New Strategies for the Elimination of Polio from India

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product was similar to that of unglycosylated GFP (Fig. 3D), and thus the glycosylation reaction occurred on the folded GFP. Thus, PglB modified a peptide displayed on a folded protein, although it is likely that the grafted loop itself is relatively flexible.

To check the folding-dependent recognition of consensus sequons by PglB, we analyzed the PglB-dependent in vitro glycosylation of the eukaryotic glycoprotein bovine ribonuclease A (RNaseA). The native N-glycosylation site N34 of this protein is located in a structured domain. Different folding variants of the RNaseA allowed us to analyze the effect of unfolding on glycosylation. Consequently, RNaseA was expressed with a single point mutation (S32D), producing a bacterial consensus site at the native N-glycosylation site N34. Oxidative refolding of RNaseS32D from inclusion bodies (*20*) yielded enzymatically active protein (fig. S3), showing that RNaseS32D was able to fold into its native conformation despite the mutation.

Chemical treatments with denaturing, reducing, oxidizing, and alkylating agents yielded two RNaseS32D oxidation isomers (Fig. 4A). In reduced and alkylated RNaseS32D (RA), all four disulfide bonds were reduced in denaturing solution, and cysteines were alkylated to inhibit further disulfide bond formation. Rapid oxidation before alkylation led to another set of oxidation isomers, "scrambled" RNaseS32D (SC). Scrambled RNases contain randomly oxidized SS bonds, producing a heterogeneous population of proteins. A third RNaseS32D form was synthesized by limited proteolysis with subtilisin (*20*), which removes the N-terminal 21 amino acids of the protein. The resulting RNase S-protein (SP), like the RA and SC forms, was enzymatically inactive (fig. S3), but retained its native disulfide bonds and thus about 50% secondary structure, whereas the SC and RA forms appeared as random coils, as judged from far-ultraviolet circular dichroism (far-UV-CD) spectroscopy (Fig. 4B) (*23*). All four forms served as substrates for PglB (Fig. 4C). Glycosylation of the active RNaseS32D occurred with low efficiency (Fig. 4C), and the small amount of glycosylated RNaseS32D was active, as indicated by the zymogram assay (*20*) (Fig. 4C). The other forms were modified quantitatively (Fig. 4C). Thus, nonstructured protein domains are better substrates for PglB glycosylation than are folded ones. No glycosylation at all was observed with the same substrate proteins that lacked the S32D mutation (fig. S4B).

Our results show that completely folded proteins can be glycosylated both in vivo and in vitro. Bacterial OTase glycosylates native AcrA protein as well as an acceptor sequence grafted into the active GFP protein. In contrast, fully folded RNaseS32D was weakly glycosylated, whereas partial or complete unfolding strongly improved substrate activity.

The observation that the folding states of the acceptor protein affects glycosylation efficiency leads us to conclude that a specific substrate conformation must be adopted during the glycosylation process, most likely the Asn-turn (*24*). This makes potential acceptor sites present in a fixed environment suboptimal substrates for the bacterial OTase. We predict that native glycosylation sites in bacterial proteins will be located in locally flexible structures.

In contrast, the coupling of glycosylation and translocation in eukaryotes releases N-glycosylation from such structural constraints and, in combination with the less stringent primary sequence requirement, results in a more versatile and general glycosylation system.

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New Strategies for the Elimination of Polio from India

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The feasibility of global polio eradication is being questioned as a result of continued transmission in a few localities that act as sources for outbreaks elsewhere. Perhaps the greatest challenge is in India, where transmission has persisted in Uttar Pradesh and Bihar despite high coverage with multiple doses of vaccine. We estimate key parameters governing the seasonal epidemics in these areas and show that high population density and poor sanitation cause persistence by not only facilitating transmission of poliovirus but also severely compromising the efficacy of the trivalent vaccine. We analyze strategies to counteract this and show that switching to monovalent vaccine may finally interrupt virus transmission.

