Title: Circadian actin dynamics drive rhythmic fibroblast mobilisation during wound healing

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One Sentence Summary: The circadian clock in fibroblasts determines the efficiency of wound healing through rhythmic regulation of actin cytoskeletal dynamics.
Abstract:
Fibroblasts are primary cellular protagonists of wound healing. They also exhibit circadian timekeeping which imparts a ~24-hour rhythm to their biological function. We interrogated the functional consequences of the cell-autonomous clockwork in fibroblasts using a proteome-wide screen for rhythmically expressed proteins. We observed temporal coordination of actin regulators that drives cell-intrinsic rhythms in actin dynamics. In consequence the cellular clock modulates the efficiency of actin-dependent processes such as cell migration and adhesion, which ultimately impact the efficacy of wound healing. Accordingly, skin wounds incurred during a mouse’s active phase exhibited increased fibroblast invasion in vivo and ex vivo, as well as in cultured fibroblasts and keratinocytes. Our experimental results correlate with the observation that the time of injury significantly affects healing after burns in humans, with daytime wounds healing ~60% faster than night-time wounds. We suggest that circadian regulation of the cytoskeleton influences wound healing efficacy from the cellular to the organismal scale.

Introduction:
Circadian rhythms allow organisms to organise behaviour and physiology to an approximately 24-hour rhythm, facilitating adaptation to the environmental cycle of day and night. Whilst circadian rhythms in mammals are most evident at an organismal level, circadian timekeeping occurs cell-autonomously (1). The clock in every cell and tissue is synchronised in vivo by systemic cues such as body temperature and glucocorticoid signalling, which are themselves co-ordinated by a master clock in the hypothalamic suprachiasmatic nuclei (2). There is mounting evidence that circadian disruption, associated with modern lifestyles as well
as aging, contributes to morbidities as diverse as cancer, cardiovascular disease and diabetes (3, 4). A major knowledge gap exists however, between the well-characterized circadian gene expression rhythms that occur in healthy peripheral tissues in vivo and the way in which different cell types exploit their innate clockwork to achieve beneficial circadian regulation of cell type-specific functions. The specific advantage conferred by the cell-intrinsic clockwork upon cellular function has not been explored for most cell types, but potentially holds the key to ameliorating the adverse effects of chronic circadian clock disruption.

The cellular clockwork is underpinned by cycles of ‘clock gene’ expression, wherein complexes containing Aryl hydrocarbon receptor nuclear translocator-like protein 1 (ARNTL/BMAL1) drive expression of E-box regulated genes, including the transcriptional co-repressor proteins Period (PER) and Cryptochrome (CRY), which eventually repress their own transcription. Beyond this core loop, BMAL1/PER/CRY-dependent expression of myriad ‘clock-controlled genes’ facilitates differential control of cellular activity across each circadian cycle (5). Indeed, in vivo the core clockwork orchestrates a considerable proportion of the transcriptome (3-16%) to a ~24-hour program, with the identity of specific ‘clock-controlled genes’ varying in a tissue-specific fashion (6).

Fibroblasts are a well-established model of the cell-autonomous clock and possess robust circadian rhythms in clock gene expression (1). However, transcriptomic analyses of cultured fibroblasts have revealed very few circadian-regulated transcripts compared with the number observed in tissues from mice (>3000 in liver compared with 11 in fibroblast cultures) (7). This indicates that in vivo a considerable part of circadian transcriptional regulation is driven systemically (7). Proteomic analyses, which additionally incorporate post-transcriptional circadian regulation, have been applied to whole tissues from mice; however, the extent to which the cell-autonomous clock impacts upon cellular protein abundance has not been
investigated. The capacity of most cell types to keep time in isolation of systemic cues suggests functionality, but the advantage that it confers upon fibroblast-specific cellular functions has not been interrogated (2).

Within the body, fibroblasts are mesenchymal cells that serve to secrete extracellular matrix, a function that is especially salient during wound healing. Upon wounding, fibroblasts respond to chemotactic cues that stimulate proliferation and migration into the affected area (8). Fibroblast motility is driven by actin polymerisation at the leading edge of the cell to form protrusive lamellipodia and filopodia (9), and by disassembly of actin filaments at the trailing edge. Mutation of the actin binding protein CAP2, for example, causes wound healing defects in mice and reduced cell motility in scratch assays, associated with altered actin cytoskeletal dynamics and abnormal cell morphology (10).

Separately, it has been reported that certain parenchymal cell types exhibit circadian rhythms in actin polymerisation, and this was suggested to be controlled by systemic circadian cues rather than the cell-autonomous clockwork (11). The putative driver of these actin rhythms has not been identified however, and neither is it clear whether circadian regulation of the actin cytoskeleton has any functional consequence. One might anticipate that if circadian regulation of the actin cytoskeleton also occurs in mesenchymal cells, then this would confer an adaptive advantage upon processes that are especially reliant upon actin dynamics, such as cell motility during wound-healing. It is established that clock gene mutant rodents exhibit impaired wound healing phenotypes, but because clock genes have multiple functions beyond timekeeping, wound healing defects cannot confidently be ascribed to circadian dysfunction and whether the actual capacity to heal shows a daily rhythm has not been investigated (12–14).
Here we demonstrate that the cell-autonomous clock in fibroblasts drives a temporal proteomic program that imposes rhythmic regulation upon the actin cytoskeleton. We explore the functional consequences of this rhythm and reveal that the cellular clock regulates wound healing in vitro, ex vivo and in vivo through circadian control of cytoskeletal dynamics. Finally, we show that our findings are mirrored by a daily rhythm in the efficacy of healing in a post hoc analysis of human clinical burn data.

**Results:**

*Defining the cell-intrinsic circadian proteome*

Although previous studies have effectively shown the extent of cell-autonomous transcriptional rhythms, it has become increasingly apparent that post-transcriptional mechanisms contribute to determining how the cellular clockwork asserts control of biological function (15–17). Primary fibroblasts exhibit well-characterised circadian rhythms in clock gene activity which persist under constant conditions for at least 6 weeks in vitro (18). To characterise the cell-autonomous circadian biology of fibroblasts we performed a proteome-wide screen for proteins where abundance changes as a function of cellular circadian timing.

We quantified proteins in extracts from quiescent, confluent primary fibroblast cultures collected over two circadian cycles using Stable Isotope Labelling by Amino acids in Cell culture (SILAC) followed by mass spectrometry. The primary fibroblasts were isolated and expanded from mice expressing PER2 fused with Luciferase (PER2::LUC) which served as a parallel reporter of cellular timekeeping (19). Of 1608 proteins identified across the timecourse, 237 exhibited a robust circadian rhythm in abundance ($P < 0.010$, Fig. 1A and fig. S1 and (20)).

Gene set enrichment analysis using the Database for Annotation, Visualization and Integrated
Discovery (DAVID) yielded several gene annotation clusters containing enriched terms (Fig. 1A-B and table S1) that related to mRNA metabolism, DNA binding, the actin cytoskeleton, transcription, protein folding, autophagy and stress signalling (21).

Cellular processes associated with several of these clusters have been previously characterised as circadian, particularly transcription and DNA binding (22). Given the importance of the cytoskeleton in directing cell motility and thus fibroblast function, we were intrigued by the number of cytoskeletal regulators, specifically actin regulators such as Cofilin 2 and RhoA, that were identified as rhythmic (Fig. 1C and fig S2). Individual terms which make up the ‘actin cytoskeleton’ cluster were not enriched ($P=0.071$ to 0.168, table S1). This indicates that, although certain proteins show abundance rhythms, there is not wholesale regulation of the pathway. However, the number of rhythmic cytoskeletal regulators is highly suggestive that activity may be clock-regulated, given that rhythmic abundance of a single control node could be sufficient to render cytoskeletal activity circadian as a result.

**Cell intrinsic clock-control of actin dynamics**

Actin dynamics exhibit circadian regulation in some peripheral tissues in vivo, and are proposed to regulate clock gene expression in response to systemic cues (11). In light of our proteomics data we asked whether actin dynamics might also be driven by the cell-intrinsic clockwork, in the absence of systemic timing cues (Fig. 2A). To test this, actin polymeric state was assayed over two circadian cycles in confluent (quiescent) monolayers of PER2::LUC fibroblasts. We observed a circadian rhythm in F:G actin ratio, with G actin generally in excess of F actin, and without any consistent rhythm in total actin abundance (Fig. 2B).

