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DEC2 modulates orexin expression and regulates sleep

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Adequate sleep is essential for physical and mental health. We previously identified a missense mutation in the human *DEC2* gene (*BHLHE41*) leading to the familial natural short sleep behavioral trait. DEC2 is a transcription factor regulating the circadian clock in mammals, although its role in sleep regulation has been unclear. Here we report that *prepro-orexin*, also known as *hypocretin* (*Hcrt*), gene expression is increased in the mouse model expressing the mutant h*DEC2* transgene (h*DEC2-P384R*). *Prepro-orexin* encodes a precursor protein of a neuropeptide producing orexin A and B (hcrt1 and hcrt2), which is enriched in the hypothalamus and regulates maintenance of arousal. In cell culture, DEC2 suppressed *prepro-orexin promoter-luc* (*ore-luc*) expression through *cis*-acting E-box elements. The mutant DEC2 has less repressor activity than WT-DEC2, resulting in increased orexin expression. DEC2-binding affinity for the *prepro-orexin* gene promoter is decreased by the P384R mutation, likely due to weakened interaction with other transcription factors. In vivo, the decreased immobility time of the mutant transgenic mice is attenuated by an orexin receptor antagonist. Our results suggested that DEC2 regulates sleep/wake duration, at least in part, by modulating the neuropeptide hormone orexin.

sleep | behavior | genetics | mouse model

The timing and duration of sleep are determined by complex mechanisms. Although the circadian clock has been shown to be an integral component of timing regulation (1), the mechanism of duration modulation is not clear. Using genetics of humans with altered sleep patterns, we have reported a number of mutations in circadian clock genes that affect the timing or duration of sleep (2–7). Among these, we reported the Pro384Arg (P384R) mutation (originally called P385R; ref. 5) in the *DEC2* gene (*BHLHE41*) that leads to the natural short sleep phenotype in one human family. DEC2 is a basic helix-loop-helix transcription factor suppressing E-box–mediated transcription (8), which is the basis of the negative feedback loop in the circadian clock (9). We have shown that mouse and *Drosophila* models carrying the h*DEC2-P384R* transgene demonstrate shortened total sleep time compared with wild-type (WT) animals (5). A recent study also reported that carriers of a Tyr362His (Y362H) variant in the human *DEC2* gene are short sleepers who are resistant to sleep deprivation (10), further supporting the important role of DEC2 in sleep homeostasis. However, an understanding of the altered molecular function of mutant DEC2 and of the normal role of wild-type (WT) DEC2 in regulating sleep duration has remained elusive. Although DEC2 is considered a clock protein, knockout of *Dec2* and double knockout of *Dec1* and *2* result in only a subtle circadian rhythm phenotype (11). Furthermore, *DEC2* mutations affect sleep rebound after sleep deprivation in mouse models and humans (5, 10), and *Dec1/2* double knockout influences sleep architecture (12). Thus, it is likely that DEC2 regulates sleep homeostasis directly rather than through clock regulation.

In the present study, we found that *prepro-orexin* [also known as *hypocretin* (*Hcrt*)] expression is enhanced in the mouse model carrying the human *DEC2-P384R* gene. *Prepro-orexin* encodes a neuropeptide precursor protein producing orexin A and orexin B (hcrt1 and 2), which are enriched in the hypothalamus and are involved in many physiological processes, including arousal

(especially consolidation of wakefulness), appetite, mood, reward, and autonomic function (13). Our results indicate that DEC2 binds to the *prepro-orexin* promoter to suppress its expression, and that the repressor function of DEC2 is altered by the P384R mutation. We conclude that DEC2 regulates the orexin signaling pathway, thus affecting human sleep behavior.

Results

To explore the mechanism underlying the behavioral phenotype of decreased sleep time in *DEC2* mutation carriers, we examined the expression of genes regulating sleep homeostasis in a mouse model of the human *DEC2* mutation. We found that *prepro-orexin* (*Hcrt*) gene expression was up-regulated in the hypothalamus of h*DEC2-P384R* transgenic (Tg) mice compared with h*DEC2-WT* Tg mice (Fig. 1A). Mammals have two receptors for orexin peptides, orexin receptor 1 (OX1R) and orexin receptor 2 (OX2R) (13). In h*DEC2* mutant mice, the expression of OX2R was also slightly increased (Fig. 1A). To confirm the up-regulation of *orexin* at the protein level, we analyzed its expression in the lateral hypothalamus at zeitgeber time 1 (ZT1) by immunohistochemistry using an antibody recognizing orexin precursor protein and the orexin A peptide. The number of orexin-positive cells was increased by the P384R mutation (Fig. 1B). We also generated FLAG-tagged h*DEC2* BAC Tg mice harboring the Y362H mutation and found that these mice showed increased *prepro-orexin* levels (Fig. S1A). Tg mice expressing either Y362H-DEC2 or P384R-DEC2 exhibited increased activity and decreased rest