The World Health Assembly committed to the global eradication of polio in 1988. Since then, the eradication initiative has achieved great successes, eliminating polio from the Americas, the Western Pacific, and Europe. However, in recent years the number of reported cases has increased after export of infection from the handful of remaining endemic countries. The difficulty in eliminating these last reservoirs of poliovirus transmission has led some to question the feasibility of eradication (*1*). Particularly worrying is the ongoing transmission in India, the source of half the world's reported paralytic cases over the past decade. Children in India have re-

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ceived many more doses of vaccine than children in other endemic countries through an intensive supplementary immunization program. Understanding the cause of polio persistence in India is a public health priority, both for the elimination efforts and as a proof of concept for the global eradication initiative. Potential explanations for persistence include gaps in routine and supplemental vaccine coverage (*2*), poor vaccine efficacy (*3*), and conditions highly favorable for the transmission of fecal-oral pathogens, including high population density and poor sanitation (*2*, *4*). Here, we examine these hypotheses formally, using detailed surveillance data from 96,421 cases of acute flaccid paralysis (AFP) collected since 1997.

The reproductive number $R(t)$ of an infection is the number of secondary infections that result from a single infectious individual in the population at time t (*5*). We estimated $R(t)$ for type 1 poliovirus transmission from the dates of onset of paralysis of laboratory-confirmed AFP cases and estimates of the incubation and infectious period (*6*). Although only ~1 in 200 cases of wild poliovirus infection result in paralysis (*7*, *8*), and not all of these cases are reported, this estimate of $R(t)$ is independent of the ratio of infections to reported paralytic cases.

The annual variation in the estimated $R(t)$ is particularly marked, with peak transmission about 150% greater than the annual average, compared, for example, with about 30% for measles in industrialized countries (*9*) (Fig. 1). Despite such strong seasonal forcing of transmission, sharp annual peaks in incidence are observed, rather than the more complex dynamics observed for some childhood infections (*10*). This is due to the long infectious period for fecal-orally transmitted polio. In contrast to Uttar Pradesh (UP) and Bihar, the estimated annual average $R(t)$ for poliovirus transmission outside these states has remained below one for the past 3 years, indicating that endemic transmission is no longer supported (Fig. 1C). This is confirmed by analysis of the genetic sequences of wild poliovirus isolates from AFP cases, with all genetic lineages circulating in India since 2003 derived from lineages circulating in UP in earlier years (*11*). The spatiotemporal dynamics of polio incidence now resemble classic "sink-source" dynamics (*12*), with the virus persisting in UP and Bihar (the "source") and expanding during the high-transmission season to infect other areas, including major cities such as Mumbai, but without the establishment of long-term transmission in these areas (Movie S1).

Why is poliovirus transmission persisting in UP and Bihar? Logistic regression reveals a significant association between continued reporting of laboratory-confirmed polio AFP cases by districts during 2000 to 2005 and (i) population density, (ii) the prevalence of diarrhea, and (iii) low routine coverage with three doses of trivalent oral polio vaccine (tOPV), after accounting for differences in the absolute number of children in each district (table S1). Those districts predicted by the regression model to have persistent poliovirus transmission are located mainly in UP and Bihar (Fig. 2). Of course, the regression simply reveals an association between these factors and continued reporting of poliovirus. This may be useful programmatically to identify those districts at higher risk of poliovirus transmission. However, there is likely to be a causal role for high population density, and the poor sanitary conditions that lead to a high prevalence of diarrhea, in the persistence of polio, consistent with the importance of these risk factors for other fecal-oral pathogens (*13*, *14*). High population density and poor sanitation can lead to more frequent infectious contacts and increase levels of excreted poliovirus in the environment. Routine immunization is also likely to be important, providing the very young with a dose of tOPV before they receive doses through supplementary immunization activities. However, routine immunization coverage relies on existing health services and is therefore confounded by socioeconomic and sanitary conditions. This is confirmed in the logistic regression, where the fraction of households reported to have a latrine was significantly associated with polio persistence when routine coverage was excluded from the analysis (table S2).

