Ubiquitin ligase Siah2 regulates RevErbα degradation and the mammalian circadian clock

Jason P. DeBruyne[a,1], Julie E. Baggs[a], Trey K. Sato[b,2], and John B. Hogenesch[b,1]

[a]Neuroscience Institute, Department of Pharmacology and Toxicology, Morehouse School of Medicine, Atlanta, GA 30310; and [b]Department of Pharmacology, Penn Genome Frontiers Institute, Institute for Translational Medicine and Therapeutics, Perelman School of Medicine at the University of Pennsylvania, Philadelphia PA, 19104

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Regulated degradation of proteins by the proteasome is often critical to their function in dynamic cellular pathways. The molecular clock underlying mammalian circadian rhythms relies on the rhythmic expression and degradation of its core components. However, because the tools available for identifying the mechanisms underlying the degradation of a specific protein are limited, the mechanisms regulating clock protein degradation are only beginning to be elucidated. Here we describe a cell-based functional screening approach designed to quickly identify the ubiquitin E3 ligases that induce the degradation of potentially any protein of interest. We screened the nuclear hormone receptor RevErbα (Nr1d1), a key constituent of the mammalian circadian clock, for E3 ligases that regulate its stability and found Seven in absentia2 (Siah2) to be a key regulator of RevErbα stability. Previously implicated in hypoxia signaling, Siah2 overexpression destabilizes RevErbα/β, and siRNA depletion of Siah2 stabilizes endogenous RevErbα. Moreover, Siah2 depletion delays circadian degradation of RevErbα and lengthens period length. These results demonstrate the utility of functional screening approaches for identifying regulators of protein stability and reveal Siah2 as a previously unidentified circadian clockwork regulator that mediates circadian RevErbα turnover.

circadian clock | RevErbα/Nr1d1 | Siah2 | ubiquitin ligase screen

Circadian rhythms originate from intracellular clocks that drive the rhythmic expression of thousands of genes that ultimately manifests in daily rhythms of physiology and behavior. In mammals, the core circadian clock mechanism is composed of two interlocked transcriptional negative feedback loops (1, 2). In the primary loop, the bHLH-PAS domain containing transcriptional activators *Bmal1* (*Arntl*) and *Clock* (or its ortholog *Npas2*) form a DNA-binding heterodimer that drives expression of the *Per1/2/3* and *Cry1/2* genes. Their protein products ultimately feed back to repress CLOCK:BMAL1 activity. This loop also drives rhythmic expression of the nuclear hormone receptors RevErbα and RevErbβ (Nr1d1 and Nr1d2, respectively), which in turn rhythmically repress expression of *Bmal1*, *Clock*, and *Npas2* (3–5). Circadian expression of core clock genes and their regulated protein degradation are essential for maintaining proper timekeeping (6, 7).

The ubiquitin–proteasome pathway is responsible for the degradation of nearly all regulated proteins, including circadian clock proteins. Deficits in this process are linked to diseases ranging from cancers to neurodegenerative disorders (8–10). The ubiquitin system requires the activity of three classes of proteins: E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases. E3 ubiquitin ligases are responsible for specifying substrates and facilitating the transfer of ubiquitin directly or indirectly from E2s to the substrate protein being targeted for degradation. There are ∼600 mouse/human genes that encode E3 ligases, and there are thousands of potential E3 ligase substrates in any given cell (11). However, identifying which E3 ligases ubiquitinate which proteins for degradation can be difficult.

In many cases, ubiquitin ligases for proteins have been recovered from random mutagenesis screens for genes that regulate a global biological process such as cell division. These screens usually are designed to identify genes involved in complex biological processes rather than a specific biochemical event such as the degradation of a particular protein. Protein interaction screening has been applied to identify E3 ligase–substrate interactions (reviewed in ref. 12). However, this approach is most applicable when the E3 ligase–substrate interactions are strong and stable and may not work for transient enzyme–substrate interactions. Recently, a couple of promising large-scale approaches have been developed to identify substrates of particular E3 complexes (13–16), but these approaches are not designed to identify the E3 ligases that ubiquitinate a specific protein for degradation. In fact, aside from protein interaction screening, large-scale approaches to identify how an individual protein is degraded are limited.

Therefore, we sought to develop a general approach to identify which E3 ligases are involved in regulating the degradation of specific proteins. The mammalian circadian system is an ideal setting to evaluate the utility of such approaches, because E3 ligases have been identified for only a few of the ∼12 core clock proteins (reviewed in refs. 7 and 17). Here, we illustrate a simple screening approach centered on revealing functional interactions between a particular substrate and E3 ligases and validate this approach with the identification of the E3 ligase Seven in absentia

Significance

Rhythmic expression of most core clock genes is believed to be essential for maintaining proper timekeeping of the circadian clock. In turn, rhythmic degradation of clockwork proteins is also crucial. However, we know comparatively little about these specific processes. Here we describe a simple screening approach aimed at identifying ubiquitin ligases that degrade proteins of interest and apply it to identifying ligases that target the rhythmically abundant nuclear hormone receptor, RevErbα (Nr1d1), for degradation. This approach found the ubiquitin ligase Seven in absentia 2 (Siah2) as a key regulator of circadian RevErbα turnover and overall circadian clock function and implicates the dynamic rhythmicity of RevErbα protein abundance in maintaining ∼24-h circadian timekeeping.

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[1]To whom correspondence may be addressed. Email: jdebruyne@msm.edu or hogenesc@mail.med.upenn.edu.

[2]Present address: Great Lakes Bioenergy Research Center, University of Wisconsin, Madison, WI 53706.

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(Siah2) as a regulator of RevErbα stability and rhythmicity and overall circadian oscillator function.

