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Positively Selected *G6PD*-Mahidol Mutation Reduces *Plasmodium vivax* Density in Southeast Asians

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Glucose-6-phosphate dehydrogenase (G6PD) deficiency—the most common known enzymopathy—is associated with neonatal jaundice and hemolytic anemia usually after exposure to certain infections, foods, or medications. Although *G6PD*-deficient alleles appear to confer a protective effect against malaria, the link with clinical protection from *Plasmodium* infection remains unclear. We investigated the effect of a common G6PD deficiency variant in Southeast Asia—the *G6PD-Mahidol*^{487A} variant—on human survival related to *vivax* and *falciparum* malaria. Our results show that strong and recent positive selection has targeted the Mahidol variant over the past 1500 years. We found that the *G6PD-Mahidol*^{487A} variant reduces *vivax*, but not *falciparum*, parasite density in humans, which indicates that *Plasmodium vivax* has been a driving force behind the strong selective advantage conferred by this mutation.

Malaria is a major cause of human mortality worldwide and is considered to be one of the strongest known forces of evolutionary selection in the recent history of the human genome (*1*). Host genetic defense mechanisms are likely to have evolved to resist malaria infection in regions where the parasites have been historically prevalent. Among malaria-causing parasites, *Plasmodium falciparum* and *Plasmodium vivax* seem to have exerted strong selective pressure on the cellular phenotype of human erythrocytes, causing increased prevalence of hemoglobinopathies and other inherited blood disorders (*1*).

Glucose-6-phosphate dehydrogenase (G6PD) is an X-linked essential enzyme that plays a key role in protecting cells from oxidative stress and is particularly important in red blood cells. G6PD deficiency, affecting more than 400 million people worldwide, is associated with several clinical disorders including neonatal jaundice, hemolytic anemia following infection by certain pathogens, and favism (*2*). The high overall frequency of *G6PD*-deficient alleles in the population is thought to result from their protective effect against malaria. Evolutionary studies of the *G6PD* locus suggest that local and recent positive selection has targeted the *G6PD*-deficient allele *G6PD*$^{A-}$ in Africa (*3*, *4*) and that this process started 2500 to 3800 years before present (YBP) (*5*). These observations are consistent with signs of recent expansion of *P. falciparum* in Africa (*6*). However, the clinical link between G6PD deficiency and malaria is less clear. Although a clinical protective effect of G6PD deficiency against human lethal malaria, *P. falciparum*, has been shown in Africa (*7*), several other reports have not found an association (*8*, *9*).

Most clinical, epidemiological and evolutionary studies of the relation between G6PD deficiency and malaria protection have focused on *falciparum* malaria, particularly in Africa. The role of *G6PD*-deficient alleles in the susceptibility, or resistance, to *vivax* malaria has not been accurately tested and remains anecdotal. Nevertheless, *P. vivax* imposes a considerable burden of disease on the human population and, historically, has been associated with considerable mortality and decreased fertility in human populations (*10*). Both *P. falciparum* and *P. vivax* coexist in Southeast Asia, with *P. vivax* accounting for over half of malaria cases. Moreover, there is increasing evidence for an ancient origin of *P. vivax* in Asia (*11*), where its presence apparently predates that of *P. falciparum* (*12*).

We investigated whether G6PD deficiency increases human survival in Southeast Asia, through its effects on *vivax* and *falciparum* malaria, in an evolutionary and epidemiological study of the *Mahidol*^{487A} mutation (MIM no. 305900.005). This *G6PD*-deficient variant occurs throughout greater Southeast Asia, including mainland China, and is most common in Myanmar [Burma] (*13*). The average allele frequency in Thailand is 12%, but there is distinct local heterogeneity with increased frequency on the western border, particularly in the Mon, Burmese, and Karen populations (*13*, *14*). This mutation is classed as a moderate-to-mild G6PD variant with a reduction of 5 to 32% of wild-type activity levels in healthy individuals (*13*, *15*).

