

Timed use of digoxin prevents heart is chemia-reperfusion injury through a REV-ERB α -UPS signaling pathway

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| 12 | through a REV-ERBα-UPS signaling pathway |
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| 35 | ABSTRACT |
| 36 | Myocardial ischemia-reperfusion injury (MIRI) induces life-threatening damages to the cardiac tissue |
| 37 | and pharmacological means to achieve cardioprotection are sorely needed. MIRI severity varies along |
| 38 | the day-night cycle and is molecularly linked to components of the cellular clock including the nuclear |
| 39 | receptor REV-ERB α , a transcriptional repressor. Here we show that digoxin administration in mice is |
| 40 | cardioprotective when timed to trigger REV-ERB α protein degradation. In cardiomyocytes, digoxin |
| 41 | increases REV-ERB $lpha$ ubiquitinylation and proteasomal degradation, which depend on REV-ERB $lpha$ ability |
| 42 | to bind its natural ligand, heme. Inhibition of the membrane-bound Src tyrosine-kinase partially |
| 43 | alleviated digoxin-induced REV-ERB $lpha$ degradation. In untreated cardiomyocytes, REV-ERB $lpha$ proteolysis |
| 44 | is controlled by several E3 ubiquitin ligases and the proteasome subunit PSMB5. Among these, only |
| 45 | SIAH2 and PSMB5 contributed to digoxin-induced degradation of REV-ERBa. Thus, controlling REV- |
| 46 | $ERB\alpha$ proteostasis through the ubiquitin-proteasome system is an appealing cardioprotective strategy. |
| 47 | Our data support the timed use of clinically-approved cardiotonic steroids in prophylactic |
| 48 | cardioprotection. |

INTRODUCTION

50 The mammalian circadian clock is a critical cell-autonomous mechanism regulating many, if 51 not all, aspects of cellular, tissular and organismal homeostasis¹. Heart physiology makes no exception 52 to this rule and electrophysiological, mechanical and metabolic functions of the cardiac tissue are 53 submitted to cyclic daily variations², which are dependent on the integrity of the molecular clock³. 54 Quite expectedly, genetic or environmental perturbations of the circadian clock in mouse models lead 55 to cardiovascular pathologies including impaired cardiac contractility, fibrosis and cardiomyopathy. 56 Human observational studies also largely concur to demonstrate that circadian rhythm misalignments 57 are associated with cardiometabolic diseases⁴. The prevalence of adverse cardiovascular events such 58 as stroke and myocardial infarction at the sleep-to-wake transition underlines the complex interplay 59 between (mechanisms of) timed cyclic blood pressure control, heart function and vascular tone 60 In addition to altering heart responsiveness to pathological events, circadian regulation. 61 (dys)regulations also affect the heart and cardiomyocyte's ability to recover from acute injury.

62 Susceptibility to myocardial ischemia-reperfusion injury (MIRI) relies on transient adaptative 63 mechanisms to hypoxia and rapid restoration of oxidative metabolism and contractile efficiency ⁵. 64 During ischemia, the myocardial tissue undergoes ATP depletion, acidosis by accumulation of lactic 65 acid and activation of a series of immediate- and late- response intracellular signaling pathways called "RISK" and "SAFE" pathways. These signaling pathways, which are also stimulated upon 66 67 preconditioning (PC), protect mitochondrial function ⁶. Reperfusion causes massive cellular production 68 of reactive oxygen species, alkalinization and calcium overload ⁷. Sensitivity to MIRI is time-of-the-day 69 (ToD)-dependent and is exacerbated during the sleep-to-wake transition in human and mouse ⁸. Genetic alteration of circadian rhythmicity in mice ⁸ or circadian rhythm (dys)synchrony in humans 70 71 correlate with increased acute cardiac injury⁹. We recently demonstrated that timed antagonism of 72 the cyclically-expressed nuclear receptor REV-ERB α , a heme-binding transcriptional repressor within 73 the molecular clock machinery, preventively protects mouse heart from deleterious effects of MIRI in a p21-dependent manner ¹⁰. This observation mechanistically linked the ToD dependency of MIRI with 74 75 the core clock gene machinery, identifying REV-ERB α as a novel target in pharmacological PC.

