

30. A. V. Budanov, A. A. Sablina, E. Feinstein, E. V. Koonin, P. M. Chumakov, unpublished data.
31. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Supporting Online Material

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Materials and Methods

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Curcumin, a Major Constituent of Turmeric, Corrects Cystic Fibrosis Defects

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Cystic fibrosis is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). The most common mutation, $\Delta F508$, results in the production of a misfolded CFTR protein that is retained in the endoplasmic reticulum and targeted for degradation. Curcumin is a nontoxic Ca-adenosine triphosphatase pump inhibitor that can be administered to humans safely. Oral administration of curcumin to homozygous $\Delta F508$ CFTR mice in doses comparable, on a weight-per-weight basis, to those well tolerated by humans corrected these animals' characteristic nasal potential difference defect. These effects were not observed in mice homozygous for a complete knockout of the CFTR gene. Curcumin also induced the functional appearance of $\Delta F508$ CFTR protein in the plasma membranes of transfected baby hamster kidney cells. Thus, curcumin treatment may be able to correct defects associated with the homozygous expression of $\Delta F508$ CFTR.

The $\Delta F508$ CFTR mutation accounts for ~69% of all cystic fibrosis (CF) alleles, and ~90% of CF patients carry at least one copy of $\Delta F508$ CFTR (1). Although it is functional as a chloride channel (2), the misfolded $\Delta F508$ CFTR protein is retained in the endoplasmic reticulum (ER) through interactions with elements of the ER's quality control chaperone machinery and targeted for subsequent degradation in the proteasome (3–5). Many ER lumen chaperones are calcium-binding proteins (6, 7), suggesting the possibility that treatments capable of reducing the concentration of calcium in the ER lumen might interfere with chaperone function and thus permit $\Delta F508$ CFTR to escape. Incubation of human cell lines expressing $\Delta F508$ CFTR with sarcoplasmic/endoplasmic reticulum calcium pump (SERCA) inhibitors results

in the release of the ER-retained $\Delta F508$ CFTR protein, permitting it to achieve its characteristic functional residence at the cell surface (8). To identify nontoxic compounds that may work through similar mechanisms, we tested the capacity of curcumin, a relatively low-affinity SERCA pump inhibitor (apparent $K_i \sim 5$ to 15 μM) (9–11), to correct aspects of the CF defect in cell lines and mice expressing $\Delta F508$ CFTR.

To determine whether oral curcumin treatment could safely effect the functional redistribution of the $\Delta F508$ protein in the context of a living CF-affected organism, gene-targeted mice homozygous for the $\Delta F508$ mutation were given 45 mg of curcumin per kilogram of body weight by mouth daily for 3 days. The dose administered was chosen to approximate, on a weight per weight basis, curcumin doses that have been well tolerated in humans in previous studies (12). The curcumin was given either as a once per day bolus or as a divided dose three times daily. Following treatment, the membrane potential difference across the nasal epithelia [nasal potential difference (NPD)] was measured (13). In untreated CF-affected animals the nasal epithelium exhibited a large, lumen-negative potential that was sensitive to amiloride, reflecting electrogenic Na^+ absorption (Fig. 1A) (8, 14). Removal of luminal Cl^-

and exposure to isoproterenol did not substantially alter the potential in untreated $\Delta F508$ animals (15, 16). After curcumin treatment, the average baseline NPD decreased from -27.9 ± 0.77 mV to -10.8 ± 0.62 mV, approaching the values in wild-type mice (-8.36 ± 0.55 mV). In addition, perfusion with the low Cl^- solution and subsequent addition of isoproterenol each resulted in hyperpolarizations of the NPD similar to those seen with wild-type animals (Fig. 1A). Thus, there was a correction of both the baseline and isoproterenol-stimulated components of the NPD trace after treatment with orally administered curcumin. In contrast, phenylbutyrate treatment of the CF mice produced an effect only on the isoproterenol-stimulated component, consistent with what has been observed in human clinical trials (17) (fig. S1).

To test the specificity of these effects for $\Delta F508$ CFTR, curcumin was administered to homozygous CFTR knockout mice (18, 19). These animals do not express any CFTR protein but retain the remaining complement of transport systems that is associated with epithelial fluid and electrolyte secretion. We found that curcumin treatment did not correct the abnormal NPD measured in the CFTR knockout mice (Fig. 1B).

To assess whether curcumin altered intestinal ion transport, we measured the rectal potential difference (RPD) in $\Delta F508$ CF mice before and after treatment with curcumin. RPD measurements obtained from $\Delta F508$ CF mice differ in two major characteristics from those derived from wild-type littermates (20). First, the baseline RPD of the $\Delta F508$ CF mice is less negative than the baseline RPD of the wild-type mice. Furthermore, the RPD of wild-type mice, but not $\Delta F508$ CF mice, hyperpolarizes in response to forskolin. The lack of response in the CF animals is believed to result from the absence of functional CFTR in the rectal mucosa. We found that after treatment with curcumin, the CF mice had a 4.46 ± 1.0 mV hyperpolarization in response to forskolin (Fig. 2A). This response is approximately 91% of the response observed in the wild-type and heterozygote mice examined in this study.