Results

Functional Screen for Substrate–E3 Ligase Interactions. The screening approach we developed is based on the straightforward principle that coexpressing an E3 ligase with its target protein usually results in the degradation of the target protein. Therefore, we developed a cell-based screen with which we could test the ability of each E3 ligase, expressed from a cDNA clone, to destabilize a specific Flag-tagged "bait" protein of our choosing (Fig. 1*A*). After trying other tags, we chose the Flag-tag because its small size reduces potential artificial responses and because commercially available Flag-antibodies are specific using immunofluorescence.

To test the basic premise of this assay, we used a known bait–E3 ligase combination, CRY1 and FBXL3 (18–20). Cells were cotransfected with Flag-tagged CRY1 and FBXL3 or control constructs. After ∼42 h, cells were treated with cycloheximide (CHX) for 6 h to block protein synthesis and were processed for anti-Flag immunofluorescence. The effect of Fblx3 on Flag-Cry1 was robust: Fbxl3 sharply reduced the number of cells with detectable Flag-Cry1 (Fig. S1). Thus, the effect of an E3 ligase on its substrate, in this context, can be quantified simply by counting the number of Flag-positive cells, a process that we automated using a high-throughput microscope and image processing.

We next prepared an E3 ligase screening library consisting of 736 full-length cDNA clones (genecollections.nci.nih.gov/). This library covers >50% of known and predicted E2 conjugating and E3 ligating enzymes and also includes cDNAs expressing a number of deubiquitinating enzymes and other proteins associated with degradation (Dataset S1) (11, 21, 22). Using this library and the conditions optimized for FBXL3/CRY1, we evaluated the assay by screening for E3 ligases that caused the degradation of GFP (nonspecific) and two clock proteins: Flag-Cry1 and Flag-RevErbα. Overall, despite plate-to-plate variability that necessitated screening in duplicate, we found that the great majority of the 736 E3 clones had little effect on the stability of GFP- or Flag-tagged protein (Fig. 1*B* and Dataset S1). Only 53 E3 clones (∼7%) appeared to destabilize two or more substrates consistently; these clones were considered false positives and were eliminated from further analysis. Most of these false positives are likely caused by poor transfection efficiencies resulting from failed E3 cDNA plasmid preparations because wells without library clone DNA also produced a false-positive result (Dataset S1). The Flag-Cry1 screen consistently identified Fblx3 as a hit and revealed only one other potential CRY1 E3 ligase (RNF128) (Fig. 1*B*), suggesting that this screen is specific under optimized conditions.

We set out to test the generalizability of this screen and test its ability to identify E3 ligases for a protein where none are known. To do so, we chose to screen for RevErbα E3 ligases using conditions optimized for Cry1-Fbxl3. Two RevErbα E3s, *Arf-bp1* (*Huwe1*) and *Pam* (*MycBp2*) have been identified recently, but we decided not to optimize the RevErbα degradation assay using these ligases because simultaneous overexpression of both is required to ubiquitinate RevErbα (23). In addition, expressible full-length cDNA clones for these E3s were not present in the Mammalian Gene Collection (MGC) and were not included in our library. Nonetheless, the Flag-RevErbα screen using standard conditions produced two hits as candidate RevErbα E3 ligases: Siah2 and splA/ryanodine receptor domain and SOCS box containing 4 (Spsb4). We focused on these RevErbα hits as a proof of concept for this general screening approach.

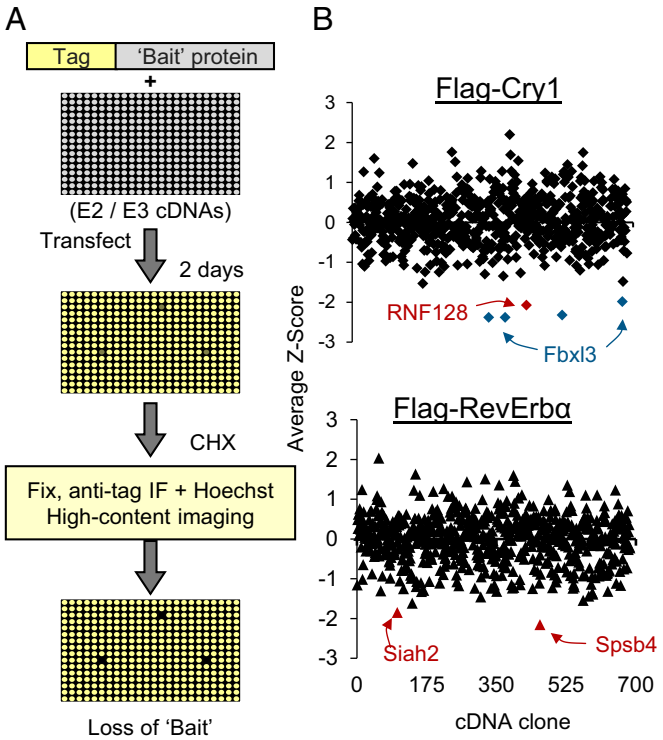

Fig. 1. A functional screen for E3 ligases that target any protein for degradation. (*A*) Schematic of the E3 ligase screen methodology. cDNAs expressing a Flag-tagged bait protein are cotransfected with individual E3 clones into AD293 cells, and after ∼2 d, cells are treated with CHX and are processed for anti-Flag immunofluorescence (IF) to determine the percentage of Flag-positive cells remaining in each well. (*B*) Screen data from Flag-Cry1 or Flag-RevErbα screens. Each point represents the average of duplicates (or quintuplets for clones 1–350). (See Dataset S1 for raw data). False positives have been removed for clarity. Positive controls are indicated in blue, and screen hits are in red.