Cytochalasin D (cytoD) (Fig. 2B) is a drug which binds both F and G actin, preventing
their interaction with cofilin, thereby decreasing the rates of both actin polymerisation as well as depolymerisation (23). We found that the rhythm in F:G actin ratio was disrupted by cytoD, indicating that fibroblast actin dynamics are normally under clock control. By phalloidin staining we confirmed that cells treated with cytoD showed disruption of the actin cytoskeleton, and noticed an increased incidence of binuclearity, but no other major morphological defects were apparent. CytoD treatment had no effect on cellular circadian rhythms, reported by PER2::LUC bioluminescence (Fig. 2B, fig. S3), and the fibroblast circadian clock was similarly resistant to other actin modulating drugs (jasplakinolide and latrunculin A). This indicates that the circadian rhythms of actin dynamics do not strongly feed back into the cell-intrinsic circadian clock mechanism; rather, they are an output from it (fig. S3), and circadian bioluminescent reporter rhythms were therefore unaffected. The periods of the F:G actin ratio rhythm and PER2:LUC were accordant, although the precise period of the F:G actin rhythm could not be determined as accurately as the PER2::LUC continuous recording, due to the comparatively lower sampling frequency (four-hourly vs. half-hourly) and its non-sinusoidal damping waveform.

Using the actin-binding silicon-rhodamine dye, SiR-Actin (24), we validated the rhythm in the total abundance of F actin in individual fibroblasts within a monolayer over 48 hours (Fig. 2C). Using harmonic regression analysis we found that 48% of cells had significant rhythms in F actin (false discovery rate <0.01 and amplitude >10% mean) (25).

Transcriptionally arrhythmic CRY-deficient fibroblasts displayed no rhythm in F:G actin ratio (Fig. 2D). Furthermore, we also observed F:G actin rhythms in another fibroblast line (NIH3T3) derived from mouse embryonic tissue (fig. S4).

To summarise, using two independent methods we detected cell-intrinsic circadian
regulation of actin polymeric state that was dependent on cycling clock gene activity. We conclude that in fibroblasts, circadian regulation of actin dynamics is driven by cell-autonomous rhythms of clock gene expression.

A circadian rhythm in fibroblast wound healing

In vivo, the rapid fibroblast expansion at tissue wounds is known to result from both migration and proliferation of fibroblasts derived from local mesenchymal cells \(^{(8)}\). To investigate the impact of actin cytoskeletal rhythms on cell migration we performed wound-healing assays on synchronised monolayers of immortalised skin fibroblasts at different circadian phases over 2 days under constant conditions (Fig. 3A). The fibroblasts showed striking circadian variations in the residual wound area after 16 hours of healing, with minimal healing elicited by wounds inflicted just after the nadir of PER2 expression, at 32-36 and then at 56-60 hours after synchronisation (Fig. 3A). In contrast, wounding at peak PER2 expression (20-24 or 44-48 hours after synchronisation) was followed by efficient, near complete healing of cell monolayers.

To monitor cell motility more closely, we followed fibroblast migration into wounds by confocal microscopy in cultures of fibroblasts at phases where we observed the maximal and minimal healing responses (24 and 32 hours after synchronisation, respectively). Monolayers wounded after 24 hours re-established more efficiently than those wounded after 32 hours, and this difference remained apparent after 60 hours of healing (Fig. 3B, movies S1 and S2). Cell division occurred infrequently in these cultures and had no circadian organisation (fig. S5), suggesting that differential cell motility underpins the time-of-wounding effect. Indeed, the initial velocity of the most motile 10% of cells was significantly greater for wounds inflicted
24 hours after synchronisation compared with those wounded 32 hours after synchronisation (3.68±0.04 µm.sec⁻¹ vs 2.78±0.03 µm.sec⁻¹, P<0.0001) (Fig. 3B). There was no discernable circadian rhythm in cell velocity during healing, although circadian rhythms in PER2 expression remained evident throughout (fig. S6). Cell motility, cortical actin distribution and cell size lacked any discernable circadian organisation in the absence of wounding (fig. S6) indicating that the circadian variation in initial cell motility we observed is only unmasked upon the insult of wounding. The circadian variation in F:G actin and wound healing response was absent in arrhythmic control fibroblasts lacking CRY proteins (fig. S7).

To assess how the circadian rhythm in actin dynamics might contribute to the time-of-wounding effect on healing we used SiR-actin to dynamically follow actin distribution in cells undergoing healing. Wound-oriented polarisation of F-actin in cells undergoing healing was consistently greater when monolayers were wounded 24 hours after synchronisation rather than 32 hours after synchronisation (Fig. 3C). This is indicative of more efficient F actin enrichment at lamellipodia, the primary means by which fibroblasts effect migration (9). The time-dependent difference in F actin polarisation was sustained for at least the first 9 hours of healing. This is consistent with the migratory capacity of cells, adjacent to a nascent wound, being determined by the actin dynamical state at the circadian phase when the wound is incurred.

**Uncoupling wound healing from the circadian clock**

Cell adhesion is also dependent on the actin cytoskeleton. We thus thought it likely that BMAL1/PER/CRY-dependent circadian regulation of actin dynamics would impact similarly on cell adhesion, which can be detected directly through measurement of cellular impedance
Accordingly, continuous recording of cellular impedance in PER2::LUC cultures revealed a CRY-dependent circadian oscillation, with greatest adhesion in-phase with PER2 expression (Fig. 4A).

To establish causality, we reasoned that if PER:CRY-dependent oscillations in the abundance of actin regulators drive the rhythmic microfilament dynamics that underlie the rhythm in F:G actin ratio, which in turn direct rhythmic adhesion and the time-of-wounding effect on migration, then cytoD disruption of the rhythm in actin dynamics (Fig. 2B) would attenuate both rhythmic outputs (27). To test this we added 0.5 µM cytoD to PER2::LUC fibroblast monolayers and monitored impedance and the response to healing 24 or 32 hours after synchronisation, as well as PER2 abundance in parallel. Compared with controls, we observed that cellular impedance rhythms were more severely damped and no longer showed a circadian rhythm in the presence of cytoD (Fig. 4A). Whilst cells still migrated after wounding in the presence of cytoD, they became insensitive to the time of wounding (Fig. 4B and fig. S8). Therefore, rhythmic wound healing and adhesion result from circadian control of actin dynamics, but do not affect the cellular clock mechanism itself. Both circadian actin rhythms (Fig. 2), and their consequences (Fig. 4), can be uncoupled from clock-control by cytoD treatment, without affecting cellular timekeeping.

We considered that Rho activity might also contribute to the circadian differences in F:G actin ratio, wound healing and cell adhesion. Using the Rho inhibitor CT04 we observed damped rhythms in cell adhesion in PER2::LUC fibroblasts with only subtle effects on circadian rhythms in PER2 abundance (fig. S9). We performed wound-healing assays in cells treated with CT04 and observed abrogation of the time-of-wounding effect (fig. S9). The effect of Rho inhibition on impedance and wound healing rhythms suggests that changes in Rho activity might also contribute towards transmitting circadian timing information to the
cytoskeleton.

Diurnal fibroblast mobilisation in vivo

To investigate the functional consequences of rhythmicity within the fibroblast actin cytoskeleton in the setting of an intact tissue, we measured fibroblast mobilisation in an ex vivo murine skin explant model. Circadian rhythms in the skin of freely behaving mice have been demonstrated previously, and our in vitro experiments suggested that these would persist after wounding (28). Skin explants from PER2::LUC pups were mounted onto membranes and bioluminescence was monitored to confirm that the peripheral circadian clock continues to function in cultured skin ex vivo (Fig. 5A). Subsequently, skin from 5-day old mouse pups was harvested in the middle of the resting period or early active phase and the explant was wounded by biopsy punch and then mounted onto culture membranes. These times of day were chosen on the basis of our cell culture assays, being the times when we would expect to observe the greatest difference in cell migration. The migration of fibroblasts into heterologous blood clots filling the biopsy wounds was assayed by immunofluorescent staining against vimentin. We found that the number and total volume of fibroblasts invading the wound area was roughly two-fold greater in skin explants collected during the active phase than those made during the resting period (Fig. 5B-D, Movies S3 and S4). Finally, we wounded freely behaving adult mice by incision, again during the mid-resting or early active phases, and allowed them to heal for 48 hours (Fig. 5E). Fibroblast enrichment at the wound edge was again quantified using vimentin staining in transverse sections. Once more we observed that fibroblast mobilisation to wounds was significantly greater when wounds were inflicted during the active phase compared with the rest phase ($P < 0.032$, Fig. 5F & G). This indicates that the degree of
fibroblast mobilisation in monolayers, skin explants and mice is dependent on the time-of-wounding.