Children in India receive the majority of their tOPV doses through supplementary immunization activities. These have been increasingly focused on UP and Bihar, such that since 2004, children in these states were reported to have on average received more doses of vaccine than children in other parts of India (Fig. 1, D and E). In fact, at the end of 2005, children under 5 years old were reported to have received on average 15 doses of tOPV in UP and Bihar, compared with 10 in the rest of India, and only 4% of children were reported to have received fewer than 3 doses, of whom 90% were under 6 months old. Even under conditions highly favorable for the fecal-oral transmission of wild poliovirus, this level of vaccine coverage should have eliminated infection. We therefore estimated the efficacy of tOPV by comparing the reported number of doses of vaccine received by polio cases with nonpolio AFP controls (*6*). We found a decline in the relative odds of infection with paralytic polio with increasing number of doses of tOPV that is consistent with a constant, but unexpectedly low, probability of protection per dose (Fig. 3A). The estimated protective efficacy against type 1 poliovirus was just 9% per dose in UP, significantly lower than an estimated 21% per dose in the rest of India (Table 1). Similar results are obtained for

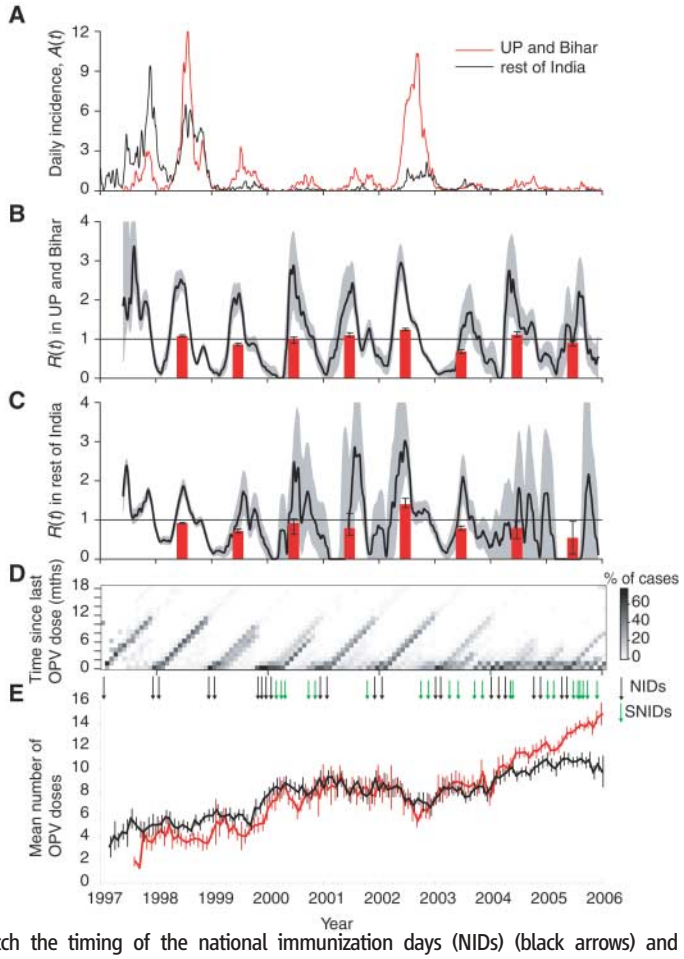

Fig. 1. Poliovirus and vaccination dynamics, 1997 to 2005. (**A**) The estimated daily incidence of laboratory-confirmed type 1 paralytic poliovirus $A(t)$ in UP and Bihar (red) and the rest of India (black). (**B** and **C**) The estimated reproductive number $R(t)$ in (B) UP and Bihar and (C) the rest of India, averaged over a sliding window of 43 days (the mean infectious period; solid line) and over a year (red bars). The gray areas and error bars present the bootstrap 95% confidence intervals about these estimates. (**D**) The distribution of reported time since last OPV dose and (**E**) average number of OPV doses received among 0- to 4-year-old children in UP and Bihar (red) and the rest of India (black), based on age-standardized nonpolio AFP cases. The "pulsed" declines in the average number of doses, and increases in time since last OPV dose, closely match the timing of the national immunization days (NIDs) (black arrows) and subnational immunization days (SNIDs) (green arrows).

type 3 poliovirus, although confidence intervals (CI) are wider, reflecting the lower number of reported cases. Estimates of vaccine efficacy were not significantly affected by the period of analysis or the year of onset of paralysis.