The E3 Ligase Siah2 Regulates RevErbα Stability. To validate the primary screen, we focused predominantly on evaluating Siah2 as a regulator of RevErbα stability because, although Spsb4 contains an SOCS domain common to some E3 ligases and analogous to F-box proteins (24, 25), Siah2 is a RING type E3 ubiquitin ligase and plays a prominent role in regulating the activation of the hypoxia pathway (26). In this role, it helps facilitate growth and metastasis of some tumors and thus may be an anticancer therapeutic target (27). Siah2 also interacts with and targets several other proteins for degradation (reviewed in ref. 28), including NcoR1 (29). NcoR1 is a corepressor required for RevErbα-mediated transcriptional repression (30), thus placing Siah2 in a potential complex with RevErbα.

In the screen, Siah2 consistently reduced Flag-RevErbα$^+$ cells by ∼50% compared with plate mean, and, as noted above, it did not affect the stability of Flag-Cry1 or GFP. To confirm this result, we retested the Siah2-induced destabilization of RevErbα on a smaller scale using the image-based assay. As expected, cotransfection of two independent Siah2 clones induced a marked destabilization of Flag-RevErbα, resulting in its maximal degradation within 4 h of CHX blocking (Fig. S2*A*). Siah2 degradation was specific to RevErbα: Two different negative control plasmids (empty Sport6 vector and Fblx3; however, see ref. 31) had no effect on Flag-RevErbα stability, and Siah2 did not alter the stability of Flag-PER1 within the same experiments (Fig. S2*A*).

The screen results also were confirmed by immunoblotting. As in the imaging studies, expression of Siah2 specifically destabilized both Flag-RevErbα and Flag-RevErbβ in transfected cells (Fig. 2*A*). Spsb4 also destabilized Flag-RevErbα in this assay (Fig. S2*B*). In contrast, the Siah2 paralog Siah1 or three other randomly selected E3 ligases (Fig. S2 *B* and *C*) could not destabilize Flag-RevErbα. Importantly, Siah2 did not alter the

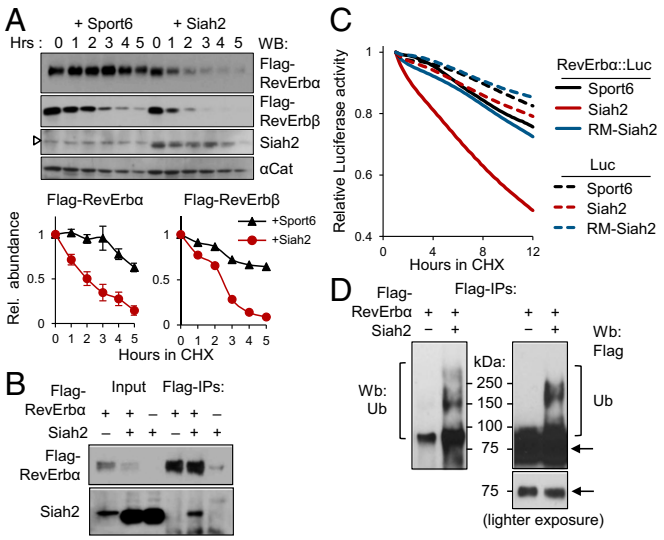

Fig. 2. Siah2 destabilizes RevErbα and RevErbβ. (*A*, *Upper*) Sample Western blots illustrating RevErbα/β stability when coexpressed with Siah2 at the indicated time (in hours) after the addition of CHX. The white triangle indicates a nonspecific band. (*Lower*) Quantified data for RevErbα (mean ± SEM, $n = 4$ independent experiments) and Flag-RevErbβ (mean ± SD, $n = 2$ independent experiments) stability. Siah2 significantly destabilizes RevErb proteins ($P < 0.001$, one-way and two-way ANOVA). (*B*) Interactions by coimmunoprecipitation of transfected AD293 cells. Cells were treated with MG-132 to allow the accumulation/stabilization of RevErbα::Siah2 interactions. (*C*) Plasmids expressing luciferase or an RevErbα::luc fusion protein were cotransfected with the indicated constructs. RM-Siah2, RING mutant Siah2 with no E3 ligase activity (26). Luciferase activity from each culture was followed at 10-min intervals after CHX treatment using an Actimetrics LumiCycle; means of $n = 3$ or 4 per group are plotted. RevErbα::luc decay rates were enhanced significantly only by wild-type Siah2 ($P < 0.0001$, ANOVA, Tukey HSD test). (*D*) Flag-RevErbα immunoprecipitates from denatured AD293 extracts were probed for ubiquitination using the indicated antibodies. Brackets indicate the polyubiquitinated forms of Flag-RevErbα, and arrows indicate the non-ubiquitinated forms. Data are representative of at least three independent experiments. IP, immunoprecipitation; WB, Western blot.

stability of other Flag-tagged core clock proteins (Fig. S2*D*). Corroboration of our screen results in these experiments confirms Siah2 and Spsb4 as hits, demonstrating the specificity and selectivity of the E3 ligase screen.

We next determined whether Siah2 might act as a RevErbα E3 ligase. In overexpression experiments, we were able to detect a small fraction of Siah2 interacting in a complex with Flag-RevErbα (Fig. 2*B*) or Flag-RevErbβ (Fig. S3*A*) immunoprecipitates. Endogenous NcoR1 also was present in Flag-RevErbα immunoprecipitates, but its presence does not appear to be required for Siah2-mediated degradation of RevErbα (Fig. S3 *B* and *C*). We next tested the ability of a ligase-dead Siah2 mutant (RING-mutant, RM-Siah2) (26) to destabilize RevErbα using luciferase-tagged RevErbα (RevErbα::Luc) in similar CHX-chase experiments (Fig. 2*C*). As expected, wild-type Siah2 destabilized RevErbα::Luc, but RM-Siah2 had no effect on RevErbα::Luc stability, indicating that Siah2 requires its E3 ligase activity to destabilize RevErbα. Finally, as expected for an E3 ligase, cell-based ubiquitination assays using denatured cell extracts (32) revealed that Siah2 substantially increases the apparent polyubiquitination of Flag-RevErbα (Fig. 2*D*). Taken together, these results suggest that Siah2, as an E3 ligase, is a proximate, if not direct, facilitator of RevErbα/β degradation.