The search for pharmacologically protective compounds in MIRI has proven difficult ¹¹. Despite their continued use in atrial fibrillation to ameliorate heart contractile properties, cardiotonic steroids (CTS) such as digoxin are not recommended in MIRI cardioprotection at therapeutic concentrations ¹². Renewed interest in CTS has however sparkled from the identification of dissociated inotropic and non-ion pumping signaling properties of Na⁺-K⁺ ATPase (NKA), a target of CTS. Isoforms of the 3 NKA subunits [α , β and γ /FXYD] associate to make up the heteromeric membrane NKA complex Inotropic

effects of CTS are essentially mediated by their ability to bind to and inhibit NKA ATPase activity ¹³, thereby triggering intracellular accumulation of Na⁺ and decreasing Ca⁺⁺ efflux through the Na⁺/Ca⁺⁺ exchanger NCX1. CTS-induced signaling responses can be induced at ATPase sub-inhibitory concentrations and stems at least in part from NKA's ability to act as a CTS plasma membrane receptor ^{14,15}. CTS binding to the NKA α 1 subunit allows the activation of multiple downstream intracellular signaling pathways, including part of RISK or SAFE pathways, hence suggesting a potential usefulness of CTS in preventive MIRI protection ¹⁶.

Thus, given the temporal gating of signaling pathways, the involvement of both the NKA signalosome and REV-ERBα in MIRI protection, and the known susceptibility of REV-ERBα activity to intracellular signaling pathways, we investigated whether CTS-induced signaling pathways could alter REV-ERBα functions and sensitivity to MIRI. Here we show that digoxin and other CTS activate signaling pathways in cardiomyocytes and trigger REV-ERBα protein degradation through the ubiquitin-proteasome system (UPS) in an SRC kinase-dependent manner. Timed in vivo treatment with digoxin prevented REV-ERBα protein accumulation and conferred protection against MIRI.

RESULTS

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99 Cardioprotection by digoxin is time of the day-dependent.

100 We previously demonstrated, using the Langendorff heart perfusion model, that isolated 101 mouse hearts display a ToD-dependent sensitivity to MIRI. Mouse hearts are intrinsically more 102 resistant to MIRI at the beginning of the rest phase [zeitgeber time 0 (ZT0), beginning of the light phase] 103 than at the end of this period (ZT12, average infarct size 27% vs 47%)(¹⁰ and Fig. 1a). We asked whether 104 ex vivo PC efficiency follows a similar pattern. Submitting ZTO isolated hearts to a short iterative 105 ischemia-reperfusion sequence (PC) prior to a prolonged ischemic period neither protected nor 106 aggravated infarct size. In contrast, MIRI-prone ZT12 hearts were protected by this local PC protocol 107 (average infarct size 31% vs 47%) (Fig. 1a). To shed light on these differential sensitivities, the 108 transcriptomic phenotype of ZTO and ZT12 hearts was investigated (Fig. 1b and Supplementary Dataset 109 1). Only a few protein-encoding genes were found to be differentially expressed (n=145, Fig. 1b), with 110 10-12% being core circadian genes including REV-ERBα, which is lowest expressed at ZTO. In addition, 111 ≈75% of differentially expressed genes are cyclically expressed with a period of 20-24 hours 112 (Supplementary Dataset 1, Fig. 1b, right panel and Fig. 1c). None of them belonged to RISK or SAFE 113 cardioprotective pathways. Thus, ZT12 hearts are intrinsically more sensitive to MIRI, responsive to 114 PC and exhibit specific molecular phenotypic features related to molecular clock-driven processes.