We examined the processing of $\Delta F508$ CFTR protein expressed in the baby hamster kidney (BHK) cell line. The ER-retained $\Delta F508$ CFTR protein is core-glycosylated (4), whereas wild-type CFTR acquires complex glycosylation (4, 21). Incubation of BHK cells expressing

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$\Delta F508$ CFTR at 26°C allows a portion of the $\Delta F508$ CFTR protein to transit the biosynthetic pathway and acquire complex glycosylation (21). This behavior was also detected in cells treated with varying concentrations of curcumin at 37°C (Fig. 3A).

Surface delivery of the $\Delta F508$ CFTR protein in curcumin-treated cells was confirmed through surface labeling experiments performed with BHK cells that express CFTR proteins carrying triple hemagglutinin (HA) tags in their fourth extracellular loops. Intact cells were incubated at 0°C with an antibody directed against HA, followed by ¹²⁵I-conjugated secondary antibody. Curcumin treatment resulted in the surface expression of a quantity of $\Delta F508$ CFTR protein that was ~25% of that which could be achieved through low-temperature incubation (Fig. 3B). The functional competence of this cohort of surface $\Delta F508$ CFTR was established by measuring iodide efflux in BHK cells expressing $\Delta F508$ CFTR (21, 22). The peak rate of cyclic adenosine monophosphate (cAMP)-stimulated iodide efflux was ~40% higher in curcumin-treated cells than it was in cells treated with dimethyl sulfoxide (DMSO) vehicle alone (Fig. 3C). These effects were not observed in cells expressing the wild-type CFTR protein. Thus, curcumin treatment is able to induce the functional plasma membrane localization of the $\Delta F508$ CFTR protein.

The $\Delta F508$ CFTR protein interacts with the ER chaperone protein calnexin, which may play an important role in its ER retention (23). To determine whether curcumin treatment influences this interaction, we performed coimmunoprecipitation studies. Calnexin was readily detected in immunoprecipitates prepared from the $\Delta F508$ CFTR-expressing Chinese hamster ovary (CHO) cells (24). Calnexin did not appear to coprecipitate with the $\Delta F508$ CFTR protein when the CHO cells were treated with 50 μ M curcumin for 3 hours before cell lysis (Fig. 3D). Thus, curcumin's capacity to release a cohort of $\Delta F508$ CFTR from the ER correlated with the dissolution of the calnexin- $\Delta F508$ CFTR interaction.

Homozygous $\Delta F508$ CFTR mice are extremely susceptible to gastrointestinal obstruction, leading to considerable mortality. It has been shown that the osmotic laxative Colyte (Schwarz Pharma, Milwaukee, WI) markedly increases these animals' survival rate (25). We treated homozygous $\Delta F508$ CFTR mice with oral curcumin and compared their survival to mice given no treatment or the standard Colyte treatment. Six of the ten mice in the no-treatment group died of intestinal obstruction within 10 weeks (Fig. 2B). This 60% mortality rate is similar to values previously reported for this CF mouse model (26). Only one mouse in each of the

Colyte- ($n = 10$) and curcumin-treated groups ($n = 10$) died. The Colyte-treated mice gained 0.99 ± 0.47 g of weight per week, which was similar to the 0.77 ± 0.13 g/week gained by the curcumin-treated group. These gains can be contrasted to the

average 0.40 ± 0.43 g/week of weight loss observed in the untreated mice (Fig. 2C).

Our previous work has demonstrated that several other structurally diverse SERCA pump inhibitors, including thapsigargin and 2,5-di-(tert-butyl)-1,4-hydroquinone (DBHQ),

Fig. 1. Nasal potential difference measurements in curcumin-treated and untreated CF mice. (A) The mean NPD observed after treatment with oral curcumin. NPD was measured for untreated wild-type mice (gray filled squares, $n = 7$), untreated $\Delta F508$ CFTR CF mice (gray filled circles, $n = 11$), $\Delta F508$ CFTR CF mice administered curcumin (45 mg/kg) by oral gavage once a day for three consecutive days (red filled circles, $n = 8$), and $\Delta F508$ CFTR CF mice administered curcumin (15 mg/kg) by oral gavage three times a day for three consecutive days (orange filled circles, $n = 10$). (B) NPD was measured in oral curcumin-treated and untreated CFTR knockout mice. Knockout mice were administered curcumin (15 mg/kg) by oral gavage three times a day for three consecutive days. Gray filled symbols represent untreated animals ($n = 4$), and orange filled symbols represent treated animals ($n = 4$). Standard error bars are indicated in all traces. Solution changes are indicated by the arrows.