It is well known that trivalent OPV tends to be less efficacious in developing countries (even after accounting for vaccine formulation, quality, and administration) because of host and environmental problems, particularly interference with seroconversion by other enteroviruses and failure of the vaccine virus to establish infection in children with diarrhea (*15*–*17*). However, the per-dose estimate of efficacy for UP is significantly lower than earlier estimates of ~30%, based on seroconversion and small case-control studies in India (*15*, *18*–*20*) [as compared with ~65% in industrialized countries (*21*)]. Pre-release potency testing of tOPV used in India has been satisfactory and loss of potency before administration is unlikely, because vaccine is distributed rapidly and vaccine vial monitors have been used since 1998 to ensure that only vaccine stored at the right temperature is used. Vaccine efficacy may be underestimated if controls are less exposed to wild poliovirus than cases, or if parents overreport the number of doses of vaccine received by their children. However, the potential for differences in exposure was minimized by closely matching cases and controls by location, age, and date of onset of paralysis, and the matching criteria were chosen such that estimates of efficacy were robust to their value (*6*). Also, although some over-reporting of doses may occur, sensitivity analysis demonstrates that a vaccine efficacy comparable to the ~30% per dose found in earlier studies would require reporting of four doses for every one dose received, which is inconsistent with detailed case investigations. Instead, the lower efficacy in UP compared with earlier, mainly urban, studies is likely to be the result of more severe environmental problems. This conclusion is supported by the significantly lower efficacy estimated for tOPV administered in UP compared with other states where population is less dense and sanitary conditions are better.

High population densities and poor sanitation therefore appear to explain the persistence of polio. These factors act to facilitate the transmission not only of poliovirus but also of other enteroviruses and diarrhea, which interfere with the live-attenuated oral vaccine. Therefore, despite the higher number of doses received by children in UP and Bihar, we estimate that only 71% of children under 5 years old in these states were successfully immunized against type 1 poliovirus in early 2005, compared with 85% in the rest of India [based on vaccine coverage estimated from the nonpolio AFP data (*6*)].

The government of India and its partners have responded to this problem with a combination of new approaches and vaccine strategies. The currently high reported coverage with tOPV and low vaccine efficacy means that benefits from increasing vaccine efficacy will outweigh those from increasing vaccine distribution (Fig. 3B). For example, doubling the efficacy of the current vaccine would be equivalent to increasing the average number of doses received by children in UP and Bihar from 15 to 28. The global eradication of type 2, and the elimination of type 3 polio cases in recent years from all of India except a cluster of districts in western Uttar Pradesh, has motivated the introduction of monovalent vaccine, effective only against type 1 poliovirus, to immunization days in selected states beginning in April 2005. Monovalent vaccine has potentially higher efficacy than the trivalent vaccine because of the absence of interference with the two other OPV types (*22*). However, exclusive use of monovalent vaccine during immunization days can put the population at risk of outbreaks of type 3 poliovirus.

Mathematical analysis shows that the optimal balance of monovalent and trivalent vaccine use depends on the relative efficacy of the monovalent vaccine and the transmissibility (basic reproductive number R_0) of type 1 compared with type 3 wild poliovirus (Fig. 3C). Earlier studies of seroconversion from developing countries, including India, suggest a relative monovalent vaccine efficacy between 2.0 and 2.5 times that for trivalent vaccine (*15*, *22*, *23*). If types 1 and 3 were to have equivalent R_0, then support for monovalent vaccine use would be borderline (Fig. 3C). However, the lower incidence of type 3 despite broadly equivalent efficacy of tOPV against types 1 and 3—both in this study (Table 1) and in developing-country studies of seroconversion after administration of "balanced" formulations of tOPV (*15*)—suggests a lower transmissibility and/or lower pathogenicity (case-to-infection ratio) for type 3 (*8*). Lower transmissibility is consistent with the observation of a lower prevalence of antibodies against type 3 in India before vaccination (*24*, *25*). In this case, use of monovalent vaccine