Siah2 Mediates Circadian Turnover of RevErbα. If Siah2 is a physiologically relevant E3 ligase for RevErbα, then disrupting Siah2 function by RNAi should stabilize endogenous RevErbα. Indeed, we found that siRNA knockdown of Siah2 slowed the overall degradation of endogenous RevErbα in U2OS cells (Fig. 3*A*; see also Fig. S4 *A* and *B*), a widely used cellular model harboring an endogenous circadian clock (33–38). In CHX-treated control U2OS cells, endogenous RevErbα was degraded to 50% of its starting level in less than 1 h and reached basal levels by 2 h after protein synthesis block. In Siah2-depleted cells, in contrast, nearly 2 h were required for RevErbα to be degraded by 50%, and 3–4 h were required for RevErbα to reach basal levels after translational block. Although RevErbα still was degraded in Siah2-depleted cells, its half-life was nearly doubled without Siah2, indicating that Siah2 plays a role in regulating endogenous RevErbα stability.

RevErbα/β mRNA and protein abundance levels cycle according to a robust circadian rhythm in most tissues and cell lines because of their rhythmic expression. However, the mechanisms underlying the degradation of RevErbα/β levels at the end of a cycle are unknown. We therefore asked if Siah2 could play a role in this circadian degradation of RevErbα, contributing to its overall rhythmic profile and presumed function. To do so, we determined the effect of RNAi-mediated Siah2 depletion on cyclical RevErbα abundance in synchronized U2OS cells. In control cultures, RevErbα protein abundance displayed a robust oscillation (one-way ANOVA and Cosinor, $P < 0.0001$) with peak levels at approximately ∼21 h after synchronization that fell sharply to trough levels about 12 h later [$P < 0.001$, Tukey honestly significant difference (HSD) post hoc test] before starting to accumulate again (Fig. 3 *B* and *C* and Fig. S4*C*). We hypothesized that Siah2 was responsible for this sharp decline in RevErbα levels by driving its degradation from peak to trough.

Indeed, depleting Siah2 substantially impaired the rhythms of RevErbα protein abundance (Fig. 3 *B* and *C* and Fig. S4*C*). We had one trial in which the RevErbα rhythms appeared visible in Siah2-depleted cells (Fig. 3*B*), but even in this case RevErbα degradation was prolonged without Siah2. Both ANOVA and Cosinor analysis on data combined from six replicates from four independent experiments indicated that, overall, RevErbα levels were not significantly rhythmic in Siah2-depleted cells ($P > 0.1$ for both tests) (Fig. 3*C* and Fig. S4*C*). RevErbα protein levels reached normal initial peak levels at ∼21–24 h and might decline slightly without Siah2, but statistically, RevErbα levels were not significantly different across the peak–trough (18–39 h) interval ($P > 0.1$ in a pairwise Tukey HSD test). We did detect overall increases in RevErbα protein levels over time in Siah2-depleted cells, comparing the 44–45 h points with the 18–21 h or 30–33 h time points (ANOVA $P < 0.001$; $P < 0.05$, Tukey HSD test); these increases likely were the consequence of the rhythmic circadian *RevErbα* gene expression, because Siah2 depletion did not appreciably alter the amplitude or shape of the rhythm of *RevErbα* mRNA abundance (Fig. S5*A*). Regression analysis focusing exclusively on the circadian decline phase (20–34 h) further revealed that RevErbα degradation without Siah2 was at least threefold slower and was not nearly as precise as in control cells (Fig. 3*D*). Thus, combined with the data above, these results implicate Siah2 as an important mediator of circadian degradation/clearance of RevErbα, likely as a RevErb E3 ligase.

Siah2 Regulates Circadian Clock Function. Several lines of evidence suggest that the robust rhythmicity of RevErbα/β protein abundance is a key determinant not only of RevErb function but also of overall circadian clock function. First, increasing the DNA-binding activity of RevErb proteins in the promoter of the *Cry1* gene can lengthen circadian periods (39). Second, circadian oscillators can be modulated by RevErbα ligands (40, 41). Finally, either continuous overexpression of RevErbα (42) or genetic loss of both RevErb proteins profoundly disrupts overall clock function in vitro and in vivo (43, 44). Combined with the observation that RevErbα/β proteins are robustly