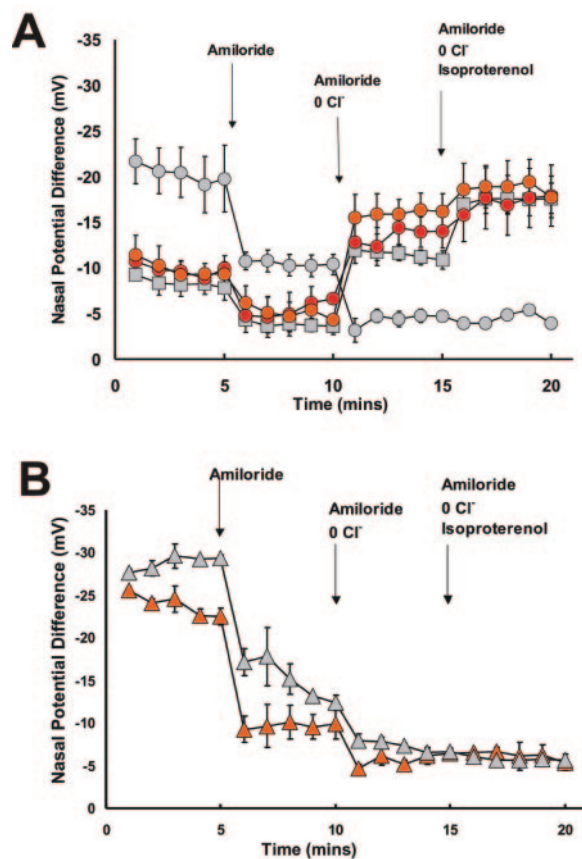
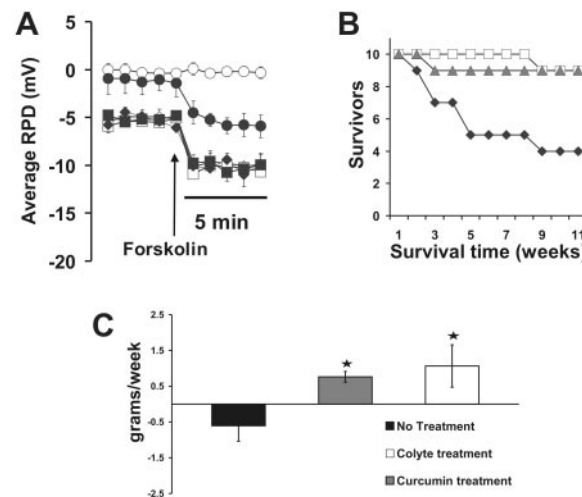


Fig. 2. Effects of curcumin treatment on response of rectal potential difference (RPD) to forskolin, on survival, and on weight gain. (A) RPD measurements were performed with Cl⁻ free solution containing amiloride and then in a solution containing low chloride, amiloride, and forskolin. Solution changes are indicated by arrows. Circles represent $\Delta F508$ CFTR mice, diamonds represent heterozygotes, and squares represent wild-type mice. Open symbols correspond to pretreatment animals, while closed symbols correspond to curcumin-treated animals ($n = 7$ for pretreatment animals and $n = 6$ for posttreatment animals for each group). Standard error bars are indicated in all traces.



(B) Survival was documented for 10 weeks after weaning. Open squares represent Colyte-treated mice ($n = 10$), gray triangles represent curcumin-treated mice ($n = 10$), and black circles represent untreated mice ($n = 10$). Mortality in each case was due to intestinal obstruction. (C) Weight gain was monitored over the course of the 10-week survival study depicted in (B). The bars represent the average weekly weight gain in grams \pm SEM, $n = 10$ mice for each group (6 mice died in the no-treatment group; therefore, the average weight gain reflects 3 to 10 mice in this group). Stars represent a significant difference from untreated animals ($P \leq 0.01$) (ANOVA, Kruskal-Wallis).

share curcumin's ability to induce the functional expression of the $\Delta F508$ CFTR protein, both in vitro and in vivo (8). A number of ER chaperone polypeptides are calcium-binding proteins (6, 7), which suggests that these compounds may exert their effects by altering the concentration of free calcium in the ER lumen, thus perturbing the capacity of calcium-dependent chaperone mechanisms to recognize and retain the misfolded $\Delta F508$ CFTR protein. It is worth noting, however, that curcumin exhibits structural similarities to isoflavanoid compounds that may bind directly to the CFTR protein and alter its channel properties (27). It is also possible, therefore, that curcumin may bind directly to CFTR and that such a direct interaction may stabilize its tertiary struc-