We first used an evolutionary approach to detect the potential molecular signature of positive selection at the *G6PD-Mahidol*^{487A} mutation. We genotyped 30 single-nucleotide polymorphisms (SNPs) (including the *Mahidol*^{487} variant) (table S1) dispersed along a 2.4-Mb region encompassing *G6PD* (Fig. 1A) in a panel of 384 unrelated individuals, the majority of whom are Karen, living in the Suan Phung district of Thailand (*16*). After reconstructing the phase of extended haplotypes (*16*), we implemented the long-range haplotype (LRH) test, which identifies alleles that have undergone recent positive selection, i.e., alleles associated with high levels of extended homozygosity at nearby markers and present at high population frequencies (*3*). The frequency of the *Mahidol*^{487A} mutation in our population sample was 24% and showed very high levels of extended homozygosity: 63% of *Mahidol*^{487A}-bearing haplotypes showed complete haplotypic conservation over the entire 2.4-Mb region (Fig. 1, A and B). The high level of homozygosity surrounding *Mahidol*^{487A} was highly significant given its frequency ($P < 10^{-4}$), when compared with the empirical distribution of allelic homozygosity versus frequency for all X-linked HapMap Phase II SNPs in Han Chinese (*17*), after matching for SNP density (Fig. 1C). However, the power of the LRH test can be challenged by uncertainties related to phase reconstruction and specific population histories (*18*). To circumvent this, we compared the observed allelic homozygosity associated with *Mahidol*^{487A} in males only (whose haplotypic phase is known) with simulations of a ~1-Mb recombining X-linked region for a population experiencing different demographic regimes (*16*). Consistently, we found a highly significant signal of positive selection at *Mahidol*^{487A}, independently of the demographic scenario considered ($P < 10^{-4}$ for the constant population size, the bottleneck and the expansion models) (Fig. 1D). The low microsatellite diversity associated with *Mahidol*^{487A} (Fig. 1E) confirmed the results based on the LRH test. Together, our evolutionary analyses show that the *Mahidol*^{487A} mutation is under recent and strong positive selection in the Karen population, indicating that this mutation has conferred a strong selective advantage in human survival.

We estimated the age of *G6PD-Mahidol*^{487A} and the selection coefficient that would be consistent with such a strong positive selection. We obtained similar estimates using two different methods (*19*, *20*), which showed that the fre-

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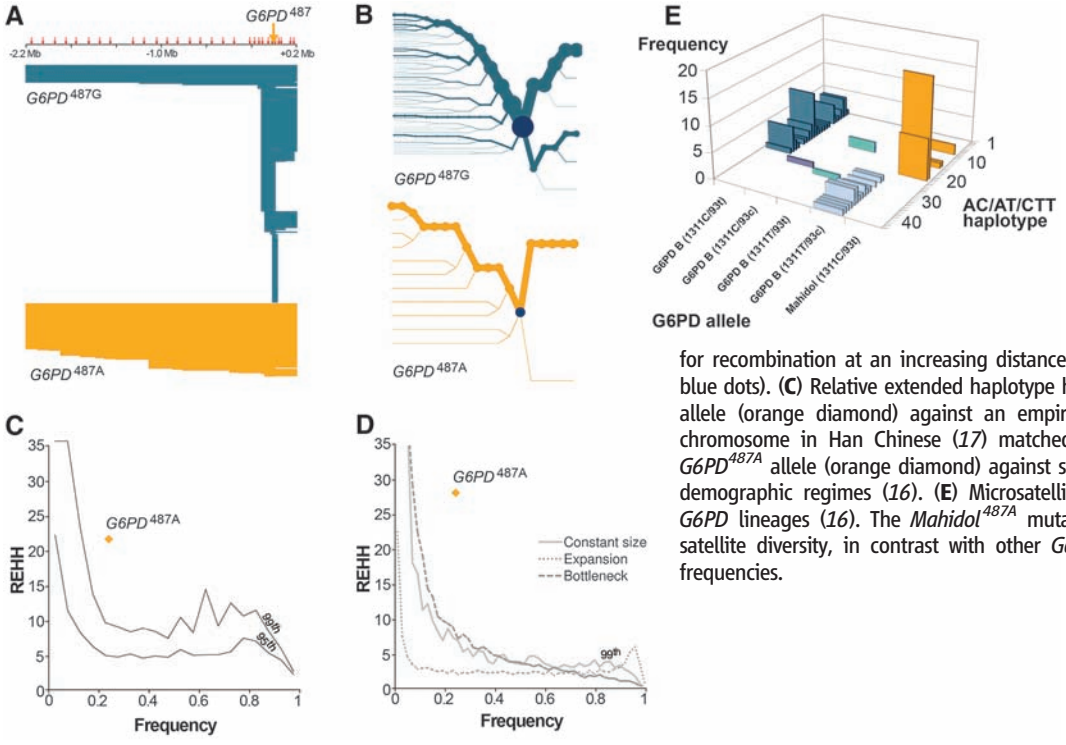