115 We then assessed whether the previously reported ex vivo PC properties of digoxin can 116 translate in vivo and whether they are ToD-dependent. We injected a single bolus of digoxin 4 hours 117 prior to organ sampling at ZTO or ZT9 at a pharmacologically well-tolerated dose (1 mpk IP¹⁷). This 118 timing is based on pharmacokinetics studies of digoxin in rodents showing plasma concentration 119 peaking within 1 hour after injection, followed by a 4- to 6-hour tissue distribution phase ¹⁸ yielding an estimated extracellular fluid concentration of about 5-10µM¹⁹. The infarct size in untreated mice was 120 121 as in Fig. 1a, larger by 20% at ZT9 when compared to ZT0 hearts (Fig. 1d), in agreement with ¹⁰. 122 Whereas digoxin pretreatment showed a clear, albeit non-significant (p=0.08) trend to increase MIRI 123 at ZTO, it significantly reduced infarct size by 20% at ZT9 (Fig. 1d). Pre-ischemic monitoring of heart 124 performances with a constant flow perfusion did not reveal significant changes in contraction force, 125 perfusion pression and myocardial contractility (Supplementary Fig. 1a-d), thus ruling out an effect of 126 digoxin treatment on coronary vascular tone, endothelial function as well as on systolic and diastolic 127 dynamics. In the MIRI model, a significant proportion of hearts displayed post-ischemic ventricular 128 arrhythmias, making the analysis of cardiac contractile performance upon recovery infeasible.

130 Cardioprotection by digoxin is REV-ERBα-dependent.

131 Since the selected time points correspond to the nadir (ZT0) and zenith (ZT9) of REV-ERBa 132 protein level, respectively (Fig. 1e and Extended data Fig. 1a), we assessed whether digoxin treatment 133 affects REV-ERBa mRNA and protein levels. Increasing doses of digoxin administered at ZT5 led to a 134 dose-dependent decrease REV-ERB α protein level at ZT9 to reach 60% at 1 mpk (Fig. 1f and Extended 135 data Fig. 1b). In contrast, the expression of the REV-ERBα-encoding Nr1d1 gene, whose expression 136 precedes REV-ERBa protein expression by approximately 2 hours (Fig. 1c and Extended data Fig. 1c), 137 was not affected by digoxin treatment. The expression of REV-ERBa target genes (Arntl/Bmal1 and 138 *Cdkn1a/p21*, Extended data Fig. 1c), which mirror the REV-ERB α protein level pattern in homeostatic conditions (Fig. 1c and Extended data Fig. 1c), were increased by digoxin treatment (Extended data Fig. 139 140 1d) as well as P21 protein levels (Extended data Fig. 1e). Digoxin did not affect REV-ERBα protein level 141 at ZTO, which was barely detectable at this time point (Extended dataFig. 1f). This ToD sensitivity to 142 digoxin was not due to changing levels during the day/night cycle of NKA isoforms ATP1A1, or ATP1A2 and ATP1A3 mRNAs or of corresponding protein levels at the time of treatment (Extended data Fig. 143 144 2a,b). Their expression was neither affected by REV-ERB KO (Supplementary Dataset 2) nor by digoxin 145 treatment (Extended data Fig. 2a). Thus, these data suggest that digoxin interferes with REV-ERBa 146 protein levels through post-transcriptional regulation.

Digoxin administered at ZT4 did not protect mouse hearts from MIRI at ZT9 in *Nr1d1^{-/-}* mice but rather increased infarct size (+11%) (Fig. 1g), phenotypically mimicking ZT0 hearts (Fig. 1d). REV-ERBα via p21 expression contributes to increased resistance to MIRI ¹⁰. A similar experiment in p21depleted mice confirmed the higher susceptibility of these mice to MIRI but digoxin treatment was still protective in these mice (-18%, average infarct size 55%)(Fig. 1g). Thus, REV-ERBα expression is required to confer cardioprotective properties to a prophylactic digoxin treatment without involving the anti-apoptotic protein P21.

154 Injected digoxin preferentially accumulates in skeletal muscles, heart and liver ²⁰. Similar to 155 the cardiac tissue, digoxin-exposed livers displayed reduced REV-ERBα protein level and increased 156 target gene expression (*E4bp4/Nfil3, Bmal1/Arntl, cdkn1a/p21*), while *Nr1d1* mRNA levels were left 157 unchanged (Supplementary Fig. 2a-e). These observations raised the question of how digoxin 158 downregulates REV-ERBα protein expression.