Significance

Sleep is essential for healthy aging, and most people need approximately 8–8-1/2 hours of sleep per night to feel good and to function optimally. We previously reported a proline-to-arginine mutation in DEC2 that leads to a life-long decrease in daily sleep need. We found that the expression of an important sleep-relevant gene, *orexin*, was increased in the DEC2 mutant mice. Further investigation revealed that DEC2 is a transcriptional repressor for orexin expression, and that mutant DEC2 exerts less repressor activity than WT-DEC2, resulting in increased orexin expression. This study represents the first step toward understanding the underlying molecular mechanism through which DEC2 modulates sleep.
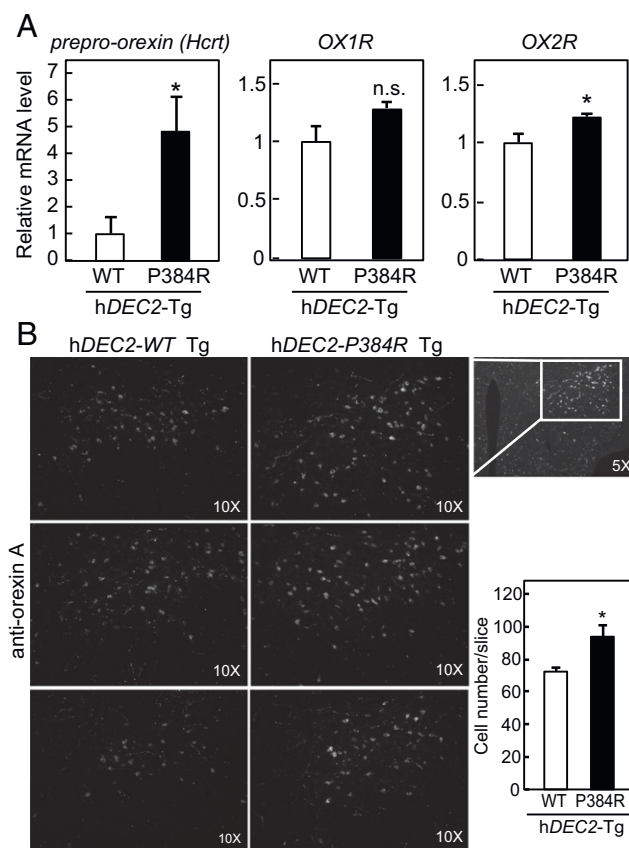
Fig. 1. Expression of orexinergic genes is up-regulated in the h*DEC2-P384R* mice. (*A*) mRNA levels of *prepro-orexin*, *orexin receptor1* (*OX1R*), and *orexin receptor2* (*OX2R*) in hypothalamus of h*DEC2* BAC Tg mice at ZT1. mRNA levels were analyzed by real-time PCR using specific primers of indicated genes and normalized to *Gapdh*. Data are shown as mean ± SEM. n = 3. *P < 0.05, Student's *t* test. (*B*) Immunohistochemistry of orexin A in the lateral hypothalamus of the BAC Tg mice at ZT1. Prepro-orexin and orexin A were immunostained with anti-orexin A antibody, and orexin A-positive cells were counted. Three representative images with the same experimental condition were shown. Data were collected from ∼30 slices and averaged for each mouse. Data are shown as mean ± SEM. n = 5. *P < 0.05, Student's *t* test.

behavior (Fig. S1B), similar to what has been shown previously in the P384R mutant mice (5).

Deletion of orexin neurons or knockout of *prepro-orexin* or orexin receptor genes results in narcolepsy in mammals, indicating that orexin signaling plays a critical role in maintaining arousal and consolidation of sleep (13). Furthermore, administration of orexin A or selective activation of the orexin neurons increases arousal and decreased sleep duration (13). Therefore, modulating orexin expression is a key step in sleep/wake regulation. Since the expression levels of *prepro-orexin* were increased in the mutant mice, we explored the possibility that DEC2 directly modulates the expression of orexin. Previous studies indicated that the 3.2-kb promoter region of *prepro-orexin* is sufficient for endogenous expression, which was confirmed by generating *prepro-orexin* promoter-driven LacZ Tg mice (14). Within this 3.2-kb region, two elements—orexin regulatory elements 1 and 2 (OE1 and OE2)—were recognized because of a high similarity between mice and humans. Of these, OE1 plays a more important role in regulating orexin expression (Fig. 2*A*) (15). To examine the role of DEC2 in orexin expression, we cloned promoter regions of the *prepro-orexin* gene including only OE1 or both OE1 and OE2. A luciferase assay using *prepro-orexin promoter luciferase* (*ore-luc*) constructs showed that DEC2 protein decreased *ore-luc* activity,

especially with the 0.6-kb region of the promoter containing OE1 (Fig. 2*B*). We then examined the occupancy of endogenous mouse DEC2 in the promoter region of the *prepro-orexin* gene in brain and observed a chromatin immunoprecipitation (ChIP) signal in the OE1 region (0.6 kb; primer sets 1 and 2) but not outside of the 3.2-kb region (primer set 3) (Fig. 2*C*).

DEC1 and DEC2 directly or indirectly (through interaction with other transcription factors) bind to DNA and modulate the transactivation of CLOCK, BMAL1, MYC, MyoD1, and E12/47, all of which are E-box–binding transcription factors (8, 9, 16–18). DEC1/2 proteins form homodimers and directly bind to class B E-boxes (CACGTG), which is the CLOCK-BMAL1 consensus sequence (8, 19). DEC1/2 also form complexes with other transcription factors, such as MyoD1 and its binding partner E12/47,
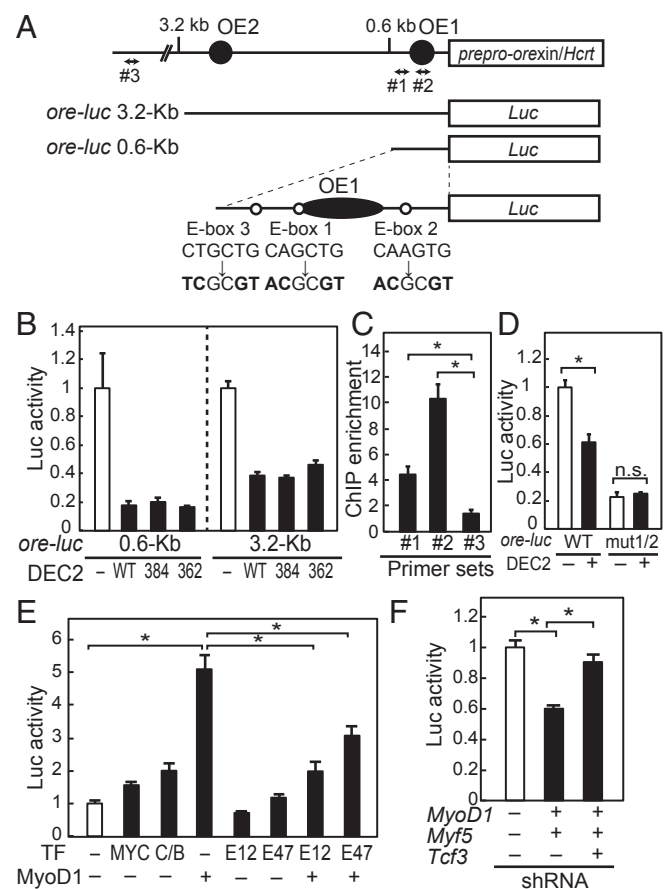