Within healing tissue, fibroblasts synthesise new extracellular matrix by deposition of collagen and fibronectin (29). Active phase wounds might exhibit enhanced scar tissue formation due to increased collagen deposition, associated with more efficient fibroblast recruitment at this time. To test this we inflicted bilateral wounds to the upper dorsal skin of mice by full-depth biopsy punch, during the resting or active phase. We then allowed the skin to heal completely over 14 days, before measuring collagen distribution around the healed volume. Epidermal collagen deposition was significantly increased above the sites of wounds that were incurred during the active phase compared with the rest phase ($P< 0.001$, fig. S10). In contrast to our observations at 2 days post-wounding, after 14 days of healing fibroblasts no longer showed any significant time-of-wounding difference (fig. S10). This is consistent with the expectation that fibroblasts numbers plateau at the wound site within 14 days (30).

Circadian rhythms in keratinocyte wound healing

Wound healing is a complex process employing multiple cell types in addition to fibroblasts. Keratinocytes migrate into nascent dermal wound areas, and any circadian modulation of this response would be expected to contribute to circadian regulation of wound healing in vivo (31). To test whether keratinocytes also display circadian wound healing responses we wounded synchronised monolayers of human keratinocytes (HaCaT) at times when fibroblast motility was at its peak or nadir, corresponding to the minimum and near maximal BMAL1 promoter activity, respectively (Fig. 6A). We observed a marked reduction in healing when cells were wounded at 32 hours after synchronisation versus those wounded
after 24 or 48 hours. There was no significant difference between the healing response when monolayers were wounded 24 or 48 hours after synchronisation, consistent with keratinocyte migration also being regulated by the circadian clock (Fig. 6A).

A time-of-wounding effect in human burn injury outcome

The observation that migration of at least two major cell types involved in wound healing is circadian regulated suggests that wound healing efficiency overall might be influenced by the biological time of wounding. Although it is unknown whether wound healing is under circadian regulation in vivo, it has been reported that mice carrying mutations of circadian clock genes have impaired wound healing phenotypes (12). If rather than being simply permissive for normal healing the circadian clock instead actively regulates tissue repair, thereby timing the most effective healing to when wounds are more likely to occur, we would expect to see time-of-day effects on wound healing. To this end, we analysed historical clinical data from the Burn Injury Database (iBID) and calculated the time required for human burn injuries to heal to 95% as a function of the time of day when the burn occurred (32). We observed an ~60% increase in healing time when burns occurred during the night compared with during the day (Fig. 6B). Whilst this post hoc analysis cannot prove that human wound healing is under clock control, we note that the time-of-day associated with optimal healing for human burns patients is consistent with our results from rodent and cellular models: all occurred at biological times when mammals would be most active, when they are most likely to incur a wound (fig. S11).
Discussion:

Using a proteome-wide screen for rhythmic protein abundance we identified a concerted regulation of 32 cytoskeletal proteins (GO:0005856). Mapping these onto the ‘Regulation of Actin Cytoskeleton’ pathway (as defined by the Kyoto Encyclopaedia of Genes and Genomes (KEGG)) revealed that several actin effector proteins, such as Cofilin2, and the key control node, RhoA, are rhythmically expressed (fig. S12). This concerted circadian regulation of the actin cytoskeleton generates rhythmic actin dynamics, which in turn modulate the ability of cells to respond appropriately at the moment of wounding – identifying a potentially important function of the cellular clock in a specific peripheral cell type. Our findings build upon an earlier link between wound healing and the circadian clock identified by Kowalska et al (12). Kowalska et al utilised per1−/−/per2brdm/brdm and bmal1−/− mutant mice, well established genetic models that do not express circadian rhythms in behaviour or gene expression. In addition to their molecular clock function however, PER1 and PER2 are immediate early genes and tumour suppressors, whereas BMAL1 is a translation factor and regulator of the antioxidant response; consequently, some elements of each mutant’s phenotype are now known to be unrelated to timekeeping function (33–35). Kowalska et al quite reasonably focused on circadian gating of cell division as a basis for understanding circadian regulation of wound healing; we propose that circadian control of migration also plays a critical role. Using a combined in vitro and ex vivo approach we demonstrate a circadian rhythm in the efficacy of wound healing, allowing us to rule out any substantial contribution of circadian gating of cell division to the differential ability of fibroblasts to enact wound healing in this context.

Our functional data show that circadian regulation of wound-evoked cell motility likely largely contributes to wound healing and epidermal collagen deposition in vivo, in skin and
other tissues. Cell-autonomous circadian regulation of migration was only unmasked in response to wounding and was underpinned by clock gene-dependent temporal organisation of actin dynamics, and associated with rhythmic cellular adhesion (fig. S11). That F actin is conducive to efficient wound healing is explained by its role in protrusive force production at lamellipodia in migrating cells (9). Rho and Rac-mediated signalling to the ARP2/3 complex and resulting cytoskeletal changes modulate cell adhesion and migration (36). As expected, the adhesion rhythms we observed were attenuated by both Cofilin and Rho inhibition, without disrupting clock gene expression cycles (Fig. 2 and fig. S9). Therefore, circadian regulation of the activity of Cofilin, Rho and other actin regulators is likely to be directly responsible for circadian actin dynamics in mammalian cells. Our findings complement reports of rhythmic Rho1 activity in the neurons of fruit flies, and lead us to speculate that circadian regulation of the cytoskeleton may be a conserved phenomenon in metazoans (37). However, rather than circadian control of a single Rho activator, we observed distributed control of actin regulatory pathways (fig. S12), incorporating multiple regulators.

The observation that wound healing is more efficient during the active phase could inform future clinical practice and has clear translational potential. Unfortunately we are currently unable to perform more in-depth analyses of the clinical dataset, to rule out severity changes over time for instance, due to the limitations of data-collection and sample size. In future, larger datasets should be gathered with circadian factors included. An experimental approach where wounds are produced in a controlled manner in a regulated environment would provide unequivocal evidence as to whether or not human wound healing is influenced by the circadian clock. We speculate that maximal healing could be promoted by pharmacological resetting of local cellular clocks prior to surgery, such as through topical application of chronoactive drugs (38).
Beyond adhesion and migration, the actin cytoskeleton is fundamental to eukaryotic cell biology; being essential to cell division, signal transduction, and pathogenesis (39–41). Its circadian regulation would therefore be likely to affect other aspects of biology of broad relevance to human health and disease.

**Materials and Methods:**

**Study design**

The primary objective was to investigate the role of circadian rhythms in controlling actin dynamics and fibroblast mobilisation during wound healing. Data was generated by mass spectrometry and western blotting on cell extracts, longitudinal bioluminescent assays, microscopic analysis of cultured cells, cellular electrical impedance assays, and immunofluorescence on fixed skin sections. For all experiments replicate numbers are outlined in the methods or figure legend. Minimum sample size was determined by power analysis based on an estimated (from preliminary work) effect size with 15% sigma and 5% type 1 error rate, replicates were included such that 1-ß=0.9. Mice in all experiments were age-matched and randomised into groups. Experimenters were not blinded to experimental groups but where possible automated analysis was used to remove bias. Adverse animal welfare issues were sufficient to halt experiments, but did not arise during this work.

**Cell culture and entrainment**

Skin tissue from *Mus musculus* strain PER2::LUC was used to establish an immortalised fibroblast cell line using the method detailed in Seluanov *et al* (19, 42, 43, 45). *cry1*+/− *cry2*+/− fibroblast cells were similarly derived from otherwise isogenic *cry1*+/− *cry2*+/− mice (46). *cry1*+/− *cry2*+/− mouse embryonic fibroblasts (MEF) and isogenic wild-type MEFs were obtained from
Akhilesh Reddy. All mouse strains were gifts from Michael Hastings and were kept under the auspices of Home Office Project Licence 70/7903, under UK Animals (Scientific Procedures) Act, 1986. NIH3T3 fibroblasts and human keratinocyte (HaCaT) cells stably expressing pBMAL1:LUC were made by transfecting cells (ATCC-CRL-1658, or HaCaT cells kindly provided by Prof. Achim Kramer (47)) with a construct encoding firefly luciferase downstream of the BMAL1 promoter in pGL4.22 (Promega) and selected for puromycin resistance. Circadian entrainment was by 12h:12h 32°C:37°C temperature cycling for 3 days. For end-point analysis, plates of cells were washed with phosphate buffered saline (PBS) and lysed at the specified time after the last entraining stimulus. Details of lysis and processing are included in Supplemental Methods. Circadian rhythmicity was assessed either by RAIN, harmonic regression or by non-linear regression to a damped sine wave (see Supplemental Methods). Cells were maintained under absolutely identical conditions to the controls used for circadian bioluminescence recording in every case - see supplementary methods and Feeney et al for additional details (44).