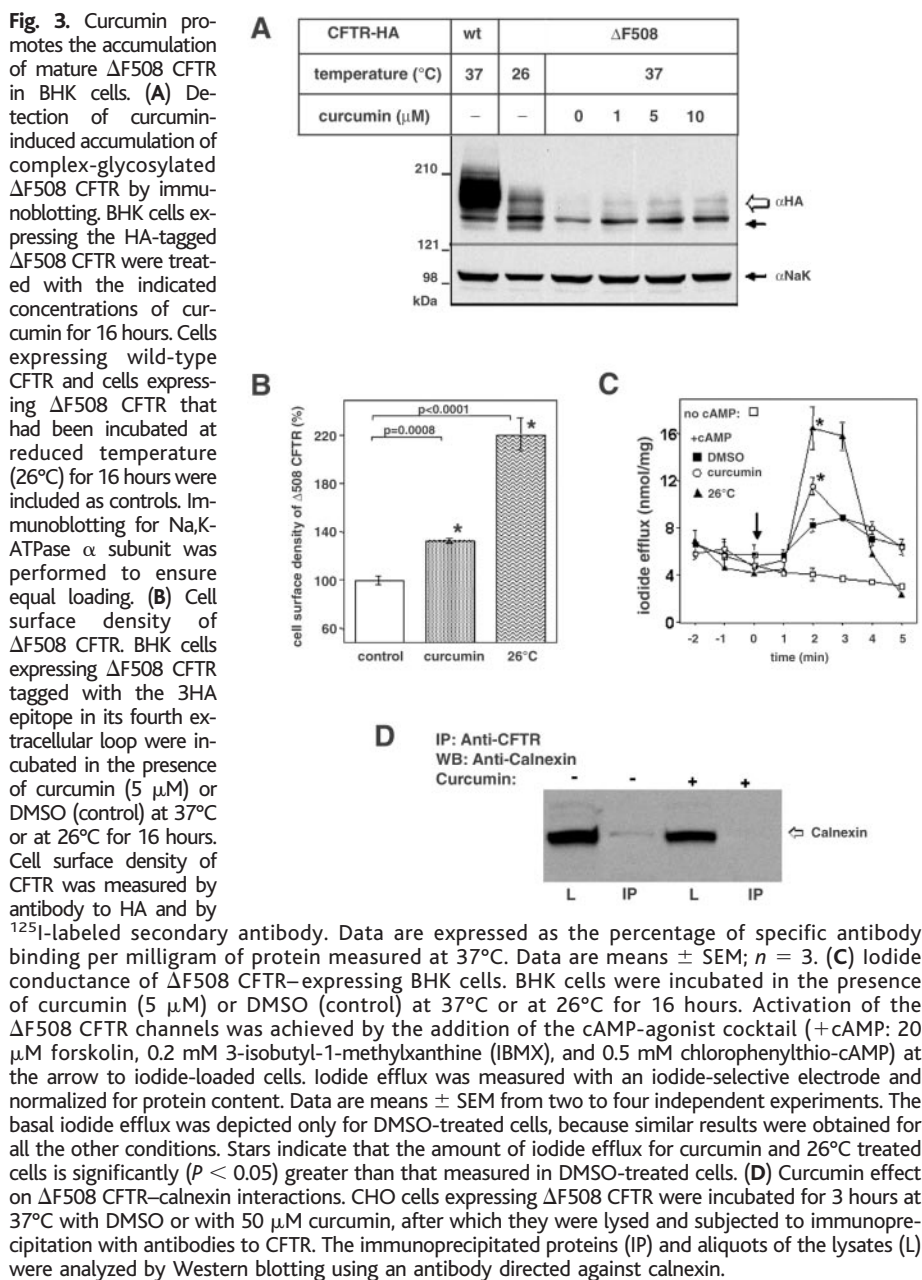
ture, thus permitting it to evade the ER quality-control machinery.

Human studies indicate that curcumin is tolerated in extremely large oral doses without apparent toxicity (12). The dose employed in the present animal experiments corresponds, on the basis of mg/kg scaling, to doses of commercially available curcumin products that are routinely consumed. This extensive experience with curcumin, both in animal models and in patients, coupled with its apparent lack of adverse effects, could facilitate the translation of the data presented here to a human clinical trial. It must, of course, be noted that the success obtained in the present animal experiments provides no guarantee that similar results will be obtained with curcumin in CF patients. Issues relating to curcumin's bioavailability, to species-specific path-

ways through which it may be metabolized, and to the extent to which the mouse model accurately recapitulates relevant features of the CF phenotype could all diminish curcumin's potential to alter $\Delta F508$ CFTR function in the setting of the human disease. The data presented here, however, suggest that curcumin and curcumin derivatives represent promising new candidate compounds that may prove useful in the search for small-molecule pharmacotherapies for CF and for other protein-folding diseases.

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 Materials and Methods
 Table S1
 References

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