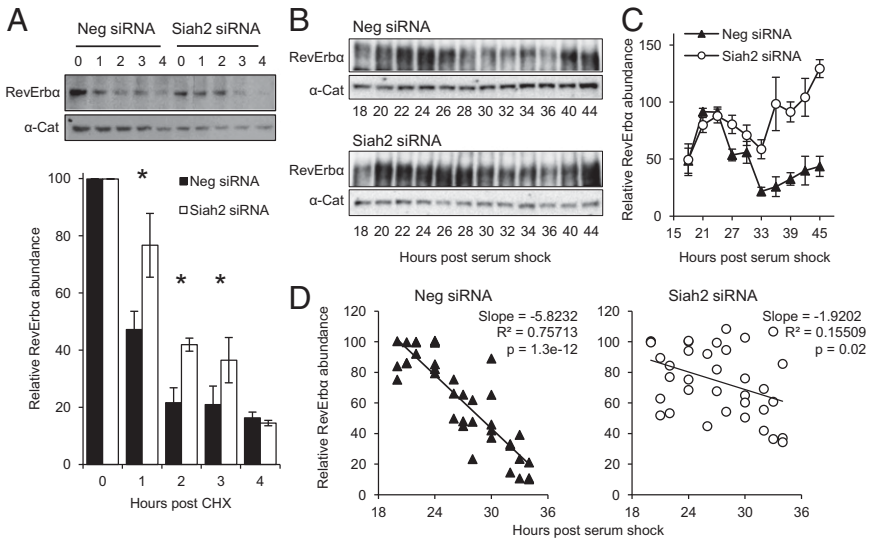

Fig. 3. Siah2 depletion impairs RevErbα degradation. (*A*, *Upper*) Representative Western blots of lysates from unsynchronized cells transfected with negative control (Neg) or Siah2 siRNA and collected at the indicated times after CHX treatment. (*Lower*) RevErbα quantification (mean ± SEM, $n = 3$ independent experiments). Two-way ANOVA revealed a significant time × siRNA interaction ($P < 0.05$); *$P < 0.05$, Bonferroni test for differences between means. (*B*) Rhythms of RevErbα protein abundance in synchronized U2OS cells transfected with the indicated siRNAs. (*C*) Data binned into 3-h intervals from six total replicates in four independent experiments (points indicate mean ± SEM, $n = 5$–9). Individual data are shown in Fig. S4*C*. Two-way ANOVA revealed a significant time × siRNA interaction ($P < 0.0001$), with specific differences from 33 to 45 h ($P < 0.05$; Bonferroni test for differences between means). One-way ANOVA and Cosinor analysis confirm that RevErbα is significantly rhythmic in control cultures ($P < 0.001$, Tukey HSD comparing hours 21–24 and 33–36) but not in Siah2-depleted cultures ($P > 0.5$, Tukey HSD comparing hours 21–24 and 33–36). (*D*) Individual data points from the peak–trough interval (hours 20–34) fit with linear regressions; the slopes, R^2, and significance of trend are shown. Analysis of covariance (ANCOVA) indicates that both the slopes and R^2 values are significantly different ($P = 0.005$ and $P < 0.0001$, respectively).

rhythmically abundant, these findings suggest that disrupting the circadian profile of RevErbα/β protein abundance by impeding their degradation may alter the function of the circadian oscillator.

To examine this potential link, we first examined the effect of Siah2 depletion on the expression of RevErbα/β target genes in U2OS cells. In synchronized U2OS cells, we found that Siah2 depletion reduced the overall expression levels of direct RevErbα/β target *Npas2* (4) in a manner consistent with the effect of RevErbα protein levels (Fig. S5*A*). Surprisingly, however, the expression of the two canonical RevErbα/β targets, *Bmal1* and *Cry1*, was not detectably altered. This apparent discrepancy could be caused by differences in how individual genes (i.e., RORs, CLOCK:BMAL1) integrate RevErbα/β and other transcriptional inputs in various tissues (45, 46). We also used Gene Dosage Network Analysis (GDNA) (35) (Fig. S5*B*) to examine how dose-dependent Siah2 knockdown alters the repression of two groups of genes: eight RevErbα/β targets and nine genes regulated primarily by CLOCK:BMAL1 (1, 2, 47, 48). This analysis revealed that five of the eight RevErbα/β targets examined were down-regulated and correlated with Siah2 mRNA knockdown, whereas only one (*Per3*) of the nine other clock-related genes was correlated (Fig. S5*B*). This striking preference of Siah2 knockdown to suppress RevErbα/β target gene expression is consistent with the findings described above suggesting Siah2 destabilizes RevErbα (Fig. 3) and indicates that Siah2 can interact with the circadian clockwork by regulating RevErbα/β function.

We next determined if Siah2 depletion alters the circadian period. To do so, we depleted Siah2 in dose–response assays in U2OS cells containing a *Bmal1-Luc* circadian reporter (35) and followed the functional consequences using kinetic luminescence imaging. A recent genome-wide screen for siRNAs that alter clock function in this cell line suggested that Siah2 knockdown lengthens period (38). Indeed, we found that Siah2 depletion dose-dependently lengthened the circadian period by up to ∼2 h (Fig. 4 *A* and *B*). Interestingly, the period lengthening was apparent only when Siah2 levels were depleted by >80% at the mRNA level (Fig. 4*C*), suggesting that Siah2 levels are normally in excess. This result was replicated in *Per2-Luc* U2OS cells (Fig. S6*A*) and was reproduced by independent siRNAs (Fig. S6*B*) and fibroblasts derived from *Siah*-knockout mice (Fig. S6*C*) (49). Thus, Siah2 is required for normal circadian oscillator function. Moreover, because Siah2 depletion appears to affect only RevErb-specific processes within the clock, our findings suggest that RevErbα/β degradation, like that of the CRY1/2 and PER1/2 proteins (6, 7), contributes to maintaining normal circadian periodicity.

Knockdown of our other RevErbα screen hit, Spsb4, also significantly lengthened periods of *Bmal1-luc* activity in U2OS, by up to ∼2 h (Fig. S7). Because both E3 ligases can target RevErbα for degradation, we asked if these E3 ligases interact genetically by determining the effect on period when both E3s were knocked down simultaneously using submaximal siRNA doses. Significantly, the effect of double Spsb4/Siah2 knockdown on period was not additive compared with the effects of individual knockdown. Instead, double knockdown reproduced the larger of the two period phenotypes seen with the knockdown of either gene individually (Fig. 4*D* and Fig. S7). This type of genetic interaction strongly suggests that Siah2 and Spsb4 act via the same pathway/mechanism to regulate clock function and provides additional validation that the E3 screening approach can identify biologically important substrate–ligase interactions. Moreover, because both E3 ligases were the most prominent hits from the RevErbα E3 ligase screen, and at least Siah2 mediates circadian clearance of endogenous RevErbα, these data strongly support the idea that RevErbα/β stability is integral to normal clock function.

