng et al., Cas9 was fused with a single guide (sg)RNA to activate exogenous reporter genes in both mouse and human cells, and succeeded in activating reporter genes in mouse zygotes. The binding of sgRNAs to promoters was most efficient with clustered sgRNAs, suggesting synergistic action. Perhaps most importantly, with the introduction of sgRNAs targeting multiple genes into cells, multiplexed endogenous gene activation was achieved with high specificity [15]. Recently Zhou et al. generated immunodeficient mouse strains through the injection of Cas9 mRNA and multiple sgRNAs into mouse embryos. The study achieved multiple gene modifications, fragment deletion, double knockout of genes localizing on the same chromosome, creating immunodeficient mouse models with heritable genetic modifications. Also, by optimizing the concentration of Cas9 and sgRNAs and designing two adjacent sgRNAs targeting one exon for each gene, the targeting efficiency and number of bi-allelic mutations increased [16]. The simplicity and accessibility of genome editing with CRISPR–Cas elements was also demonstrated in flies by Gratz et al. [17].

Wanner and colleagues raised questions as to how society will respond to the rapidly progressing field of human genetic engineering [5]. These social and ethical concerns, while not addressed in our review, are important to bear in mind as our understanding of CRISPR–Cas systems moves from theoretical to therapeutic. Although mainstream

CRISPR–Cas therapies may still be far off on the horizon, genetic engineering is progressing rapidly, and it will be interesting to see how wider society greets future changes in medicine. Ultimately, we are optimistic for future progress in CRISPR–Cas research.

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