Fig. 2. DEC2 inhibits E-box–mediated transcription of *prepro-orexin*. (*A*) Schematized model of the human *prepro-orexin* gene promoter. OE1–3 are the primer sets used for ChIP assays in *C*. (*B*) Luciferase assay in HEK293 cells. DEC2 expression inhibits *ore-luc* activity. Firefly luciferase activity was normalized to *Renilla* luciferase activity. (*C*) ChIP with mouse brain and anti-DEC2 antibody. Brains (including hypothalami) were collected at ZT8 and homogenized. ChIP signals were determined by real-time PCR using the primer sets shown in *A*. Signals were normalized to normal mouse IgG. Data are shown as mean ± SEM. n = 4. *P < 0.05, Tukey's test. (*D*) Luciferase assay using E-box mutant constructs. Mutations are shown at the bottom of *A*. DEC2 repressor activity was not observed for E-box mutant *ore-luc*. Data are shown as mean ± SEM (n = 3). *P < 0.05, two-way ANOVA followed by post hoc test. (*E*) The effect of transcription factors known to bind E-boxes. FLAG-tagged transcription factors (TF) were expressed in HEK293 cells. C/B indicates FLAG-tagged CLOCK and BMAL1. Data are shown as mean ± SEM. n = 4. *P < 0.05, Tukey's test. (*F*) The effect of *MYOD1* and *MYF5* knockdown by shRNA on *ore-luc* activity. shRNA of *MYOD1/MYF5* (with/without *TCF3* shRNAs) were transfected into HEK293 cells. Data are shown as mean ± SEM. n = 4. *P < 0.05, Tukey's test.

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to bind to another type of E-box sequence (CAGCTG) (18, 20) found in promoter region of orexin (Fig. 2A). Thus, we introduced mutations in putative E-boxes in the 0.6-kb region to examine the effect on DEC2 repressor activity (Fig. 2A). The mutations at E-boxes 1 and 2 attenuated DEC2 activity, suggesting that DEC2 represses *ore-luc* activity via these two elements (Fig. 2D). We then examined which binding partner of DEC2 is involved in orexin regulation mediated by these E-box sequences. Among E-box–binding transcription factors, MyoD1 dramatically activated *ore-luc* (Fig. 2E). In contrast, the transactivation of *ore-luc* by MYC and CLOCK/BMAL1 was much less than that by MyoD1.

Although MyoD1 is known for its role in regulating muscle cell differentiation and muscle regeneration, it is nonetheless widely expressed in many tissues, including brain. In the regulation of muscle differentiation, MyoD1 cooperates with E12 and E47 proteins, which are splice variants encoded by the *TCF3* gene (16, 17, 20, 21). MyoD1/E47 heterodimer activates *creatine kinase, M-type* (*Ckm*) gene expression (a physiological target of MyoD1) (20), and DEC2 serves as repressor for MyoD1/E47 activity on *Ckm* expression. Thus, we asked whether E12/E47 also influences MyoD1 activity on *ore-luc*. In contrast to the case for *Ckm*, we found that overexpression of E12 and E47 inhibited MyoD1 activation of *ore-luc*. We then knocked down *MYOD1* and its homolog (*MYF5*), which has a redundant role in muscle differentiation (22). Double knockdown of *MYOD1* and *MYF5* by shRNAs resulted in decreased *ore-luc* activity, while additional knockdown of *TCF3* eliminated the reduction almost completely (Fig. 2F), consistent with E12 and E47 serving as repressors of MyoD1 for the *prepro-orexin* promoter. Taken together, these results suggest that MyoD1, E12, and E47 participate in the regulation of *prepro-orexin* expression, with E12 and E47 acting as repressors of MyoD1.