Fig. 1. Positive selection at the *G6PD-Mahidol*^{487A} mutation. (**A**) Comparison of extent of homozygosity flanking the *G6PD*^{487A} (Mahidol) and the *G6PD*^{487G} (non-Mahidol) alleles over a 2.4-Mb region. Positions of the 30 genotyped SNPs are tagged by red arrows. (**B**) Haplotype bifurcation plots for the two *G6PD*^{487} alleles. Line thickness represents the number of haplotypes in our sample. Each successive bifurcation corresponds to evidence for recombination at an increasing distance from the core *G6PD*^{487} alleles (dark blue dots). (**C**) Relative extended haplotype homozygosity (REHH) of the *G6PD*^{487A} allele (orange diamond) against an empirical distribution of REHH for the X chromosome in Han Chinese (*17*) matched for SNP density. (**D**) REHH of the *G6PD*^{487A} allele (orange diamond) against simulated distributions under different demographic regimes (*16*). (**E**) Microsatellite diversity associated with different *G6PD* lineages (*16*). The *Mahidol*^{487A} mutation was associated with low microsatellite diversity, in contrast with other *G6PD* mutations at similar population frequencies.

quency of the *Mahidol*^{487A} mutation started to increase at ~1500 YBP, with a selection intensity of ~0.23 (Table 1). The selection coefficients of *Mahidol*^{487A} are among the strongest detected so far in the human genome, including human immunodeficiency virus (HIV)–protective haplotypes (~0.30) (*21*), malaria-protective *G6PD*$^{A-}$ (~0.2) (*5*, *20*) and *β-globin* variants (~0.26 and ~0.08) (*22*, *23*), as well as lactase persistence (~0.1) (*24*).

We investigated the nature of the selective advantage conferred by the *G6PD-Mahidol*^{487A} mutation by testing its influence on the outcome of infection with either *P. falciparum* or *P. vivax*. To this end, we conducted a community-based longitudinal study in the Suan Phung district of Thailand, which has a total population of 5368. From the 3484 participants of the malaria epidemiology study (*25*), we obtained genotypes at the *G6PD-Mahidol*^{487} position from 925 individuals (*16*). Between 1998 and 2005, there were 1090 *P. falciparum* clinical episodes in 460 of these individuals, and 524 *P. vivax* clinical episodes in 262 of these individuals. Reliable parasite density data was available for 823 observations of *P. falciparum* parasite density in 400 individuals and for 417 observations of *P. vivax* parasite density in 227 individuals. Details on the sample selection procedures are provided in (*16*) and summarized in fig. S1.

To test for a genetic association between *G6PD-Mahidol*^{487A} and the number of *Plasmodium* species clinical cases or parasite density, we performed a family-based association test (FBAT), which corrects for spurious association due to population stratification (*16*). Seventy-seven families were informative for the association with the number of *Plasmodium* species clinical cases, 44 of which were informative for the association with *P. falciparum* density and 35 for the association with *P. vivax* density (i.e., some families were infected by different *Plasmodium* species at different times) (*16*). We found that *Mahidol*^{487A} had no effect on the number of cases of clinical malaria due to either *P. vivax* or *P. falciparum* reported for each individual during the 7-year observation period (*16*). This is likely due to the high heterogeneity of exposure to infection in this area of low transmission intensity, where virtually all infections by either parasite species lead to symptomatic episodes (*25*). Our analyses also revealed that *Mahidol*^{487A} was not significantly associated with *P. falciparum* density, using both annual values of parasite density (*16*) or mean density values across all years under any genetic model (table S2). By contrast, *Mahidol*^{487A} was significantly associated with reduced *P. vivax* density using both annual values of parasite density (χ^2 test, $P = 0.029$) and mean density values across years [χ^2 test; dominant model $P = 0.011$, additive model $P = 0.016$, recessive model $P = 0.048$ (table S2)] taking into account age and environmental covariates that affect parasite density (*16*, *26*). A permutation test with 100,000 iterations confirmed that these results were significant (χ^2 test, $P = 0.017$).

Table 1. Joint estimates of the age and the selection coefficient of the *Mahidol*^{487A} mutation. One generation is considered equivalent to 25 years. ML, maximum-likelihood; CI, confidence interval.