159

160 Digoxin affects REV-ERBα proteostasis in a cell-autonomous manner.

161 Analysis of bulk cardiac tissue as above may overlook outcomes stemming from multiple inter-162 or intracellular crosstalks. We therefore asked whether REV-ERBa protein degradation occurs in 163 isolated digoxin-treated cardiomyocytes. We first performed experiments using the human cardiomyocyte cell line AC16 from adult human ventricular heart tissue²¹. Since digoxin and other CTS 164 have significant pro-apoptotic properties ²², we investigated whether digoxin induces AC16 cell death. 165 166 AC16 cells were synchronized at T₋₂ by a dexamethasone pulse (2 hours) which transiently induces Per1 167 gene expression 23 and subsequently incubated with digoxin at T₀. Cell viability was only mildly affected 168 by digoxin at tested concentrations (Extended data Fig. 3a). Probing apoptosis-related protein levels 169 in AC16 whole cell extracts did not reveal any significant change in the apoptotic cascade (Extended 170 data Fig. 3b). In these conditions, REV-ERBα reached a maximal expression 24 hours after 171 synchronization (T_{24}). Digoxin blunted REV-ERB α expression at all time-points (Fig. 2a and Extended 172 data Fig. 3c). REV-ERB α protein levels were normalized to HSP90 α levels, whose half-life is 36-40 173 hours, vs 1 hour for REV-ERB α . NKA subunits α 1 and α 2 mRNAs and protein levels were steadily 174 detectable in these conditions (Extended data Fig. 2c,d). Digoxin's effect on REV-ERBa protein level 175 was fully prevented by an anti-digoxin antibody (Extended data Fig. 3d), excluding any artifactual 176 modulation of cellular pathways by contaminants. The digoxin effect was dose-dependent with an 177 observed EC_{50} = 96nM (Extended data Fig. 3e), which fits with the reported binding constant of digoxin to the human NKA α 1 subunit (K_D=87nM), the major CTS signalosome relay ^{24,25}. It is also consistent 178 179 with the observed efficiency of the non-glycosylated CTS bufalin [EC₅₀<0.1µM (Extended data Fig. 3f), 180 K_D =42nM²⁴]. The structurally-related ouabain also decreased REV-ERB α stability (Extended data Fig. 181 3g).

Under basal conditions, expression levels of *NR1D1* and of the REV-ERBα target gene *ANRTL/BMAL1* showed, as expected, an antiphasic transcript expression in untreated AC16 cells (Fig.
2b). Digoxin increased at T₂₄ both *NR1D1* and *ANRTL/BMAL1* mRNAs, both known to be directly and
cyclically repressed by REV-ERBα (Fig. 2b) and *ANRTL/BMAL1* promoter activity became acyclic (Fig.
2c). P21 protein level increased upon digoxin treatment (Fig. 2D), alike after in vivo digoxin injection
(Extended data Fig. 1e).

Digoxin's effect was also assessed using the human osteosarcoma U2OS cell line, a commonly used and robust circadian model (Extended data Fig. 4a-c). REV-ERBα expression peaked 16-20 hours after synchronization, and digoxin blunted REV-ERBα protein level at all time-points (Extended data Fig. 4a). The expression of RORα, a transactivator counteracting REV-ERBα activity, remained unchanged (Extended data Fig. 4a) whereas *NR1D1* and BMAL1/*ARNTL* transcripts were higher expressed (Extended data Fig. 4b), with *ANRTL/BMAL1* promoter activity becoming acyclic (Extended data Fig. 4c). Similar results were obtained using human primary cardiomyocytes (Fig. 3) and HepG2 cells (Supplementary Fig. 2f). Thus, CTS strongly decrease REV-ERBα protein level in several
 representative cardiomyocyte models and unrelated cell lines (U2OS, HepG2), suggesting a highly
 conserved mechanism involving NKA.