Discussion

Regulated protein degradation is increasingly appreciated as a major contributor to the functioning of critical cellular pathways. Ubiquitin-mediated proteasomal degradation is vital to

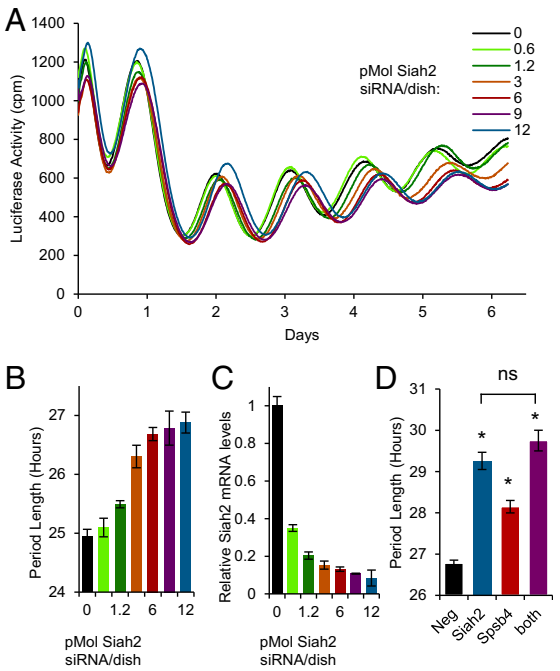

Fig. 4. Siah2 depletion dose-dependently slows circadian oscillator function. (*A*) Average bioluminescence rhythms produced by *Bmal1-Luc* U2OS cells transfected with a dosage series of *Siah2* siRNAs (mean, $n = 4$). The indicated dose is the amount of Siah2 siRNAs in a mixture with negative control siRNA for a total of 24 pmol siRNA per well. (*B*) Circadian periods (mean ± SEM, $n = 4$) of the cultures in *A*. (*C*) *Siah2* mRNA levels (mean ± SEM) from three additional replicates harvested at time 0. Circadian periods in *B* are highly correlated with Siah2 mRNA levels in *C* (Spearman $R = -0.96$, $P < 0.001$), both of which are dose-dependent (ANOVA, $P < 0.001$). (*D*) Average periods (± SEM, $n = 10$–12 combined from three independent experiments) of bioluminescence rhythms produced by *Bmal1-Luc* U2OS after knockdown of Spsb4, Siah2, or both Spsb4 and Siah2 (6 pmol each). See also Fig. S7. *$P < 0.0001$ vs. negative controls, ANOVA, Tukey HSD test; ns, not significant ($P = 0.24$, Tukey HSD test).

nearly all aspects of cellular function, and deficits in this process are linked to a wide variety of diseases. This contribution is especially clear in dynamic processes such as the cell cycle and the circadian clock, which require active degradation of many of their constituents. However, despite the importance of these interactions, identifying E3 ligase/substrate pairs has been difficult, because generic screening approaches have lagged. As discussed above, physical interaction screens require strong physical interactions that may depend on posttranslational modifications. Recently, cell-based functional screening approaches have been developed to identify substrates based on differential ubiquitination (14, 15) or stability proteins expressed as GFP fusions (13, 16) in cells where a specific E3 ligase has been mutated or inhibited. Although these approaches are immensely useful for determining the function of specific E3 ligases, they are not directly amenable for identifying the E3 ligases of a specific substrate protein of interest. The screen we demonstrate here addresses this gap: It can identify E3 ligases that regulate the stability of specific substrate proteins without requiring stable protein–protein interactions. Determining the substrate protein's inherent stability and the minimal amount of transfected cDNA that maintains consistent substrate expression are the only prerequisites for this screen. Once these parameters are optimized, one or more substrates can be screened in less than a week to identify biologically relevant regulators of a protein's stability. Thus, this approach can be fast, specific, and generalizable to identify potential E3 ligases for nearly any protein.

Using this approach, we recovered two potential RevErb E3 ligases, Spsb4 and Siah2. Biochemical validation studies have focused on Siah2 to this point, and our results indicate that it is a bona fide regulator of RevErbα stability and overall clock function. Overexpression experiments suggest Siah2 and RevErb proteins can interact physically, whereby Siah2 causes the ubiquitination of and, via its E3 ligase activity, the degradation of RevErbα/β proteins. Although it is possible that these actions may be indirect, our results are consistent with the notion that Siah2 is a RevErb E3 ligase. Moreover, of the circadian clockwork proteins, only Siah2 is capable of targeting the RevErbα/β proteins for degradation in these assays. Thus, our screen assay appears to be adept in uncovering specific functional E3 ligase–substrate interactions.

Our knockdown studies implicate Siah2 as an important regulator of endogenous RevErbα stability. We found that Siah2 depletion profoundly altered the rhythmic profile of RevErbα protein abundance in synchronized cells. Without Siah2, the rate of RevErbα degradation was substantially slower and was much less precisely controlled, particularly during the period of the normal circadian decline. This result appears to be a bit more exaggerated in synchronized cells than in unsynchronized cells, perhaps suggesting that Siah2 has a specific role in the circadian regulation of RevErbα. It is not yet known if RevErb proteins are rhythmically degraded—that is, if the degradation rate is constant and outpaced by its synthesis or changes throughout the day. In either case, our data strongly suggest that Siah2 plays an important role in mediating the circadian degradation of RevErbα/β required for its robust rhythmicity.