**Live cell actin analysis**

Cells were labelled with 1 µM CellTracker Green (Thermo) and 100 nM Silicon-Rhodamine (SiR)-actin (Cytoskeleton.org) was included in the media throughout entrainment and image acquisition. A Leica SP8 confocal microscope with a 10x/0.40 objective was used to capture 7 µm z-sections covering the entire monolayer thickness every 60 minutes. Average z-projections were generated for each time-point and individual cells tracked using the Nikon Elements General Analysis suite. The SiR-actin signal from each cell track over 24 hours long was subjected to harmonic regression analysis to determine rhythmicity (25).

For analysis of F-actin distribution during healing, cells were delimited manually. The centres of mass for CellTracker Green (cell body) and SiR-actin (F-actin) were calculated using
ImageJ (National Institutes of Health). The difference in position was calculated for each time-point and used as a measure of the degree of actin polarisation in the cell. We were not able to conduct a detailed analysis of stress fibres in these cultures due to limitations in spatial resolution and signal:noise ratio, both of which are essential to reliable identification of bona fide stress fibres.

**Monolayer healing assays**

Live-cell and endpoint scratch assays were performed by wounding synchronised monolayers at defined timepoints with a 200 µL plastic pipette tip and allowing healing at constant 37°C. Healing was assessed by the relative size of residual wound areas or using CellTracker dye-based assays on a confocal microscope as detailed in our supplementary methods.

**Ex vivo wound healing assays**

PER2::LUC pups were raised in 12hr:12hr L:D cycles from birth. For ex vivo assays, on P5 they were sacrificed by cervical dislocation at Zeitgeber Time (ZT) 5 or ZT13 where ZT0 is ‘lights on’. Skin explants ~0.5 cm² were obtained from each mouse and 1.5 mm circular holes made in the explants using a biopsy punch. The explants were then mounted onto 0.4 µm millicell cell culture inserts (Millipore) and 4 µL of platelet rich plasma (PRP) +10% (see supplemental methods) calcium carbonate was added to each wound area and allowed to clot for 5 minutes. The explants were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) + 10% Hyclone III serum at 37 °C, 5% CO₂ for 48 hours prior to fixation with 3% paraformaldehyde and immunostaining for vimentin to identify fibroblasts

**In vivo wound healing**

PER2::LUC mice were raised in 12hr:12hr L:D cycles from birth. Age-matched (3-5 month-old) mice were shaved and wounded by a 1 cm incision at ZT5 or ZT13 where ZT0 is
‘lights on’ and allowed to heal for 48 hours in 12hr:12hr L:D cycles. Age-matched (1-2 month-old) mice were subjected to two bilateral full-thickness 1.5mm circular biopsy punches to the upper back and allowed 14 days of healing in 12hr:12hr L:D cycles. Mice were sacrificed and wounded skin areas were excised and fixed with 4% PFA on 0.4 µm millicell cell culture inserts (Millipore). 60 µm transverse sections were made in frozen gelatin prior to immunostaining. Fibroblasts were identified by immunostaining with rabbit anti-vimentin, mouse anti-PDGFRα or rabbit anti-CD26 antibody (Abcam) and Alexa647 conjugated donkey anti-rabbit/mouse IgG (Thermo). Fibroblast enrichment at the wound edge after 48 hours was measured as follows. The longest continuous wound edge from each section was manually traced. The mean vimentin signal from the edge to 300 µm into the tissue was quantified along the length of the wound giving vimentin signal versus distance from wound edge (Fig.5F). The enrichment of vimentin was defined as the area under the curve (AUC) using the mean vimentin signal between 200-300 µM from the edge as the baseline (Fig.5G). Fibroblast distribution after 14 days of healing was analysed by mean fluorescent intensity from the epical edge of the signal at the centre of the wound to a depth of 200 µm. Vimentin and PDGFRα stained the dermis, CD26 stained the epidermis and dermis. The total signal for each fibroblast marker was quantified by AUC to a depth of 100 or 200 µm.

Collagen deposition was measured after 14 days by staining the sections for 3 hours with Col-F (ImmunoChemistry laboratories). The Col-F signal was quantified along the full thickness of the epidermis for >2 mm, centred on the wound. The Col-F was quantified by calculating the AUC using the distal signal as a baseline.

**Human data**

Anonymised records were obtained from a specialized burn database (iBID) which records outcomes from all the major burns units in England and Wales (32). In 2012, time of injury
and number of days until 95% healing began to be recorded. Burns were included if a 95% heal time was recorded on the database and the subject had been admitted via the emergency department for their burn. Subject inclusion criteria were 18-60 years old with a body mass index (BMI) between 20-30 kg/m². Subject records were excluded if they had any previous diseases or a skin graft was used in the treatment of their burn, leaving 118 patients in total (2012-2015).

**Statistical analysis**

For this study we utilised the following statistical methods; GO enrichment analysis using DAVID, RAIN, the extra sum-of-squares F test for comparison of nonlinear regression fits, harmonic regression, two-way ANOVA with and without Tukey’s multiple comparisons correction, two-way ANOVA with the Holm-Sidak adjustment, one-way ANOVA, Student’s and Welch’s t-test. They are each detailed along with the relevant parameters in our methods and supplemental methods and figure legends. In general $P < 0.05$ was considered significant and $P < 0.01$ highly significant.

**Supplementary Materials:**

Supplemental Methods.

fig. S1. Cell-intrinsic rhythms in the fibroblast proteome

fig. S2. Validation of Cofilin2 and RCC2 circadian abundance.

fig. S3. Circadian clocks are robust against actin modulating drugs

fig. S4. Cell-intrinsic rhythms in actin polymerisation in NIH3T3 cells

fig. S5. Cell division in healing fibroblast monolayers

fig. S6. Cell area, velocity and cortical actin distribution are not circadian in confluent
cultures with circadian PER2:LUC activity.

fig. S7. Circadian rhythms in actin dynamics and wound healing efficacy are cryptochrome dependent in embryonic fibroblasts

fig. S8. Fibroblast monolayer healing in the presence of cytoD

fig. S9. Rho inhibition disrupts impedance rhythms and time-of-wounding effects without disrupting the circadian clock

fig. S10. Collagen deposition and fibroblast distribution after 14 days of healing.

fig. S11. A model of clock control of wound healing

fig. S12. Circadian Rhythmicity of the ‘Regulation of Actin Cytoskeleton’ Pathway.

table S1. DAVID clustering of rhythmic proteins identified by RAIN ($P<0.01$)

table S2. Individual subject-level data for N < 20

Movie S1. Healing of fibroblast monolayers wounded 24 hours post-synchronisation

Movie S2. Healing of fibroblast monolayers wounded 32 hours post-synchronisation

Movie S3. Fibroblast invasion of wounds made in the active phase after 48 hours of healing

Movie S4. Fibroblast invasion of wounds made in the inactive phase after 48 hours of healing

References and Notes


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Figure and Table Legends:

Fig. 1. The cell-intrinsic fibroblast circadian proteome contains numerous cytoskeletal regulators

A. Protein annotation clusters generated by DAVID containing terms enriched (P <0.10) with rhythmic protein abundances identified by Rhythmicity Analysis Incorporating Nonparametric methods (RAIN) (P<0.01) from analysis of primary lung fibroblasts from PER2::LUC mice. B. The 10 largest Gene Ontology (GO) (cellular compartment) terms within the rhythmic dataset by protein number. C. Mean abundance (Light:Heavy (L:H) ratio) of
rhythmic proteins from the ‘actin cytoskeleton’ cluster determined by 3 SILAC experiments with 3 parallel PER2::LUC measurements indicating the circadian phase (heat map).