198

199 Digoxin induces multiple intracellular signaling pathways.

200 Digoxin's effect on REV-ERBa levels are detected after a short treatment (210 minutes, 201 Extended data Fig. 3h). Therefore, digoxin (at $5x EC_{50}=0.5\mu M$) and other modulators were, in further 202 experiments, added at T₁₈, 6 hours prior to the REV-ERBα protein peak in order to minimize secondary 203 responses to treatment(s) (Fig. 4a, top panel). In these conditions, we assessed the involvement of 204 NKA as a bifunctional protein regulating ion fluxes and exerting signaling functions, as the calculated 205 in vivo concentration of digoxin (5-10 μ M, Fig. 1d) activates the signalosome-related PKC ε in mouse 206 heart ²⁶. In vitro, 3,4,5,6 tetrahydroxyxanthone (THX) inhibits NKA ATPase activity with an efficiency 207 similar as ouabain (EC₅₀=1.6 μ M), while being unable to activate Src ²⁷. We therefore compared the 208 activity of THX to that of digoxin on REV-ERBa stability. In contrast to digoxin, THX treatment did not 209 alter REV-ERBα levels in AC16 (Fig. 4a and Supplementary Fig. 3a) and U2OS cells (Supplementary Fig. 210 **3b**), suggesting that the ion pumping activity of NKA is dispensable for digoxin-induced REV-ERB α 211 decrease. Pharmacological perturbation of calcium fluxes through inhibition of voltage-dependent 212 Ca⁺⁺ channels by diltiazem and verapamil (Supplementary Fig. 3c), through SERCA inhibition by 213 thapsigargin (Supplementary Fig. 3d) or through blockade of the Na⁺-Ca⁺⁺ exchange system NCX by KB-214 R7943 (Supplementary Fig. 3e) did not regulate REV-ERBα protein levels. Taken together, this data 215 rules out a contribution of the inotropic arm of NKA.

216 The ability of digoxin to trigger intracellular pathways at low and moderate concentrations 217 (0.5µM and 5.0µM) was thus evaluated using serine/threonine (S/T) kinase arrays (Fig. 4b-d). Digoxin 218 activated multiple S/T kinases belonging mostly to the human CMGC (Cyclin-dependent kinases, 219 Mitogen-activated protein kinases, Glycogen synthase kinases, CDC-like kinases), CAMK (Calcium and 220 Calmodulin-regulated Kinases) and the related AGC (PKA, PKG, PKCs) groups (Fig. 4b-d). The IKB kinase 221 complex was also detected as being significantly activated. Activation of representative kinases of the 222 CMGC (ERK1&2) and the AGC (AKT1&2) groups was validated by an immunoblotting approach 223 (Supplementary Fig. 4a,b). To disentangle downstream effects of digoxin-mediated signalosome 224 activation, a genome-wide transcriptomic analysis was performed in similar conditions. As reported 225 in other systems ²⁸, digoxin increased the expression of immediate-early genes (IEGs) such as EGR-1, 226 *c-FOS* and *c-JUN* both at low and high concentrations (Fig. 4e and Supplementary Dataset 3). Gene set 227 enrichment analysis of upregulated genes identified the NFκB pathway as most robustly induced (Fig.

4f), in line with the phosphorylation of IκB kinase subunits (Fig. 4b, c). Monitoring gene expression in
digoxin-exposed vs control mouse ZT9 hearts (Supplementary Dataset 4) showed an induction of *Egr- 1, c-Fos* and *c-jun* and other immediate early genes, with a 31% (12/38) overlap (Fig. 3e, see *) between
in vitro, digoxin-upregulated genes in AC16 cells and in vivo digoxin-upregulated genes in mouse heart.

232 Digoxin has been ascribed multiple effects owing to its ability to modulate the activity and/or 233 expression of several proteins: it modulates transcriptional activity of the nuclear receptor RORy 29, 234 and interacts with PKM2 30 , a target gene of HIF1 α whose translation is inhibited by digoxin 31 . Modulation of these pathways in AC16 cells by either a RORy inverse agonist (ML209, Supplementary 235 236 Fig. 5a), by knocking down *PKM2* (Supplementary Fig. 5b) or through $HIF1\alpha$ overexpression 237 (Supplementary Fig. 5c) or activation (Supplementary Fig. 5d) did not blunt or exacerbate digoxin-238 induced REV-ERBα degradation. Thus, these 3 established digoxin targets are not effectors of digoxin 239 action on REV-ERBα.