Interestingly, although DEC2 strongly inhibited *ore-luc* activation by MyoD1 (Fig. 3A), DEC2 repressor activity was completely abolished by the expression of E12 or E47 (Fig. 3A), with no effect on DEC2 expression levels (Fig. S2). It is possible that in the formation of functional complexes of transcription factors (MyoD1, E12/47, and DEC2), excessive E12/47 can interfere with proper DEC2 interaction and abolish DEC2 function. To confirm that E-box1 is the target of MyoD1, we used the mutated E-box sequences described above (Fig. 2 A and D). The mutations in E-box1 reduced MyoD1 transactivation and attenuated the DEC2 repressor activity of MyoD1 (Fig. 3B). Consistent with this, E-box1 is identical to the consensus sequence for MyoD1 rather than other DEC2-binding proteins, such as CLOCK and BMAL1. Moreover, sequence analysis revealed that only E-box1 is conserved in humans and mice *prepro-orexin* promoters, implying that E-box1 is more likely to be functional in vivo. However, mutations spanning all E-boxes within the 0.6-kb region (E-box1, E-box2, and E-box3) did not completely abolish MyoD1 transactivation (Fig. S3), suggesting that additional *cis* elements for MyoD1 exist in this region. We then examined the effect of the P384R mutation on DEC2 activity for *ore-luc*. We and another group have previously shown that the P384R mutation decreased the DEC2 repressor activity of CLOCK-BMAL1–mediated *Per1-luc* and *Per2-luc* transactivation (5, 10). Similarly, the P384R mutation reduced DEC2 repression of MyoD1 activity on promoter elements in both 0.6- and 3.2-kb *ore-luc* constructs (Fig. 3C). These data indicate that DEC2 and its binding partner MyoD1 inhibit and activate orexin expression, respectively. More importantly, the human mutation of DEC2 resulted in reduced DEC2 repressive activity and thus increased *ore-luc* activity. We also confirmed these findings in another cell line, SH-SY5Y, a neuroblastoma-derived cell line (Fig. 3D), because responses of the orexin promoter can vary among different cell lines (23). Taken together, our in vitro data are congruent with the finding
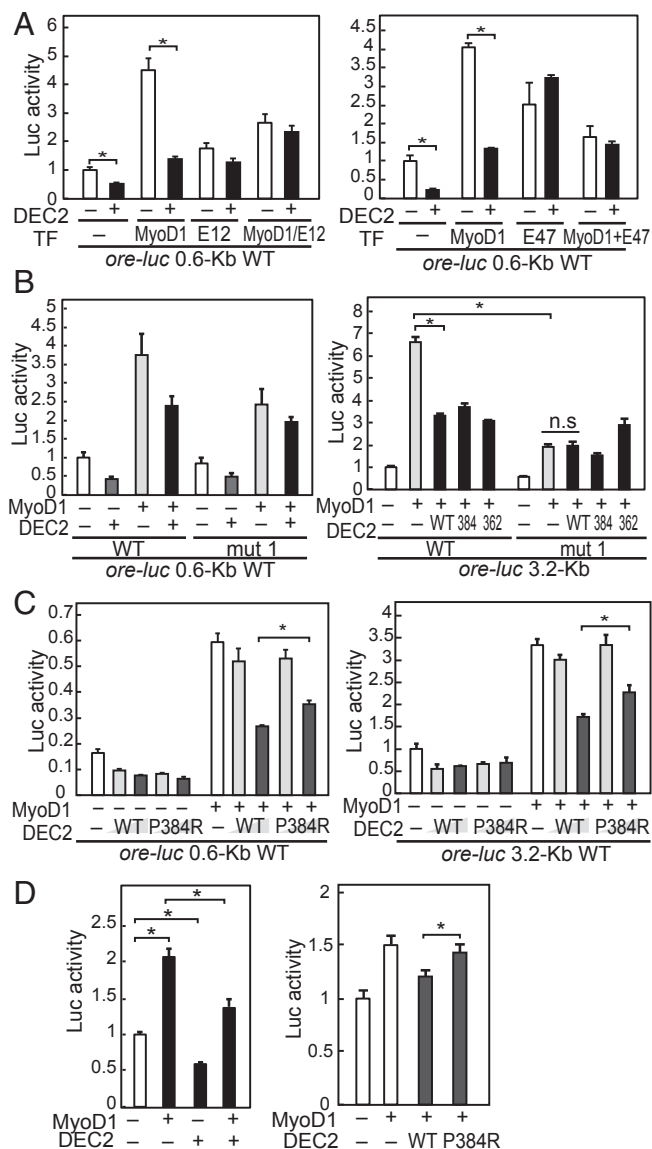

Fig. 3. The P384R mutation decreases DEC2 repressor activity. (A) Overexpression of E12 (*Top*) and E47 (*Bottom*) abrogated the effect of DEC2. HEK293 cells were transfected with the indicated plasmids. Data are shown as mean ± SEM. n = 4. *P < 0.05, Tukey's test. (B) The mutation in E-box1 reduced MyoD1 activity and DEC2 repressive activity in both 0.6- and 3.2-kb *ore-luc* constructs. Data are shown as mean ± SEM. n = 4. *P < 0.05, Tukey's test. (C) The effect of the P384R mutation on DEC2 activity in HEK293 cells. Data are shown as mean ± SEM. n = 4. *P < 0.05, Student's t test for HEK293. (D) The effect of MyoD1 and DEC2 in SH-SY5Y cells. Data are shown as mean ± SEM. (*Left*) n = 4–8. *P < 0.05, Student's t test. (*Right*) n = 4. *P < 0.05, Tukey's test.

that orexin expression is up-regulated in the mouse model of the human mutations (Fig. 1A).

We next asked how the mutation reduces DEC2 activity. We found that the protein levels of DEC2-WT, DEC2-P384R, and DEC2-Y362H were comparable, as has been reported previously (10). The degradation rate of DEC2 was not affected by either mutation (Fig. S4). A previous study demonstrated that the interaction with MyoD1 in vitro is reduced by deletion of the Gly-Ala rich domain of DEC2 (8, 20), in which the two mutations reside. Thus, the mutations may affect the interaction of DEC2 with other transcription factors. A coimmunoprecipitation experiment showed that the interaction of DEC2 with E12 was greatly strengthened by the expression of MyoD1 (Fig. 4A), implying that

DEC2, MyoD1, and E12 form a complex, and that DEC2 binds to a MyoD1/E12 complex. We found that the complex formation of DEC2, MyoD1, and E12 was weakened by the P384R mutation, but not by the Y362H mutation, in cell culture (Fig. 4 *A* and *B*). It is possible that the reduced interaction of DEC2-P384R with E12/47 could impact the occupancy of DEC2 on the *prepro-orexin* promoter. To compare the DNA-binding affinity of WT and mutant DEC2 in brain, we used *H11* locus-specific *DEC2* Tg mice, in which a conditional allele of the gene was integrated into the *H11* locus, so that we can exclude an effect of copy number and expression level of transgenes (Fig. S5*A*). After crossing these mice with *Nestin-Cre* Tg mice, the expression of Myc-tagged DEC2 proteins was driven by a constitutive promoter—the cytomegalovirus