Fig. 2. CRY-dependent cell-intrinsic rhythms in actin polymerisation

A. Schematic depicting rhythms in actin polymerisation, which may be cell intrinsic (blue arrow; driven by circadian gene expression) in addition to systemic cues (red arrow). B. Immunoblots using anti-actin antibody against fractionated and total protein from PER2::LUC fibroblasts at the indicated time after synchronisation in the presence of DMSO (i) or cytoD (0.5 µM) (ii). F:G actin ratio is quantified below, with best-fit curves from a comparison of fits (n=3 mean±Standard Error (SEM)). 3 parallel bioluminescent measurements (heat map) are included as a marker for the circadian clock. As G actin was in excess, exposures between western panels are not equivalent. C. Live cell recordings of actin abundance (SiR-actin) in cells labelled with Celltracker Green (i,ii, scale bar =100 µm). iii. SiR-actin intensity over time for 8 individual tracks (orange lines) with mean (black) ±SEM (grey) overlayed. Tracks with a circadian harmonic regression FDR (q value) <1% and amplitude >10% of the mean are highlighted and quantified (iv). D. F:G actin ratios from wild type (WT, black line) or cry1-/- cry2-/- (orange line) fibroblasts at the indicated time after synchronisation, with best-fit curves from a comparison of fits (n=3, mean±SEM, RAIN p-values indicated).

Fig. 3. A circadian rhythm in fibroblast wound healing response

A. Fibroblast monolayers derived from adult PER2::LUC mouse skin were entrained and wounded after 20-64 hours in free run (i). (ii) Images of wound healing assays; time at wounding is indicated, residual wound is indicated by pink highlighting (scale bar = 500 µm).
(iii) Quantification of the residual wound after 16 hours of wound healing (line, mean±SEM, n=4) with 3 parallel PER2::LUC measurements (heat maps). RAIN p-value indicated. Bi. Fibroblasts labelled with CellTracker Red healing after wounding at the indicated time after synchronisation (t). Scale=100 µm. Wound healing (ii) and leading cell velocity (iii) are quantified (n=4 or 5, ±SEM). P values from Tukey’s multiple comparisons test after 60 hours of healing (tH60) (ii) or tH0 (iii) are indicated. Ci Fibroblasts labelled with SiR-actin (red) and CellTracker Green (cyan) treated as in B (scale bar = 50 µm). A single cell for each condition has been highlighted in white with time healing indicated. The centre of mass for each label (ii) was determined over 9 hours of healing and the mean (±SEM) degree of polarisation (∆x) is indicated (iii). The P value from a two-way analysis of variance (ANOVA) is indicated.

Fig. 4. *Actin polymerisation rhythms are required for circadian regulation of adhesion and wound healing efficacy by fibroblasts*

A. Impedance measurements from cry1−/−cry2−/− (blue) or WT fibroblasts treated with DMSO (black) or cytoD (orange) with simultaneous PER2::LUC measurements (heat maps) (mean±SEM, n= 6-8). B. Quantification of mean fibroblast monolayer healing after wounding at the indicated time post-synchronisation (t) in the presence of 0.5 µM cytoD or vehicle (n=6-12, ±SEM). p-values from an ANOVA with Tukey’s test for multiple comparisons are indicated.

Fig. 5. *Diurnal variation in wound healing outcome and fibroblast mobilisation*

A. Bioluminescent recording of PER2 expression in neonatal (P5) skin explants from
PER2::LUC mice (mean±SEM n=6). B. Mouse skin wounds before and after 48 hours of healing. Fibroblasts were identified by anti-vimentin reactivity (red) and morphology, and quantified by number (C) and volume (D) (mean±SEM, n=6-7, Holm-Sidak’s adjusted $P$ value is indicated). Scale bar =200 µm. E. 60 µm transverse sections of mouse wounds made during the active and resting phases stained using anti-vimentin (magenta) and Hoescht (blue). Cross-sectional vimentin staining across wound edges was quantified (F, mean±SEM) and Area Under Curve (AUC) was calculated using distal vimentin as a baseline (G) (mean±SEM, n=16 (active) or 20 (resting), $P$ from a student’s t test is indicated).

Fig. 6. A circadian rhythm in keratinocyte wound healing and a diurnal variation in human burn healing outcome

A. Synchronised human HaCaT keratinocyte monolayers expressing luciferase under control of the BMAL1 promoter (i, mean±SD, n=24) were wounded at the indicated times (vertical lines) and healing monitored by confocal microscopy (ii). Relative fluorescence in the wound area (iii, mean±SEM n=4) was calculated and maximal healing after 15 hrs was compared by Tukey’s multiple comparisons test (iv, $P$ values are indicated) B. Mean time to 95% healing ±SEM from 118 human burn incidents separated by time of burn occurrence in 4 (left) or 12 hour (right) bins. ANOVA $P$ value is indicated, as is the $P$ value for Welch’s t-test comparing daytime vs night-time wounds. $P$ values from Holm-Sidak’s test versus the 0000-0359 bin are indicated below.
A Bi

**Intrinsic Cues**

CLOCK

BMAL1

PER

CRY

**Systemic Cues**

ACTIN

F:G actin ratio

time (hours)

24 36 48 60 72

0.0

0.5

1.0

1.5

2.0

P = 0.366

24 36 48 60 72

0.0

0.5

1.0

1.5

2.0

P = 0.004

24 28 32 36 40 44 48 52 56 60 64 68 72 76 80

time (hours)

F-actin

G-actin

total actin

P = 0.004

P = 0.366

**C**

**iii**

**iv**

159 rhythmic tracks

n=331

**D**

WT  P=0.017 period=24.0±3.1

cry1-/-cry2-/-  P=0.082

normalised F:G actin ratio

time (hours)

0.0 0.1 0.2 0.3 0.4

10-15

10-10

10-5

100

amplitude

q value

time (hours)

WT  P=0.017 period=24.0±3.1

cry1-/-cry2-/-  P=0.082
Ai

- **time at wounding (hours)**
- **mean leading cell velocity** (µm/sec^-1)

<table>
<thead>
<tr>
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<tr>
<td>20</td>
<td>24 32 36 48</td>
</tr>
<tr>
<td>44</td>
<td>48 52 56 60</td>
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</table>

B

- **time healing (t_H) (hours)**
- **residual wound area (10^5 µm^2)**

<table>
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</table>

C

- **time at wounding (hours)**
- **F actin polarisation**

<table>
<thead>
<tr>
<th>P</th>
<th>0 1 2 3 4 5</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0 0.5 1.0 1.5</td>
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</table>

- **centre of mass (CellTracker)**
- **centre of mass (SiR-Actin)**

<table>
<thead>
<tr>
<th>P</th>
<th>0 1 2 3 4 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0 0.5 1.0 1.5</td>
</tr>
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</table>
A BiPER2::LUC

WT cry1-/-cry2 -/- WT + cytoD

0 24 48 72 96 120

time (hours)

normalised cell index (AU)

0.0 0.5 1.0

0 12 24 36 48

0.0 0.5 1.0

time (hours)

healing

t 24 DMSO

t 24 CytoD

t 32 CytoD

P = 0.711

P = <0.001

t 24 DMSO t 32 DMSO t 24 CytoD t 32 CytoD

0.0 0.5 1.0

healing after 48h

(wound / non wound intensity)
PER2 expression in neonatal skin

fibroblasts in wound

fibroblast volume in wound

fibroblast staining at wound edge: cross-section

fibroblast staining at wound edge: AUC
Holm-Sidak

0.283 0.005 0.013 0.006 0.037

P = 0.017

P = 0.008

P = 0.744

0.0 0.5 1.0

0 5 10 15

0.0

0.5

1.0

0.0

0.5

1.0

0.0

0.5

1.0
Supplemental Materials:

Supplementary Methods:

Cell Culture and Entrainment

Skin tissue from *Mus musculus* strain PER2::LUC was used to establish an immortalised fibroblast cell line using the method detailed in Seluanov *et al* (19, 42, 43, 45). *cry1<sup>-/-</sup> cry2<sup>-/-</sup> fibroblast cells were similarly derived from otherwise isogenic *cry1<sup>-/-</sup> cry2<sup>-/-</sup>* mice (46). *cry1<sup>-/-</sup> cry2<sup>-/-</sup>* mouse embryonic fibroblasts (MEF) and isogenic wild-type MEFs were obtained from Akhilesh Reddy. All mouse strains were gifts from Michael Hastings and were kept under the auspices of Home Office Project Licence 70/7903, under UK Animals (Scientific Procedures) Act, 1986.