Method	Age (generations)	95% CI	Selection coefficient	95% CI
ML deterministic method (*19*)	60.8	53.7–73.6	0.235	0.20–0.30
Bayesian method (*20*)	64.4	38–94	0.228	0.16–0.40

To verify this association, we performed a population-based association study between *Mahidol*^{487A} and *Plasmodium* parasite density in the whole population, excluding all individuals used in the FBAT analyses (*16*). *Mahidol*^{487A} significantly reduced *P. vivax* parasite density (the most significant P value was obtained for the dominant model, χ^2 test, $P = 0.006$), whereas no association was observed with *P. falciparum* parasite density for any genetic model (table S2). When considering both the family-based and the population-based data sets, *P. vivax* parasite density decreased with age (χ^2 test, $P < 0.001$), indicative of the acquisition of antiparasite immunity (Fig. 2A). Mean *P. vivax* density was reduced by 30% in females heterozygous for the *Mahidol*^{487} mutation (non-*Mah*/*Mah*) and 61% in females homozygous for *Mahidol*^{487A} (*Mah*/*Mah*) compared with non-*Mah*/non-*Mah* females; parasite density was reduced by 40% in hemizygous males for *Mahidol*^{487A} (*Mah*/Y) compared with non-*Mah*/Y males (Fig. 2B). *Mahidol*^{487A} accounted for 3.3% of the observed variation in *P. vivax* density. Although increasing age was again associated with decreasing *P. falciparum* density (χ^2 test, $P < 0.001$) (Fig. 2C), there was

no significant effect of *Mahidol*^{487A} on *falciparum* density (Fig. 2D).

A *Mahidol*^{487A} gene dose effect on *P. vivax* density was observed, whereby parasite density tends to be lower in *Mah*/*Mah* females with respect to both non-*Mah*/*Mah* females and *Mah*/*Y* males (Fig. 2B). Two main factors can explain the observed tendency. First, G6PD activity in heterozygous non-*Mah*/*Mah* females depends on the random inactivation of the X-chromosome: Each heterozygous female will harbor two populations of red blood cells (expressing *Mahidol*^{487A} or *Mahidol*^{487G}), and their relative abundance is unpredictable. Indeed, when measuring G6PD activity in Karen individuals using the fluorescence spot test (*16*), we observed that the non-*Mah*/*Mah* genotype can express either normal or deficient phenotypes (table S3). Second, the *Mahidol*^{487A} mutation markedly increases protein thermal instability with consequences for enzyme activity (*15*). Hence, in *P. vivax* infection, which induces high fever, G6PD deficiency may be more pronounced than in noninfected individuals, which contributes to the reduction in parasite density. Because genotype-phenotype correlations for G6PD can be complex, we analyzed our data using G6PD phenotypes rather than genotypes. We confirmed that both age and G6PD-deficient phenotype significantly decreased *P. vivax* density (χ^2 test, $P < 0.001$ and $P = 0.039$, respectively) (*16*).