Individually, depletion of either Siah2 or Spsb4 slows clock function, and double-depletion studies suggest these E3 ligases affect the clockwork by regulating the same pathway or target. It is conceivable that Siah2 and Spsb4 are part of the same E3 ligase complex, because Siah2 is a RING-E3 ligase, and Spsb4 is similar to an F-box protein, although this possibility remains to be determined. Collectively, our data strongly suggest that RevErbα (and RevErbβ) is the target of Siah2 and Spsb4 in the clockwork. We propose that without Siah2, RevErb proteins can repress their target genes longer, thereby delaying the activation of these target genes within the clockwork and the start of the next circadian cycle. This same concept explains why loss of rhythmic PER1/2 and CRY1/2 degradation slows circadian clock function (6, 7). Consistent with this idea, Ueda and coworkers have shown that strengthening the binding of RevErbα to its response element within the *Cry1* promoter can prolong the repression of *Cry1* expression and lengthen circadian periods (39). Although Siah2 depletion did not have a detectable effect on *Cry1* or *Bmal1* mRNA levels in U2OS cells, it did cause the suppression of other RevErbα/β clockwork targets, suggesting that other RevErbα targets also may regulate circadian periodicity (50). Importantly, Siah2 depletion altered expression of only RevErb target genes within the clockwork, suggesting that Siah2 interacts specifically with the RevErb/Ror loop of the circadian clockwork. Interestingly, knockdown of RevErbα in U2OS cells also lengthens period (35), implying that its cycling dynamics and overall levels have different roles in the clock. Nonetheless, determining the precise role of RevErb degradation in overall clock function will require examining the effect of stabilizing *RevErbα/β* knock-in mutations. Our results predict that these mutations will alter clock function, because our data strongly suggest that precisely timed degradation of RevErbα/β, by Siah2 in particular, may be a rate-limiting regulatory step underlying normal circadian timekeeping.

Materials and Methods

The E3 ligase screens were performed by reverse transfection (Fugene HD) in AD293 cells with 10 ng Flag–bait and 40 ng E3 ligase cDNA per well in optically clear, black-walled 384-well plates. Immunofluorescence was performed using standard procedures, and images were captured and analyzed using the ImageXpress Micro XLS system (Molecular Devices). Other cDNA transfections were performed in six-well plates using Fugene HD according to the manufacturer's instructions. siRNA transfections and RNA extraction/quantitative PCR (qPCR) were performed as described previously (35) using Siah2 (Hs00192581_m1) or Gapdh (control) (Hs999999905_m1) TaqMan detectors (Applied Biosystems). RevErbα was detected in Western blots using validated antibodies from Abnova (4F6 clone) (Fig. S4). Kinetic bioluminescence assays were performed as described previously (51) using a LumiCycle (Actimetrics), and periods were determined using WaveClock (52). Details can be found in *SI Material and Methods*.