NIH3T3 fibroblasts and human keratinocyte (HaCaT) cells stably expressing *pBMAL1*:LUC were made by transfecting cells (ATCC-CRL-1658, or HaCaT cells kindly provided by Prof. Achim Kramer (47)) with a construct encoding firefly luciferase downstream of the *BMAL1* promoter in pGL4.22 (Promega) and selected for puromycin resistance. Cells were grown in DMEM (Thermo) supplemented with 10% Fetalclone Hyclone III serum and penicillin/streptomycin antibiotic mixture (100U/ml and 100µg/ml respectively) at 37°C and 5% CO₂. Confluent, quiescent fibroblast monolayers were entrained by incubating them for 12 hours at 32°C followed by 12 hours at 37°C for three days, to ensure that all cells were synchronised to the same circadian phase at the beginning of each experiment. ‘Synchronisation’ was defined as the last shift to 37°C, which coincided with a change into ‘recording’ media, thereafter cells were held at constant 37°C. Recording medium was identical to DMEM except that 20mM HEPES pH 7.4, 0.3mM luciferin, 2% B27 (Thermo) were included and the concentration of sodium bicarbonate concentration was reduced to 0.035%.
**Scratch Assays**

At the designated time, synchronised fibroblast cultures in ‘recording media’ were wounded by scratching with a plastic pipette tip. Cells were kept on isothermal pads (Deltaphase) whilst wounding to mitigate temperature fluctuations. After a further 16 hours at 37°C and atmospheric CO₂ the cells were removed from the incubator and simultaneously fixed and stained with 3% paraformaldehyde + 0.15% Crystal Violet 1% Methanol at 4°C overnight. Cells were imaged using a Nikon HCA microscope using a 4x/0.20 objective with 4x4 binning. Residual wound areas from a 2.7mm² section of scratched monolayer for 4 cultures were quantified using the MRI wound healing tool for ImageJ (National Institutes for Health) (48). Bioluminescence rhythms from cells entrained by the same protocol as the scratch assay cultures were recorded using an ALLIGATOR (Cairn Research).

**Live Cell Wound Healing Assays**

PER2::LUC fibroblasts were grown to confluence in 8-well µslides (Ibidi). Confluent cells were labelled for half an hour with 1μM CellTracker Red (Thermo) and entrained using temperature cycles prior to incubation at constant 37°C, wounding and imaging of the healing chamber after 24 or 32 hours. Isothermal pads (Deltaphase) were used during cell transfers to the recording environment to mitigate temperature fluctuations. Entrainment and recording medium was 0.035% sodium bicarbonate DMEM supplemented with 20mM HEPES pH 7.4 and 2% B27 (Thermo). Cells were held at constant 37°C at atmospheric CO₂ concentration. A confocal microscope (Leica SP8) with a 10x/0.40 objective was used to capture 7μm z-sections covering the entire monolayer thickness every 30 or 60 minutes. Maximum z-projections were generated for each time-point and used for wound healing assessment.

The movement of cells into the wound area was monitored by pixel intensity where:
\[ \text{Healing} (y) = \frac{(i^w_x - i^w_0)}{(i^n_x - i^n_0)} \]

Where \( i \) is the mean intensity of the wound (superscript \( w \)) or non-wound (superscript \( n \)) area at time \( x \) or \( 0 \) (subscript). Healing was compared between datasets by ANOVA with Tukey’s multiple comparisons test.

Mean leading cell velocity was calculated using the Imaris (Bitplane) track spots function – instantaneous velocity measurements were averaged across the 90th percentile of motile cells (determined by total distance travelled throughout the timecourse) and then averaged across 4 replicates. Velocity versus time was fit to an exponential decay curve.

For drug assays media was supplemented with 0.5µM cytoD (Sigma) or CT04 (2µg.ml\(^{-1}\)) after loading of CellTracker Red dye and the wound area was defined as a rectangle extending 120µm from the edge of the non-wound area. The efficacy of cytoD treatment was confirmed by fixing cells with 4% formaldehyde after treatment with 0.5µM cytoD. Fixed cells were stained with Cytopainter-488 (Abcam), DAPI and CellMask Deep-Red according to the manufacturer's instructions and imaged using a confocal microscope as described above.

PER2::LUC rhythms in monolayers prior to, and after, wounding were analysed using an LV200 microscope (Olympus) with a 40x/0.6 lens using 200x EM gain. Recording media was supplemented with 1mM luciferin to enable bioluminescent detection and cells were grown in a coverglass-bottomed dish (Mat-ték), otherwise experimental conditions were identical to the confocal setup above.

**Ex Vivo Wound Healing**

PER2::LUC pups were raised in 12hr:12hr L:D cycles from birth to P5 when they were sacrificed by cervical dislocation at ZT5 or ZT13 where ZT0 is ‘lights on’. Skin explants ~ 0.5cm\(^2\) were made from each
mouse and 1.5mm circular holes made using a biopsy punch. The explants were then mounted onto 0.4μm millipore cell culture inserts (Millipore) and 4μl of platelet rich plasma (PRP) +20mM calcium carbonate was added to each wound area and allowed to clot for 5 minutes. PRP was obtained by using a protocol modified from Franco et al (49). Briefly, fresh mouse blood was centrifuged at 200g for 5 minutes at 4°C using 3.2% sodium citrate anticoagulant. Aliquots of platelet rich plasma made at ZT0 and ZT12 were mixed 1:1 prior to freezing. The explants were grown in DMEM + 10% Hyclone III serum at 37°C, 5% CO₂ for 48 hours prior to fixation with 3% paraformaldehyde.

Fibroblasts were identified by immunostaining with rabbit anti-vimentin antibody (Abcam) and Alexa647 conjugated donkey anti-rabbit IgG (Thermo). Fibroblasts in the wound area were quantified using Imaris (Bitplane) segmentation routines. Fibroblasts were identified by their spindle-like morphology (sphericity), anti-vimentin reactivity and size.

**Actin Fractionation**

Synchronized and confluent quiescent fibroblast monolayers in DMEM +10% HyClone III serum (Thermo) at constant 37°C and 5% CO₂ were harvested every 4 hours from 12 hours post-synchronisation onwards for 48 hours. Cells were washed in PBS prior to lysis for 15 minutes at 37°C in 50mM PIPES (pH6.9), 50mM NaCl, 5mM MgCl₂, 5mM EGTA, 5% glycerol, 0.1% Nonidet P40, 0.1% Triton X-100, 0.1% Tween-20, ATP 1mM + EDTA-free protease inhibitors (Roche). Cell debris was removed by centrifugation at 500g for 5 minutes. 200μl of lysate was then centrifuged at 150,000g for 1 hour. The supernatant was then flash frozen and the pellet dissolved in 200μl 4M urea, 15% glycerol, 1% SDS, 50mM DTT, EDTA-free protease inhibitors (Roche). The relative abundance of actin in each fraction was determined by western blotting with an anti-actin antibody (Abcam ab3280).
**Gel Electrophoresis and Western Blotting**

Protein samples were denatured in LDS buffer by heating at 70°C for 10 minutes. Where necessary, protein samples were reduced with 50mM DTT. Protein was separated on precast 4-12% polyacrylamide gels prior to transfer to nitrocellulose membranes. Membranes were blocked and probed in 0.25% dried milk, 0.25% BSA in TBST. Primary antibody incubation was conducted overnight at 4°C prior to secondary antibody incubation at 21°C for 1 hour. Membranes were washed in TBST and bands visualized by chemiluminescence or infrared detection. The signal from reactive bands was quantified using ImageJ and expressed as a fraction of the average signal across the timecourse. F:G actin ratio was determined from fractions normalized in this manner. Brightness and contrast were altered in the displayed blots to highlight circadian variation; quantitation was done without these alterations.

**Cell Morphometry**

For assessment of cellular morphology and actin distribution cells were transfected with a plasmid expressing the Calponin homology domain of Utrophin fused to GFP (UtrCH-GFP) (50) and imaged 48 hours later, following a medium change. A confocal microscope (Leica SP8) with a 10x/0.40 objective was used to capture 7µm z-sections covering the entire monolayer thickness every 21 minutes for >60 hours. Maximum z-projections were generated for each time-point and cells identified and measured using the QUIMP plugins for ImageJ (National Institutes for Health) defining the cortex as 7µm from the cell edge (51). 51 cells were tracked across 11 µslide wells (ibidi).