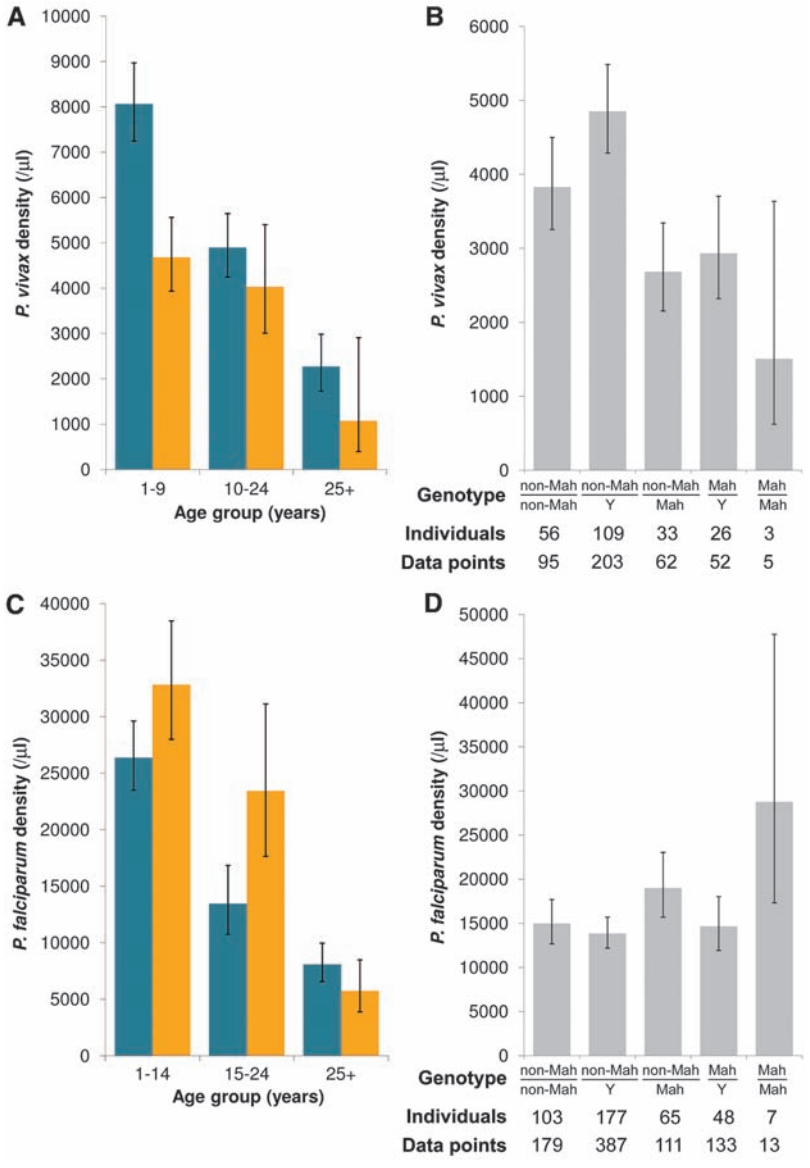

Fig. 2. Effect of *G6PD* status on parasite densities. (**A**) *P. vivax* and (**C**) *P. falciparum* parasite densities (means ± SD) according to age group and *G6PD-Mahidol*^{487} status (blue, non-Mahidol; orange, Mahidol). (**B**) *P. vivax* and (**D**) *P. falciparum* parasite densities (means ± SD) according to *G6PD-Mahidol*^{487} genotype status taking into account age (*16*). Data points refer to the total number of observations of parasite densities obtained for each group of individuals. Note that all data were used, including the FBAT informative families.

Although the precise mechanism underlying this protective effect is unknown, it is likely to be related to the effects of G6PD deficiency on red cell physiology, particularly by increasing oxidative stress. Young red cells (i.e., reticulocytes) contain more antioxidant enzymes than mature red cell populations (*27*). The preference shown by *P. vivax* for reticulocytes suggests that *P. vivax* is more sensitive to oxidative stress than *P. falciparum*, which is known to have no red-cell preference (*28*). Under these conditions, reduced G6PD activity would have a greater effect on *P. vivax*.

The historical expansion of malaria has been linked to that of agriculture, which generated breeding grounds for mosquitoes and increased human population density (*29*), thereby facilitating human-mosquito contact and the conditions for stable malaria transmission. In East Asia, farming is mainly associated with the development of rice culture in China ~8000 YBP. Although there is evidence of rice cultivation in Southeast Asia dating back to 4200 YBP, it developed mainly over the last 2000 years (*30*). The Karen people, belonging to the Sino-Tibetan language group, are thought to descend from Tibetan people who entered Myanmar ~1500 YBP (*31*). It is noteworthy that the estimated age of *Mahidol*^{487A}, at ~1500 YBP, coincides with the proposed arrival of the Karens into the region and with the time at which rice started to be extensively cultured. This supports a link between the selective advantage conferred by the *G6PD-Mahidol*^{487A} mutation and its protective effect against *vivax* malaria.

In conclusion, we showed that the *G6PD-Mahidol*^{487A} mutation has been under strong positive selection for the last 1500 years and that it reduces *P. vivax* parasite density in humans. These findings provide evidence that *vivax* malaria has been a driving force behind the selective advantage conferred by the *Mahidol*^{487A} mutation and supports the notion that *P. vivax* historically had a considerable impact on human health (*10*), at least in Southeast Asia. Indeed, *P. vivax* infection is not only responsible for clinical malarial attacks, but has also been implicated in causing low birth weight (*32*) and malnutrition in children (*33*), both of which have strong impacts on childhood survival. The significant health burden imposed by *P. vivax* has been seriously underestimated, and increased global efforts to combat malaria should encompass *P. vivax* as well as *P. falciparum* malaria.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/326/5959/1546/DC1
Materials and Methods
Fig. S1
Tables S1 to S3
References