(CMV) early enhanced/chicken beta actin (CAG) promoter—and comparable amounts of DEC2 proteins were detected in mouse brain (Fig. S5*B*). We confirmed that in the H11 mice, DEC2-Myc binds to the E-box–containing promoters of *prepro-orexin* and *Dbp* genes, as expected (Fig. S5*C*); however, weakened binding of mutant DEC2 to the *prepro-orexin* promoter was observed in H11 *P384R-DEC2* Tg mice (Fig. 4*C*). In contrast, there was no obvious effect of the Y362H mutation on the interaction of DEC2 with E12 in cell culture (Fig. 4 *A* and *B*), suggesting that a different molecular mechanism is responsible for the reduced repressor activity of DEC2-Y362H.

To further investigate the dynamics of the interaction of E12/47 with DEC2, we examined the effect of *TCF3* knockdown on
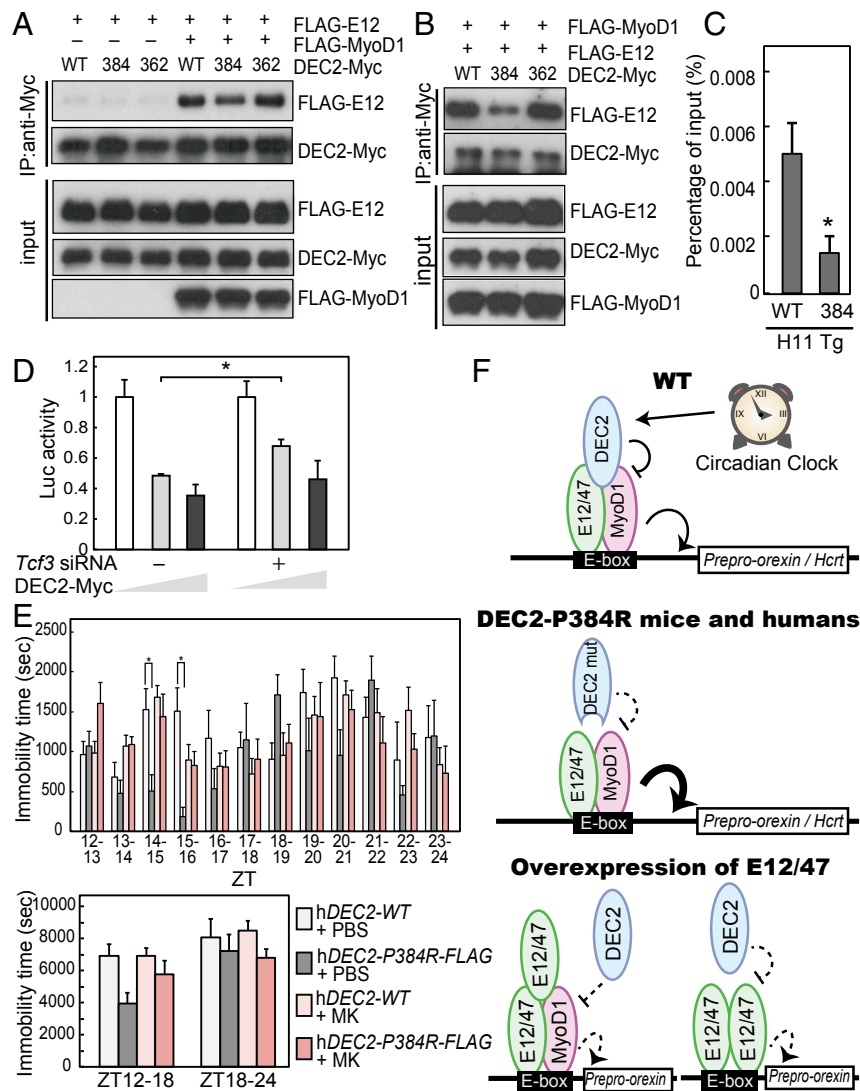

Fig. 4. The P384R mutation decreases DEC2 binding to E12 and DNA. (*A*) Co-IP of E12 and DEC2 expressed in HEK293 cells. DEC2-Myc-His was immuno-precipitated with anti-Myc antibody. Results were obtained from three independent experiments. (*B*) The effect of the mutation on the interaction of DEC2 with E12 in HEK293 cells. (*C*) ChIP assay of DEC2 in mouse brain from H11 Tg mice. DEC2-Myc proteins were precipitated with anti-DEC2 antibody, and ChIP signals were determined using real-time PCR. Data are shown as mean ± SEM. *n* = 4. **P* < 0.05, Student's *t* test. (*D*) The effect of *TCF3* knockdown on DEC2 repressor activity of *ore-luc*. Data are shown as mean ± SEM. *n* = 4. **P* < 0.05, Student's *t* test. (*E*) Locomotor activity of FLAG-h*DEC2-P384R*, and h*DEC2-WT* mice after injection of orexin receptor antagonist. MK-6096 (25 mg/kg) or PBS were administered p.o. at ZT11, and recording started from ZT12. Mouse movement was tracked by an infrared video camera from ZT12-24 in LD 12:12. The immobilization time was plotted every 60 min (*Top*) or every 6 h (*Bottom*). Data are shown as mean + SEM. *n* = 6. **P* < 0.05, two-way ANOVA. (*F*) Proposed model for regulation of *orexin* expression by DEC2. DEC2 binds and competes with transcription factors (MyoD1, E12, and E47) and suppresses the transcriptional activity of *orexin*. Overexpressed E12 or E47 likely interfere with the binding of DEC2 to MyoD1/DNA. The P384R mutation in DEC2 decreased the interaction with E12 and DNA-binding affinity, leading to up-regulation of orexin.