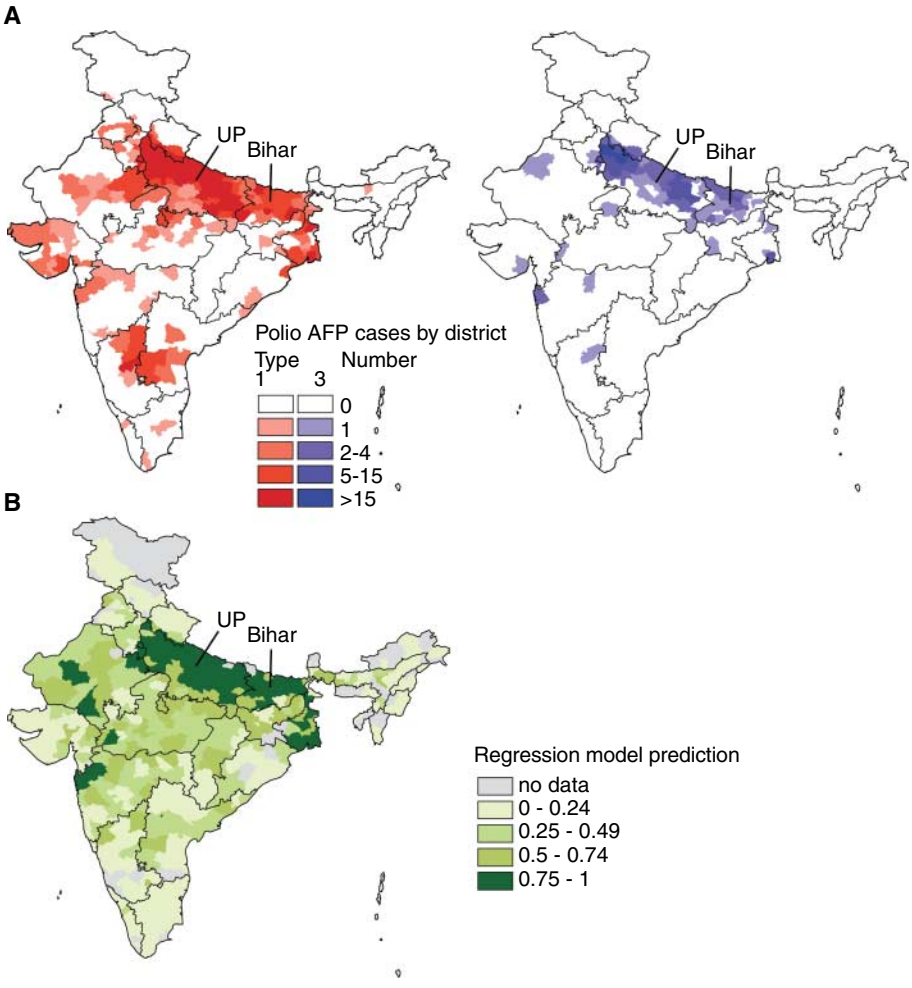

Fig. 2. Location of persistent poliovirus transmission in India. (**A**) Incident laboratory-confirmed type 1 and type 3 polio cases by district for the period 2000 to 2005. (**B**) Regression model estimate of the probability of poliovirus persistence (≥1 case of polio over 2000 to 2005) in each district, based on the number and density of children, the reported prevalence of diarrhea, and routine coverage with three doses of tOPV.

against type 1 is supported, with the amount distributed depending on the relative transmissibility (Fig. 3C). In districts where type 3 has been absent for several years, a greater fraction of vaccine doses distributed can be monovalent, depending on the risk of importation of type 3. In these districts, monovalent vaccine use has the potential to halve the population susceptible to type 1, assuming that coverage is maintained at its current level, substantially increasing the probability of interrupting transmission.

With new vaccine strategies based on careful use of monovalent vaccine targeted at districts with high population densities and poor sanitation, the analyses presented here suggest that wild poliovirus could soon be eliminated from India. Achieving this goal may also be facilitated by future improvements in sanitation, which can reduce transmission of both poliovirus and other infections that interfere with tOPV. Critical to the success of these new strategies will be continued dialogue and engagement with local communities to ensure high coverage with the appropriate vaccine.