**Circadian Proteomics of Primary Fibroblasts**

Primary fibroblast cells were obtained from the lung tissue of a two month old male PER2::LUC C57BL/6J mouse (45). Cell cultures were maintained in 5% CO₂ humidified incubators. The primary fibroblasts were expanded to P4, hereafter cells were seeded in 60mm dishes and left for two days to create
a confluent monolayer. Cell dishes were then placed under temperature entrainment for three cycles (37˚C to 32˚C) before going into 24 hours of free-running (37˚C) and from that point onwards three dishes were harvested every third hour for more than two consecutive cycles (54 hours, 19 time points).

To generate a SILAC spike-in sample, we used immortalised fibroblasts, which were passaged for ten doublings in SILAC DMEM (Invitrogen), 10 % FBS (1k Da dialysed, Dundee Cell products) with 0.39mM $[^{13}C_6,^{15}N_2]$-L-Lysine and 0.79mM $[^{13}C_6,^{15}N_4]$-L-Arginine (Cambridge Isotope Laboratories). To ensure representation of all peptides, cells were synchronised and harvested every six hours in one circadian cycle, after which the four resulting samples were mixed into one spike-in sample. For all samples, cells were lysed in 2 % SDS, snap frozen in liquid N$_2$ and kept at -80˚C until further use.

Protein concentrations were determined by BCA assay (Pierce) before mixing 25µg of SILAC spike with 25µg of protein lysate from each biological replicate. Samples were digested by the FASP method using N-ethylmaleimide (NEM) as an alkylation reagent and a trypsin ratio of 1:25 (52). Peptides were dried and reconstituted in 0.1% formic acid. The peptides were analysed as single runs by nano-scale capillary LC-MS/MS using an Ultimate U3000 HPLC (Thermo) to deliver a flow of approximately 300 nL/min. A C18 Acclaim PepMap100 5µm, 100µm x 20mm nanoViper (Thermo), trapped the peptides prior to separation on a C18 Acclaim PepMap100 3µm, 75µm x 250mm nanoViper (Thermo). Peptides were eluted with a 120-minute gradient of acetonitrile (2% to 60%). The analytical column outlet was directly interfaced via a nano-flow electrospray ionisation source, with a hybrid quadrupole orbitrap mass spectrometer (Q-Exactive Plus Orbitrap, Thermo). Data dependent analysis was carried out, using a resolution of 30,000 for the full MS spectrum, followed by ten MS/MS spectra. MS spectra were collected over a m/z range of 300–2000. MS/MS scans were collected using a threshold energy of 27 for higher energy collisional dissociation (HCD). The FASP step through to spectrum collection was completed in duplicate on separate occasions, the data from each run was amalgamated and quantified with MaxQuant 1.5 (53). NEM was set as a fixed modification while N-acylation and methionine oxidations were set as variable. ‘Light’ to ‘Heavy’ protein isotope ratio was computed and normalised within each sample so that
the median ratio was equal to 1.

RCC2 and Cofilin2 (CFL2), proteins of interest found to be rhythmic (p<0.01) with sufficiently high amplitude to enable detection of rhythms by chemiluminescent detection were validated by western blotting and RAIN (fig. s2).

Assessment of Cellular Growth Rate

To assess the rate of cell division in confluent cultures undergoing wound healing we loaded cells with 1µM Celltracker Red or 1µM CellTracker Green and mixed them at a 10:1 ratio. The cells were visualised as in the Live Cell Wound healing protocol above except that cell division events in CellTracker Green labelled cells were manually defined and used to calculate the probability of such events occurring per time frame. Cells were imaged every hour, which was sufficient to capture cell divisions mid-mitosis. This was confirmed by the lack of green labelled cells appearing mid-way through the timecourse without an observed associated mitotic event.

Assessment of Rhythmicity

To assess the rhythmicity of our western blotting and residual wound area timecourse data we used the RAIN algorithm (20). Outliers were identified and excluded from RAIN analysis using the ROUT method in Prism (Graphpad) with a coefficient, Q, of 1% (54). We constrained the algorithm to search for 24-hour periodicity with symmetrical peak-trough profiles.

For bioluminescence recordings, DMEM+10% HyClone III was supplemented with 1mM luciferin and maintained at 37°C in an ALLIGATOR (Cairn Research). The CO2 concentration was maintained at atmospheric levels or at 5% with appropriate adjustment of bicarbonate concentration to match that of any parallel assays. 30min-1 hour exposures were taken continuously and cosmic ray artefacts were removed by outputting minimum pixel values from adjacent images into a denoised dataset. Luminescence was
calculated from mean pixel intensity and detrended by subtraction of a 24-hour moving average. Where necessary, data was fit by least squares regression to a damped sine with a linear baseline:

\[ y = (mx + c) + a e^{-kx} \sin \left(\frac{2\pi x - r}{p}\right) \]

Where \( m \) is the gradient of the baseline and \( c \) is the \( y \) offset. \( k \) describes the rate of damping, \( a \) the amplitude, \( r \) the phase and \( p \) the period. Damping is generally thought to occur at a population level in fibroblasts due to desynchronisation of cells (55, 56).

To illustrate the degree of rhythmicity of WT, cryptochrome mutant and cytoD treated cellular F:G actin ratio (Fig. 2), an extra-sum of squares test was used to choose between the above equation and a straight line. The preferred fit is plotted alongside the data.

For the SILAC dataset the RAIN algorithm was then allowed to search for periodicities of 24±3 hours. Due to the partial proteome coverage the dataset included missing timepoints for many proteins. To be included in the RAIN analysis proteins must be detected in at least 2 biological replicates at 12 different timepoints.

**Gene Set Enrichment Analysis**

A list of proteins identified as rhythmic was compared to a population background of all proteins detected (meeting the above inclusion criteria). The DAVID platform v6.8 functional annotation clustering tool was employed with UP_KEYWORDS, GOTERM_BP_DIRECT, GOTERM_CC_DIRECT and GOTERM_MF_DIRECT as clustering features (21). Clusters containing enriched terms were sorted by size and given our own nomenclature based upon the terms included.

**Measurement of Cellular Impedance**

Cells were grown to confluence in 16 well E-plates and entrained using the above protocol prior to
continuous measurement of cell index in our standard recording medium (see above) using an xCelligence RTCA DP device (ACEA) at constant 37°C and atmospheric CO₂. Cell Index (CI) is an arbitrary measurement of impedance calculated by the ratio of cell-electrode impedance at each time-point versus a background reference taken prior to seeding cells. CI was calculated every 30 minutes and smoothed by a 7-point (3.5hr) moving average. For drug assays the recording media was supplemented with 0.5µM cytoD or 2µg.ml⁻¹ Rho Inhibitor I (CT04, Cytoskeleton Inc).

Measurement of PER2::LUC in skin biopsies

Age-matched female PER2::LUC mice were housed in a 12h:12h LD cycle and allowed to acclimatise for 2 weeks. ~500mg skin was collected from 3 mice every 4 hours throughout a 24-hour period and snap frozen. Thawed skin was homogenised in 500μl of lysis buffer (100mM potassium phosphate pH 7.8, 1mM EDTA, 7mM 2-mercaptoethanol, 1%(v/v) Triton X-100, 10% (v/v) glycerol and complete EDTA-free protease inhibitors (Roche)). The homogenised tissue was clarified by centrifugation at 10,000g for 2 minutes. 10μl of the resultant supernatant was added to 100μl of luciferase buffer (15mM MgSO₄, 30mM HEPES pH7.8, 300uM luciferin, 1mM ATP) and luminescence measured immediately for 10 seconds on a Berthold CentroPRO LB 962. Luminescence normalized to protein concentration was determined by BCA assay kit (Thermo).
Fig. S1. Cell-intrinsic rhythms in the fibroblast proteome
Abundance (L/H ratio) of rhythmic proteins (RAIN p<0.01) determined by SILAC with 3 parallel PER2::LUC measurements (heat map). Proteins are ordered top to bottom by phase determined by RAIN. Note that HIST1H2B is not differentiable from HIST1H2BB, HIST1H2BF, HIST1H2BK, HIST1H2BA, HIST3H2BA and HIST3H2BB.
**fig. S2. Validation of Cofilin2 and RCC2 circadian abundance.** Immunoblots using antibodies targeting the indicated proteins in whole protein extracts from synchronised PER2::LUC fibroblasts sampled at the indicated time after synchronisation. p values from a RAIN analysis are indicated.
fig. S3. Circadian clocks are robust against actin modulating drugs
A. Synchronised cells were fixed 30 min after treatment with DMSO or cytoD as indicated. Cells were stained with DAPI, CellMask-deep red and Cytopainter-488 (phalloidin). Scale=50 μm. B. Luminescence quantitation from PER2::LUC expressing mouse fibroblasts (Arbitrary Units, AU) ±SEM in the presence of cytoD, jasplakinolide or latrunculin A. Dose response curves of the period parameter from a damped sine wave least square fit are plotted. The Pearson correlation r statistic is indicated.
fig. S4. Cell-intrinsic rhythms in actin polymerisation in NIH3T3 cells