DEC2 repressor activity. *TCF3* knockdown by siRNA decreased DEC2 repressive activity in a small, but statistically significant way (Fig. 4D). These results suggested that E12/47 helps to recruit DEC2 to the complex and to E-boxes. To test whether orexin mediates the sleep phenotype altered in the *DEC2* mutant mice, we orally administered orexin receptor antagonist to the Tg mice and recorded their wake/rest behavior. As shown previously (Fig. S1B), h*DEC2-P384R* mice have decreased total rest time compared with h*DEC2-WT* Tg mice, especially during the first half of the dark phase (Fig. 4E). Interestingly, the administration of MK-6096, a nonselective orexin receptor antagonist, partially cancelled the phenotype of decreased sleep time observed in h*DEC2-P384R* mice (Fig. 4E).

Taken together, the results presented here indicate that MyoD1 activates *prepro-orexin* gene expression, and that DEC2 is a repressor of this activation (Fig. 4F). *Prepro-orexin* expression and orexin levels show daily rhythms in hypothalamus and cerebrospinal fluid (24, 25). *DEC2* expression oscillates in a circadian manner (9, 26) and thus may contribute to the expression rhythms of *prepro-orexin*. Our results suggest that E12/47, MyoD1, and DEC2 form a complex to regulate *prepro-orexin* expression. This complex formation is reduced by the P384R mutation, leading to orexin up-regulation. Overexpression of E12 or E47 attenuates DEC2 repressor activity, possibly because the interaction of DEC2 and E12 depends on MyoD1 (Fig. 4A), and excessive accumulation of E12/47 may prevent the DEC2–MyoD1 interaction (Fig. 4F, *Bottom*).

Discussion

Here we have demonstrated that DEC2 protein physically binds to the orexin promoter in mouse brain and regulates expression of the gene through E-box *cis* elements. The 3.2-kb 5′ UTR of the *prepro-orexin* promoter containing the E-box is sufficient for the endogenous expression of orexin (14), and activation of the cells driven by this promoter region using optogenetic manipulation was sufficient to induce wakefulness (13, 27). Therefore, our findings provide insight into how orexin expression, which is involved in many biological functions, is regulated at a molecular level. We previously reported that the human *DEC2* mutation contributes to short sleep in humans and decreased sleep time in other animals (flies and mice) (5), but the detailed mechanism of these sleep phenotypes has remained elusive. In the present study, we found that the mutation leads to increased orexin expression in mice, contributing to our understanding of how the mutation alters sleep architecture. Interestingly, we also observed an increased level of orexin receptor 2 in the mutant mice. A previous study showed that pharmacologic activation of REV-ERB activity resulted in decreased *prepro-orexin* and orexin receptor expression, while knockout of *Reverbβ* increased expression (28). Thus, *prepro-orexin* and orexin receptor genes likely are regulated by a similar mechanism. Administration of orexin A peptide directly into the brain increased wakefulness (13, 29), indicating the wake-promoting effect of orexin. Overexpression of *Hcrt* caused consolidation of the active state and reduced rest in zebrafish (30). This Tg zebrafish also showed an insomnia-like phenotype, manifested as a decreased arousal threshold (30). Furthermore, CAG promoter-driven *prepro-orexin* in mice causes fragmentation of sleep without affecting total sleep duration (31). The behavioral output of chronically increased orexin expression might be more complex and makes comparisons of acute and chronic models difficult to interpret. It is possible that increased orexin expression within a physiological range (not overexpression) at a specific time point (e.g., ZT1) may decrease the total duration of sleep. Importantly, the sleep phenotype (in Tg mice) is attenuated by an orexin receptor antagonist, further confirming the connection between DEC2 and the orexin pathway (Fig. 4E).

As described above, despite the growing list of studies characterizing physiological changes resulting from modulation of orexin signaling, our understanding of the regulation of orexin expression at a molecular level remains very limited. Previous studies have shown that two transcription factors—Foxa2 and NR6A1—enhance orexin expression, whereas the physiological significance of these proteins in sleep regulation has not yet been determined (32, 33). IGFBP3 has been shown to decrease orexin expression in cell culture, and h*IGFBP3* Tg mice have exhibited decreased orexin levels and wake times (23), consistent with our results (i.e., increased orexin and increased daily active time). Here we have identified additional proteins (DEC2, MyoD1, and E12/47) as regulators of orexin promoter activity. MyoD1 elevates *ore-luc* activity, while the binding partners E12 and E47 inhibit MyoD1-mediated transactivation (Fig. 2E).

Our results suggest that MyoD1, E12, and DEC2 form a complex, because the interaction of DEC2 with E12 was significantly strengthened by MyoD1 expression (Fig. 4A). Our in vitro studies show that the reduction in DEC2 repressor activity due to the P384R mutation resulted from altered binding to E12 and DNA. Thus, it is likely that MyoD1/E12 functions in the recruitment of DEC2 (Fig. 4 D and F, *Top*). However, we also observed that overexpression of E12 or E47 cancelled the effect of DEC2 (Fig. 3A). We hypothesize that overexpressing E12/47 can compete with MyoD1 binding to E-boxes, inhibiting the ability of DEC2 to bind to E-boxes, because DEC2 requires MyoD1/E12 heterodimer for recruitment (Fig. 4F, *Bottom*, *Right*). Another possibility is that overexpressed E12/47 cannot be replaced by DEC2 in binding to E-boxes (Fig. 4F, *Bottom*, *Left*), suggesting complex dynamics of transcription factors. Orchestration of these transcription regulators is expected to contribute to the time-dependent regulation of the sleep hormone, orexin, while temporal dynamics of these regulators on the E-box will require further study.