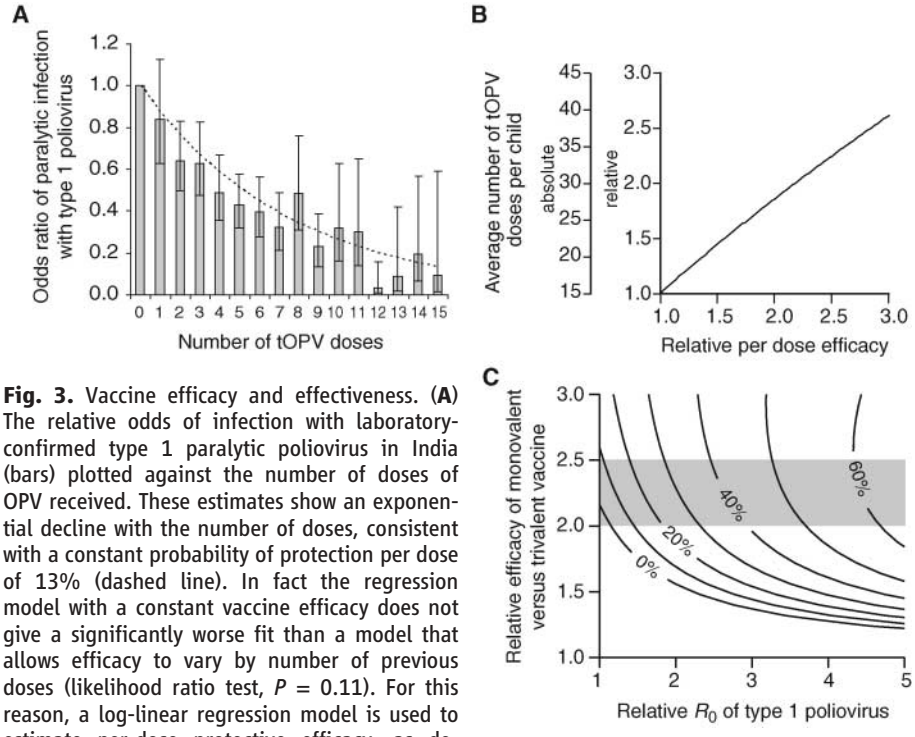

Fig. 3. Vaccine efficacy and effectiveness. (**A**) The relative odds of infection with laboratory-confirmed type 1 paralytic poliovirus in India (bars) plotted against the number of doses of OPV received. These estimates show an exponential decline with the number of doses, consistent with a constant probability of protection per dose of 13% (dashed line). In fact the regression model with a constant vaccine efficacy does not give a significantly worse fit than a model that allows efficacy to vary by number of previous doses (likelihood ratio test, $P = 0.11$). For this reason, a log-linear regression model is used to estimate per-dose protective efficacy, as described in (*6*). The error bars indicate 95% confidence intervals. (**B**) The absolute and relative increase in the average number of doses of OPV received by children less than 5 years old that would be required to achieve the same reduction in the effective reproductive number for poliovirus transmission as a given increase in vaccine efficacy. (**C**) A contour plot indicating the percentage of all doses of OPV administered that should be monovalent type 1 to minimize the average effective reproductive number of wild poliovirus types 1 and 3, for different relative efficacies of the monovalent versus trivalent vaccine and different relative transmissibility R_0 of type 1 versus type 3 (*6*). The expected relative efficacy of the monovalent vaccine is between 2 and 2.5 and is highlighted on the plot by the gray rectangle. Below the 0% contour, all doses should be trivalent. In (B) and (C), vaccine efficacy is assumed to be 9%, with an average child having received 15 doses of vaccine, in agreement with data from UP at the end of 2005.

Table 1. Estimates of trivalent OPV efficacy in India, 1997 to 2005. The per-dose protective efficacy of the vaccine was estimated from the reported number of OPV doses received by polio AFP cases compared with matched nonpolio AFP controls, using conditional logistic regression (*6*). Regression model 1 provides an estimate for all India, whereas model 2 includes an interaction term between efficacy and location.

Poliovirus	Regression model	Location	Cases	Matches	Vaccine efficacy (%) (95% CI)
Type 1	Model 1	All India	4421	1627	13 (10–16)
	Model 2	Rest of India	1512	361	21 (15–27)
		Bihar	387	158	18 (9–26)
		Uttar Pradesh	2522	1108	9 (6–13)*
Type 3	Model 1	All India	1204	474	13 (7–18)
	Model 2	Rest of India	221	79	21 (8–33)
		Bihar	136	53	22 (4–36)
		Uttar Pradesh	847	342	9 (3–15)

*Significantly different from rest of India, $P < 0.01$

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Supporting Online Material

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