Immunoblots using anti-actin antibody against total or fractionated protein from NIH3T3 fibroblasts at the indicated time after synchronisation. F:G actin ratio is quantified below (n=3 mean±SEM) with 3 parallel pBMAL-LUC measurements (heat map). As G actin was in excess, exposures between western panels are not equivalent. RAIN p-value is indicated.
**fig. S5. Cell division in healing fibroblast monolayers**

A montage of z-projections from a confocal microscopy timecourse of synchronized fibroblast cells after monolayer wounding 24 hours after synchronisation. Cells were labelled in a 1:10 ratio with CellTracker Green and Red. The green signal was used to manually define cell division events. An example division event 36 hours into the healing phase (tₕ) is highlighted. The temporal resolution of 1 hour was sufficient to image all division events mid-mitosis, confirmed by visual monitoring for nascent green cells appearing mid-way through the timecourse. The proportion of green cells undergoing division at each time-point is quantified in B for 3 replicates.
fig. S6. Cell area, velocity and cortical actin distribution are not circadian circadian in confluent cultures with circadian PER2::LUC activity.
A. Whole field mean bioluminescent recording of PER2 expression in a PER2:LUC monolayer during microscopic recording. After wounding by scratching the recording was restarted. The phase of the fitted damped-cos wave is indicated ±SEM in the phase diagrams below. B. Graphs depicting mean 2-dimensional cell area (i), cellular velocity (ii) and percentage of total actin in the cell cortex (iii) ±SEM at the indicated time since synchronisation from 51 unwounded cells imaged by confocal microscopy in 11 independent wells. Cells were transfected two days prior to imaging with UtrCH-GFP to specifically mark F-actin. An example micrograph is shown depicting the cell boundary and cortical boundary (iii).
fig. S7. Circadian rhythms in actin dynamics and wound healing efficacy are cryptochrome dependent in embryonic fibroblasts

Ai. Immunoblot analysis using anti-actin antibody against fractionated protein from CRY1 CRY2 (wild type, WT), or cry1<sup>-/-</sup> cry2<sup>-/-</sup> mouse embryonic fibroblasts (MEF) at the indicated time since synchronisation. ii. Quantitation of the F:G actin ratio as a mean of 3 replicates ±SEM. A binomial line was fit to each dataset to remove longitudinal trends from the data. The residuals ±SEM are shown in Aiii. RAIN p-values are indicated. B. Monolayer healing after wounding at the indicated time after synchronisation in WT (i) or cry1<sup>-/-</sup> cry2<sup>-/-</sup> (ii) MEFs (n=3, ±SEM). p-values indicate Tukey's multiple comparisons test at t<sub>48</sub>.
**fig. S8. Fibroblast monolayer healing in the presence of CytoD**

Fibroblasts labelled with CellTracker Red before ($t_0$) and after ($t_{48}$) healing. Monolayers were wounded at the indicated time after synchronisation in the presence of 0.5 μM cytoD or vehicle. Scale=100 μm. Wound healing from the full timecourse is quantified in Fig.4C.
fig. S9. Rho inhibition disrupts impedance rhythms and time-of-wounding effects on healing without disrupting the circadian clock
A. Impedance measurements from PER2::LUC fibroblasts treated with DMSO (blue) or 2µg.ml⁻¹ CT04 Rho Inhibitor (green) with simultaneous PER2::LUC measurements (heat-maps) (means ±SEM, n=4). B. Quantification of mean fibroblast monolayer healing after wounding at the indicated time post-synchronisation (t) in the presence of 2µg.ml⁻¹ CT04 or vehicle (n=3-5, ±SEM). p-values from an ANOVA with Tukey’s test for multiple comparisons are indicated.
Fig S10. Collagen deposition and fibroblast distribution after 14 days of healing.
A. Col-F staining of collagen of 60μm transverse sections of residual wounds after 14 days of healing. Wounds were made at ZTS (resting, black bars/lines) or ZT13 (active, orange bars/lines) (scale bar = 100μm). Staining along the epidermis, centred on the wound (w) is quantified in B and C (mean±SEM, n=6, p values from a t test are indicated). D. Fibroblast marker distribution in residual wound areas was assayed by immunostaining using antibodies against the markers shown in wounds made as in A (scale bar = 100μm). The fluorescent signal was quantified for a 100μm wide section of wound extending from the apical edge of the signal to 200μm into the wound. Vimentin and PDGFRα stained the dermis, CD26 stained the epidermis and dermis. Intensity is quantified in E (mean±SEM, n=6) and the total fluorescence (AUC) calculated for 200μm (F) and 100μm depths (G) (mean±SEM, n=6, P values from a t test are indicated).
**fig. S11. A model of clock control of wound healing**

A. Rhythmic gene expression is established by the PER:CRY gene expression feedback loop, which generates rhythmic levels of actin regulatory proteins. The action of these proteins serves to generate a rhythm in actin dynamics reflected in the F:G actin ratio, which drives the observed changes in cell adhesion and migratory potential with respect to wound healing. B. PER protein expression, which is at its peak at subjective dusk in mouse skin or at 28 hours in fibroblasts, coincides with the peak of F actin, cell adhesion and migratory potential (wound healing) Data is taken from Fig.3A and 4A and B. C. The relative timings in tissue culture (Cellular Time (CT)) and mice (Zeitgeber Time (ZT), where lights on is ZT0) are indicated on a clock diagram where 360° is 1 day. The chart shows mean normalised PER2 expression data from Fig.2C converted to CT where 1 cycle equals 24 hours (red, ±SD, n=3 ) vs PER2 expression in mouse skin biopsies made at the indicated ZT (mean±SEM, n=3).
fig. S12. Circadian Rhythmicity of the ‘Regulation of Actin Cytoskeleton’ Pathway
Proteins identified by SILAC mass spectrometry are mapped onto the KEGG pathway ‘Regulation of Actin Cytoskeleton’ and colour coded according to their rhythmicity. Rhythmic proteins (green boxes) tend to be effector proteins but also include Rho. Arrhythmic proteins (orange boxes) tend to be signalling proteins. Proteins with insufficient peptide detection by mass spectrometry are indicated in grey boxes.
<table>
<thead>
<tr>
<th>Table 1: Unique Proteins in Section 6</th>
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**Note:** The table continues with similar entries.
Table S1. DAVID clustering of rhythmic proteins identified by RAIN (p<0.01)
Movie Legends:

Movie S1

A video depicting maximum z-projection micrographs from live cell confocal microscopy of fibroblasts labelled with CellTracker Red undergoing monolayer healing after wounding at 24 hrs post synchronisation. Time healing is indicated at the top right. This video plays twice and is summarised in Figure 2d.

Movie S2

A video depicting maximum z-projection micrographs from live cell confocal microscopy of fibroblasts labelled with CellTracker Red undergoing monolayer healing after wounding at 32 hrs post synchronisation. Time healing is indicated at the top right. This video plays twice and is summarised in Figure 2d.

Movie S3

A surface rendering of confocal Z-projections of a representative skin wound made at ZT13 and allowed to heal ex vivo for 48 hours. Fibroblasts were identified by anti-vimentin antibody reactivity and spindle-shaped morphology. Fibroblasts are coloured red. The surrounding non-wound area was identified by diffuse DAPI staining and rendered in cyan.

Movie S4

A surface rendering of confocal Z-projections of a representative skin wound made at ZT5 and allowed to heal ex vivo for 48 hours. Fibroblasts were identified by anti-vimentin antibody reactivity and spindle-shaped morphology. Fibroblasts are coloured red. The surrounding non-wound area was identified by diffuse DAPI staining and rendered in cyan.