Collectively, our results suggest that the role of DEC2 in sleep regulation is via orexin, at least in part. Consistent with the capacity of orexin to modulate sleep duration is recent evidence that orexin antagonists, now available for the clinical treatment of insomnia, reliably increase total sleep time in both insomnia patients (34–36) and healthy controls (37). Our findings demonstrate that DEC2 is a target for modulating orexinergic signaling.

Materials and Methods

Mice. All experimental protocols were conducted according to the National Institutes of Health guidelines for animal research and were approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco. Male mice were entrained to a 12-h light:12-h dark cycle (LD 12:12) for at least 10 d with free access to food and water. For expression rhythms of *prepro-orexin* and *orexin receptors*, mice were killed at ZT1, and hypothalami were collected for mRNA extraction. For protein expression of hDEC2-Myc in H11 Tg mice, mouse brains were collected at ZT1 and fractionated into cytosolic and nuclear fractions as described previously (7).

Generation of BAC Tg Mice. We engineered a human BAC clone, CTD-2116MB, containing the entire DEC2 gene in a 125-kb genomic insert to generate BAC Tg mice. The BAC clone was modified by homologous recombination to introduce the P384R or Y362H mutation and to add a FLAG tag sequence to the C terminus of DEC2. All relevant segments generated by PCR and recombination were sequence-confirmed. BAC DNA was injected into C57B/6J embryos following standard procedures. Tg lines were maintained by backcrossing to C57B/6J mice.

Generation of H11 Tg Mice. For generation of H11 knockin Tg mice (38), we used pBT346.2 plasmid provided by Devine Patrick and Benoit Bruneau, University of California, San Francisco. A *phosphoglycerate kinase* (*PGK*) promoter-neomycin (*Neo*) cassette sandwiched by *loxP* sequences was inserted behind the CAG promoter in the pBT346.2 vector. A cDNA encoding Myc-tagged h*DEC2* was inserted after the *PGK-Neo* cassette. Plasmid was twice-purified by phenol-chloroform and then injected into embryos of TARGATT knockin mice (38) in the FVB background together with φC31 integrase mRNA. Mice were crossed with *β-actin-Flp* Tg mice to remove the

plasmid backbone before crossing to *Cre* Tg lines. For experiments, mice were crossed with *Nestin-Cre* mice [Tg (Nes-cre)1Kln; The Jackson Laboratory] to activate expression of DEC2.

Luciferase Assay. HEK293 cells and SH-SY5Y cells were transfected with *prepro-orexin-luc* (*ore-luc*) expression vector (pGL4.18; Promega), *Renilla-luc* (pGL4.75; Promega) control vector, and indicated vectors. The luciferase assay was performed using the Promega Dual-Luciferase Reporter Assay System according to the manufacturer's protocol. Bioluminescence was detected with a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek). The bioluminescence of firefly Luc was normalized to that of *Renilla* Luc.

Immunoprecipitation. HEK293 cells were transfected with plasmid vectors containing hDEC2-His-Myc, FLAG-MyoD1, or FLAG-hE12. At 48 h after transfection, the cells were lysed in IP buffer (20 mM Hepes pH 7.8 at 4 °C, 137 mM NaCl, 2 mM EDTA, 10% glycerol, and 0.1% Triton-X100), and the cell lysate was incubated with anti-Myc antibody (Santa Cruz Biotechnology) overnight. Protein G Sepharose (GE Healthcare) was added to the lysate, incubated for 2 h, and then washed three times, followed by Western blot analysis.

ANY-Maze Analysis of Tg Mice. All mice tested were ~12-wk-old males maintained on a C57BL/6J background. Mice were kept in individual cages with free access to food and water. Mice were monitored by an infrared camera and tracked by an automatic video tracking system (Stoelting). For Fig. 4*E*, mice were entrained to LD 12:12. PBS (control) or MK-6096 (25 mg/kg) was injected with a 20 G animal feeding needle to mice at ZT11, and locomotor activity was recorded from ZT12. For Fig. S1B, mice were entrained to LD 12:12, and locomotor activity was recorded for 4 d. Walking distance and immobility times were calculated using ANY-maze software.

Statistical Analysis. All error bars in the figures represent SEM. No statistical analysis was used to predetermine the sample sizes. Experiments were not randomized and were not analyzed blindly. Data were statistically analyzed using R software. To assess statistical significance, data were obtained from at least three independent experiments. For the comparison of two groups with homogeneity of variance (evaluated by the F test), the two-tailed paired Student's *t* test was used. One-way or two-way ANOVA followed by Tukey's test was used for multiple comparisons with an assumption of normal distribution. A *P* value <0.05 was considered to represent a statistically significant difference.

More details on the methodology of this study are provided in *SI Materials and Methods*.