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MicroRNA-206 Delays ALS Progression and Promotes Regeneration of Neuromuscular Synapses in Mice

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Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by loss of motor neurons, denervation of target muscles, muscle atrophy, and paralysis. Understanding ALS pathogenesis may require a fuller understanding of the bidirectional signaling between motor neurons and skeletal muscle fibers at neuromuscular synapses. Here, we show that a key regulator of this signaling is miR-206, a skeletal muscle–specific microRNA that is dramatically induced in a mouse model of ALS. Mice that are genetically deficient in miR-206 form normal neuromuscular synapses during development, but deficiency of miR-206 in the ALS mouse model accelerates disease progression. miR-206 is required for efficient regeneration of neuromuscular synapses after acute nerve injury, which probably accounts for its salutary effects in ALS. miR-206 mediates these effects at least in part through histone deacetylase 4 and fibroblast growth factor signaling pathways. Thus, miR-206 slows ALS progression by sensing motor neuron injury and promoting the compensatory regeneration of neuromuscular synapses.

Amyotrophic lateral sclerosis (ALS) is the most common adult motor neuron disease (*1*). Symptoms of the disease include atrophy and paralysis of lower limb and respiratory muscles because of the degeneration of motor neurons. There is currently no effective treatment. Thus, identification of the signaling pathways and cellular mediators of ALS remains a major challenge in the search for novel therapeutics (*2*).

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In light of recent studies implicating microRNAs (miRNAs) in stress responses in muscle (*3*), we investigated whether disease progression in a mouse model of ALS was accompanied by changes in expression of miRNAs. We compared miRNA expression in skeletal muscles from the lower limbs of normal adult mice and G93A-SOD1 transgenic mice (*4*, *5*) that express a low copy number of a mutant form of superoxide dismutase (SOD1) in which glycine-93 is replaced with alanine (G93A-SOD1), as seen in a subset of human ALS patients. These mice recapitulate the progression of human ALS symptoms (*4*, *5*). Of 320 miRNAs tested, the muscle-specific miRNA miR-206 (*6*, *7*) was the most dramatically up-regulated in G93A-SOD1 muscles (Fig. 1A and fig. S1, A and B) (*8*). Up-regulation of miR-206 coincided with the onset of neurological symptoms, as indicated by levels of miR-206 in healthy G93A-SOD1 transgenic mice being similar to those in wild-type littermates (Fig. 1A and fig. S1B).

Because ALS leads to denervation of skeletal muscle (*1*), we determined whether miR-206 up-regulation was a consequence of denervation. Indeed, 10 days after severing the sciatic nerve of wild-type mice to denervate lower leg muscles, levels of mature and primary miR-206 (pri-miR-206) transcripts were robustly increased in three muscles that contain predominantly fast-twitch fibers, extensor digitorum longus (EDL), tibialis anterior (TA), and gastrocnemius/plantaris (G/P) (Fig. 1B and fig. S2) (*9*). miR-206 levels were higher in normally innervated soleus, which contains predominantly slow myofibers, and up-regulation after denervation was correspondingly less substantial (fig. S2).

miR-206 is a skeletal muscle–specific miRNA in humans and mice that is generated from a bicistronic transcript that also encodes miR-133b (fig. S3, A and B) (*7*, *10*). Two other homologous miRNA pairs, miR-1-1/133a-2 and miR-1-2/133a-1, are encoded on separate chromosomes and are expressed in skeletal and cardiac muscle (*6*, *10*). Consistent with its transcription from the same promoter, miR-133b was also up-regulated after denervation, whereas miR-1 and miR-133a were down-regulated (Fig. 1B and fig. S2).

Previous studies have implicated the myogenic basic helix-loop-helix (bHLH) proteins MyoD and myogenin in denervation-dependent gene expression (fig. S4A) (*11*). Three evolutionarily conserved E-boxes (CANNTG), which are binding sites for MyoD and myogenin, are located between –910 and –765 base pairs (bp) upstream of the start of the precursor (pre-miR-206) stem loop, within a genomic region that was previously shown to be enriched for MyoD binding by using chromatin from muscle cells (Fig. 1C and fig. S4B) (*7*). Heterodimers of MyoD and its bHLH partner E12 bind these sites (fig. S4C). In cultured cells, MyoD activated the