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1. Partch CL, Green CB, Takahashi JS (2014) Molecular architecture of the mammalian circadian clock. *Trends Cell Biol* 24(2):90–99.
2. O'Neill JS, Maywood ES, Hastings MH (2013) Cellular mechanisms of circadian pacemaking: Beyond transcriptional loops. *Handb Exp Pharmacol* (217):67–103.
3. Preitner N, et al. (2002) The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* 110(2):251–260.
4. Crumbley C, Wang Y, Kojetin DJ, Burris TP (2010) Characterization of the core mammalian clock component, NPAS2, as a REV-ERBalpha/RORalpha target gene. *J Biol Chem* 285(46):35386–35392.
5. Crumbley C, Burris TP (2011) Direct regulation of CLOCK expression by REV-ERB. *PLoS One* 6(3):e17290.
6. Brown SA, Kowalska E, Dallmann R (2012) (Re)inventing the circadian feedback loop. *Dev Cell* 22(3):477–487.
7. Stojkovic K, Wing SS, Cermakian N (2014) A central role for ubiquitination within a circadian clock protein modification code. *Front Mol Neurosci* 7:69.
8. Ciechanover A (1998) The ubiquitin-proteasome pathway: On protein death and cell life. *EMBO J* 17(24):7151–7160.
9. Petroski MD (2008) The ubiquitin system, disease, and drug discovery. *BMC Biochem* 9:(Suppl 1):S7.
10. Hoeller D, Dikic I (2009) Targeting the ubiquitin system in cancer therapy. *Nature* 458(7237):438–444.
11. Li W, et al. (2008) Genome-wide and functional annotation of human E3 ubiquitin ligases identifies MULAN, a mitochondrial E3 that regulates the organelle's dynamics and signaling. *PLoS One* 3(1):e1487.
12. Ruffner H, Bauer A, Bouwmeester T (2007) Human protein-protein interaction networks and the value for drug discovery. *Drug Discov Today* 12(17-18):709–716.
13. Yen H-CS, Xu Q, Chou DM, Zhao Z, Elledge SJ (2008) Global protein stability profiling in mammalian cells. *Science* 322(5903):918–923.
14. Burande CF, et al. (2009) A label-free quantitative proteomics strategy to identify E3 ubiquitin ligase substrates targeted to proteasome degradation. *Mol Cell Proteomics* 8(7):1719–1727.
15. Merbl Y, Kirschner MW (2009) Large-scale detection of ubiquitination substrates using cell extracts and protein microarrays. *Proc Natl Acad Sci USA* 106(8):2543–2548.
16. Emanuele MJ, et al. (2011) Global identification of modular cullin-RING ligase substrates. *Cell* 147(2):459–474.
17. Mehra A, Baker CL, Loros JJ, Dunlap JC (2009) Post-translational modifications in circadian rhythms. *Trends Biochem Sci* 34(10):483–490.
18. Godinho SIH, et al. (2007) The after-hours mutant reveals a role for Fbxl3 in determining mammalian circadian period. *Science* 316(5826):897–900.
19. Siepka SM, et al. (2007) Circadian mutant Overtime reveals F-box protein FBXL3 regulation of cryptochrome and period gene expression. *Cell* 129(5):1011–1023.
20. Busino L, et al. (2007) SCFFbxl3 controls the oscillation of the circadian clock by directing the degradation of cryptochrome proteins. *Science* 316(5826):900–904.
21. Christensen DE, Klevit RE (2009) Dynamic interactions of proteins in complex networks: Identifying the complete set of interacting E2s for functional investigation of E3-dependent protein ubiquitination. *FEBS J* 276(19):5381–5389.
22. Ye Y, Rape M (2009) Building ubiquitin chains: E2 enzymes at work. *Nat Rev Mol Cell Biol* 10(11):755–764.
23. Yin L, Joshi S, Wu N, Tong X, Lazar MA (2010) E3 ligases Arf-bp1 and Pam mediate lithium-stimulated degradation of the circadian heme receptor Rev-erb alpha. *Proc Natl Acad Sci USA* 107(25):11614–11619.
24. Linossi EM, Nicholson SE (2012) The SOCS box-adapting proteins for ubiquitination and proteasomal degradation. *IUBMB Life* 64(4):316–323.
25. Kile BT, et al. (2002) The SOCS box: A tale of destruction and degradation. *Trends Biochem Sci* 27(5):235–241.
26. Nakayama K, et al. (2004) Siah2 regulates stability of prolyl-hydroxylases, controls HIF1alpha abundance, and modulates physiological responses to hypoxia. *Cell* 117(7):941–952.
27. Qi J, et al. (2010) Siah2-dependent concerted activity of HIF and FoxA2 regulates formation of neuroendocrine phenotype and neuroendocrine prostate tumors. *Cancer Cell* 18(1):23–38.
28. Nakayama K, Qi J, Ronai Z (2009) The ubiquitin ligase Siah2 and the hypoxia response. *Mol Cancer Res* 7(4):443–451.
29. Zhang J, Guenther MG, Carthew RW, Lazar MA (1998) Proteasomal regulation of nuclear receptor corepressor-mediated repression. *Genes Dev* 12(12):1775–1780.
30. Yin L, Lazar MA (2005) The orphan nuclear receptor Rev-erbalpha recruits the N-CoR/histone deacetylase 3 corepressor to regulate the circadian Bmal1 gene. *Mol Endocrinol* 19(6):1452–1459.
31. Shi G, et al. (2013) Dual roles of FBXL3 in the mammalian circadian feedback loops are important for period determination and robustness of the clock. *Proc Natl Acad Sci USA* 110(12):4750–4755.
32. Bloom J, Pagano M (2005) Experimental tests to definitively determine ubiquitylation of a substrate. *Methods Enzymol* 399:249–266.
33. Vollmers C, Panda S, DiTacchio L (2008) A high-throughput assay for siRNA-based circadian screens in human U2OS cells. *PLoS One* 3(10):e3457.
34. Hirota T, et al. (2008) A chemical biology approach reveals period shortening of the mammalian circadian clock by specific inhibition of GSK-3beta. *Proc Natl Acad Sci USA* 105(52):20746–20751.
35. Baggs JE, et al. (2009) Network features of the mammalian circadian clock. *PLoS Biol* 7(3):e52.
36. Hirota T, et al. (2010) High-throughput chemical screen identifies a novel potent modulator of cellular circadian rhythms and reveals CKIα as a clock regulatory kinase. *PLoS Biol* 8(12):e1000559.
37. Chen Z, et al. (2012) Identification of diverse modulators of central and peripheral circadian clocks by high-throughput chemical screening. *Proc Natl Acad Sci USA* 109(1):101–106.
38. Zhang EE, et al. (2009) A genome-wide RNAi screen for modifiers of the circadian clock in human cells. *Cell* 139(1):199–210.
39. Ukai-Tadenuma M, et al. (2011) Delay in feedback repression by cryptochrome 1 is required for circadian clock function. *Cell* 144(2):268–281.
40. Meng QJ, et al. (2008) Ligand modulation of REV-ERBalpha function resets the peripheral circadian clock in a phasic manner. *J Cell Sci* 121(Pt 21):3629–3635.
41. Solt LA, et al. (2012) Regulation of circadian behaviour and metabolism by synthetic REV-ERB agonists. *Nature* 485(7396):62–68.
42. Kornmann B, Schaad O, Bujard H, Takahashi JS, Schibler U (2007) System-driven and oscillator-dependent circadian transcription in mice with a conditionally active liver clock. *PLoS Biol* 5(2):e34.
43. Cho H, et al. (2012) Regulation of circadian behaviour and metabolism by REV-ERB-α and REV-ERB-β. *Nature* 485(7396):123–127.
44. Bugge A, et al. (2012) Rev-erbα and Rev-erbβ coordinately protect the circadian clock and normal metabolic function. *Genes Dev* 26(7):657–667.
45. Korenčič A, et al. (2014) Timing of circadian genes in mammalian tissues. *Sci Rep* 4:5782.
46. Meireles-Filho ACA, Bardet AF, Yáñez-Cuna JO, Stampfel G, Stark A (2014) cis-regulatory requirements for tissue-specific programs of the circadian clock. *Curr Biol* 24(1):1–10.
47. Feng D, et al. (2011) A circadian rhythm orchestrated by histone deacetylase 3 controls hepatic lipid metabolism. *Science* 331(6022):1315–1319.
48. Wu N, Yin L, Hanniman EA, Joshi S, Lazar MA (2009) Negative feedback maintenance of heme homeostasis by its receptor, Rev-erbalpha. *Genes Dev* 23(18):2201–2209.
49. Frew IJ, et al. (2002) Normal p53 function in primary cells deficient for Siah genes. *Mol Cell Biol* 22(23):8155–8164.
50. Fang B, et al. (2014) Circadian enhancers coordinate multiple phases of rhythmic gene transcription in vivo. *Cell* 159(5):1140–1152.
51. Yamazaki S, Takahashi JS (2005) Real-time luminescence reporting of circadian gene expression in mammals. *Methods Enzymol* 393:288–301.
52. Price TS, Baggs JE, Curtis AM, Fitzgerald GA, Hogenesch JB (2008) WAVECLOCK: Wavelet analysis of circadian oscillation. *Bioinformatics* 24(23):2794–2795.