1. Borbély AA, Daan S, Wirz-Justice A, Deboer T (2016) The two-process model of sleep regulation: A reappraisal. *J Sleep Res* 25:131–143.
2. Toh KL, et al. (2001) An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. *Science* 291:1040–1043.
3. Xu Y, et al. (2005) Functional consequences of a CKIdelta mutation causing familial advanced sleep phase syndrome. *Nature* 434:640–644.
4. Xu Y, et al. (2007) Modeling of a human circadian mutation yields insights into clock regulation by PER2. *Cell* 128:59–70.
5. He Y, et al. (2009) The transcriptional repressor DEC2 regulates sleep length in mammals. *Science* 325:866–870.
6. Zhang L, et al. (2016) A PERIOD3 variant causes a circadian phenotype and is associated with a seasonal mood trait. *Proc Natl Acad Sci USA* 113:E1536–E1544.
7. Hirano A, et al. (2016) A cryptochrome 2 mutation yields advanced sleep phase in humans. *eLife* 5:e16695.
8. Kato Y, Kawamoto T, Fujimoto K, Noshiro M (2014) DEC1/STRA13/SHARP2 and DEC2/SHARP1 coordinate physiological processes, including circadian rhythms in response to environmental stimuli. *Curr Top Dev Biol* 110:339–372.
9. Honma S, et al. (2002) Dec1 and Dec2 are regulators of the mammalian molecular clock. *Nature* 419:841–844.
10. Pellegrino R, et al. (2014) A novel BHLHE41 variant is associated with short sleep and resistance to sleep deprivation in humans. *Sleep* 37:1327–1336.
11. Bode B, Shahmoradi A, Taneja R, Rossner MJ, Oster H (2011) Genetic interaction of Per1 and Dec1/2 in the regulation of circadian locomotor activity. *J Biol Rhythms* 26:530–540, and correction (2012) 27:180.
12. Baier PC, et al. (2014) Mice lacking the circadian modulators SHARP1 and SHARP2 display altered sleep and mixed state endophenotypes of psychiatric disorders. *PLoS One* 9:e110310.
13. Mieda M, Sakurai T (2012) Overview of orexin/hypocretin system. *Prog Brain Res* 198:5–14.
14. Sakurai T, et al. (1999) Structure and function of human prepro-orexin gene. *J Biol Chem* 274:17771–17776.
15. Moriguchi T, Sakurai T, Takahashi S, Goto K, Yamamoto M (2002) The human prepro-orexin gene regulatory region that activates gene expression in the lateral region and represses it in the medial regions of the hypothalamus. *J Biol Chem* 277:16985–16992.
16. Azmi S, Ozog A, Taneja R (2004) Sharp-1/DEC2 inhibits skeletal muscle differentiation through repression of myogenic transcription factors. *J Biol Chem* 279:52643–52652.
17. Azmi S, Sun H, Ozog A, Taneja R (2003) mSharp-1/DEC2, a basic helix-loop-helix protein functions as a transcriptional repressor of E-box activity and Stra13 expression. *J Biol Chem* 278:20098–20109.
18. St-Pierre B, Flock G, Zacksenhaus E, Egan SE (2002) Stra13 homodimers repress transcription through class B E-box elements. *J Biol Chem* 277:46544–46551.
19. Koike N, et al. (2012) Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. *Science* 338:349–354.
20. Fujimoto K, et al. (2007) Transcriptional repression by the basic helix-loop-helix protein Dec2: Multiple mechanisms through E-box elements. *Int J Mol Med* 19:925–932.
21. Kee BL (2009) E and ID proteins branch out. *Nat Rev Immunol* 9:175–184.
22. Rudnicki MA, et al. (1993) MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell* 75:1351–1359.
23. Honda M, et al. (2009) IGFBP3 colocalizes with and regulates hypocretin (orexin). *PLoS One* 4:e4254.
24. Desarnaud F, et al. (2004) The diurnal rhythm of hypocretin in young and old F344 rats. *Sleep* 27:851–856.
25. Justinussen JL, Holm A, Kornum BR (2015) An optimized method for measuring hypocretin-1 peptide in the mouse brain reveals differential circadian regulation of hypocretin-1 levels rostral and caudal to the hypothalamus. *Neuroscience* 310:354–361.
26. Noshiro M, et al. (2005) Tissue-specific disruption of rhythmic expression of Dec1 and Dec2 in clock mutant mice. *J Biol Rhythms* 20:404–418.
27. Adamantidis AR, Zhang F, Aravanis AM, Deisseroth K, de Lecea L (2007) Neural substrates of awakening probed with optogenetic control of hypocretin neurons. *Nature* 450:420–424.
28. Amador A, et al. (2016) Pharmacological and genetic modulation of REV-ERB activity and expression affects orexigenic gene expression. *PLoS One* 11:e0151014.
29. Mieda M, Sakurai T (2013) Orexin (hypocretin) receptor agonists and antagonists for treatment of sleep disorders: Rationale for development and current status. *CNS Drugs* 27:83–90.
30. Prober DA, Rihel J, Onah AA, Sung RJ, Schier AF (2006) Hypocretin/orexin overexpression induces an insomnia-like phenotype in zebrafish. *J Neurosci* 26:13400–13410.
31. Willie JT, et al. (2011) Ectopic overexpression of orexin alters sleep/wakefulness states and muscle tone regulation during REM sleep in mice. *J Mol Neurosci* 43:155–161.
32. Silva JP, et al. (2009) Regulation of adaptive behaviour during fasting by hypothalamic Foxa2. *Nature* 462:646–650.
33. Tanaka S, et al. (2010) Transcriptional regulation of the hypocretin/orexin gene by NR6A1. *Biochem Biophys Res Commun* 403:178–183.
34. Michelson D, et al. (2014) Safety and efficacy of suvorexant during 1-year treatment of insomnia with subsequent abrupt treatment discontinuation: A phase 3 randomised, double-blind, placebo-controlled trial. *Lancet Neurol* 13:461–471.
35. Herring WJ, et al. (2016) Suvorexant in patients with insomnia: Results from two 3-month randomized controlled clinical trials. *Biol Psychiatry* 79:136–148.
36. Black J, et al. (2017) Efficacy and safety of almorexant in adult chronic insomnia: A randomized placebo-controlled trial with an active reference. *Sleep Med* 36:86–94.
37. Sun H, et al. (2013) Effects of suvorexant, an orexin receptor antagonist, on sleep parameters as measured by polysomnography in healthy men. *Sleep* 36:259–267.
38. Tasic B, et al. (2011) Site-specific integrase-mediated transgenesis in mice via pronuclear injection. *Proc Natl Acad Sci USA* 108:7902–7907.

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