Thus, digoxin induces multiple signaling pathways, in murine cardiac tissue and cell lines, without engaging previously identified CTS targets. The causal link between digoxin-mediated induction of intracellular signaling pathways and altered REV-ERBα protein levels was further investigated.

244

245 The protein kinase SRC contributes to REV-ERBα stability.

246 We first pharmacologically modulated the known digoxin-sensitive SRC tyrosine kinase and the 247 top-ranking digoxin-activated serine/threonine kinases identified above. We employed, in conjunction 248 or not with digoxin, widely-used chemical modulators for SRC (PP2), Pl₃K (ZSTK474), ERK1&2 249 (SCH772984), AKTs (MK2206), CAMK1&2 (KN93), MEK1&2 (PD98059), PKG1&2 (RP8-pCPT-cGMPS), 250 PKD (CRT0066101), p70S6K1 (PF-4708671), RSK1&2&3 (BRD7389) and for components of the NFκB 251 pathway such as LUBAC (Bay11-7082), HOIP (HOIPIN-8), IKK α/β (BMS345541), IKK β (TCPA-1), IKK ϵ and 252 TBK1 (amlexanox) (Fig. 5 and Extended data Fig. 5). This approach proved highly efficient at inhibiting 253 kinase activity (see Supplementary Fig. 4c as an example). Results were subsequently validated for a 254 number of targets using either overexpressed constitutively active kinase mutants, other inhibitors or 255 siRNAs (Extended data Fig. 6,7).

Stabilizing effects on basal REV-ERBα levels were observed when inhibiting PI3K, ERKs or MEKs
(Fig. 5). However, this was not confirmed using distinct inhibitors or siRNAs with the exception of PI3K
(Extended data Fig. 6). The latter effect was however not detected in U2OS cells (Supplementary Fig.

259 6a). Taken together, these data show that functional interference with this set of kinases did not
 260 significantly affect REV-ERBα stability in basal conditions.

Blunting ERKs, PI3K, AKTs, CAMKs, MEKs, PKGs, PKD1, P70S6K1, RSKs, IKKs or TBK activity did not impair digoxin's ability to trigger REV-ERBα degradation, a conclusion validated through the use of alternative approaches (Extended data Fig. 6,7). In contrast, inhibition of SRC (with PP2, Fig. 5a and with saracatinib, Extended data Fig. 6a, 7a) blunted digoxin-induced REV-ERBα protein decrease, an effect which, albeit less pronounced, was also observed in U2OS cells (Supplementary Fig. 6a). This data nevertheless suggested the involvement of SRC in the chain of events leading to decreased REV-ERBα levels.

268 Bay 11-7082 efficiently stabilized REV-ERBα in non-challenged cells and significantly protected 269 REV-ERBa from digoxin-induced decreased expression in AC16 (Fig. 5j and Extended data Fig. 5j) and 270 U2OS cells (Supplementary Fig. 13b). Initially described as a linear ubiquitin chain assembly complex (LUBAC) inhibitor, this compound also significantly inhibit several E2 ligases and the proteasome ³². 271 272 The use of a more specific inhibitor of the LUBAC complex, HOIPIN-8³³ (Fig. 5k and Extended data Fig. 273 5k), of a HOIL-targeting siRNA (Extended data Fig. 6j, 7j) and the overexpression of the OUT domain-274 containing deubiquitinase with linear linkage specificity (OTULIN, Extended data Fig. 6k, 7k) show no 275 significant effects in our assay, thereby excluding the LUBAC complex as contributing to the REV-ERBa 276 degradation process.

277 Since the NFkB pathway was activated upon digoxin treatment, we assessed the involvement 278 of this transcription factor in several ways. We first searched for a transcriptomic blueprint of the activated NF κ B pathway in cardiac tissues from Nr1d1^{-/-}mice. A comparative analysis of Nr1d1^{-/-} vs 279 *Nr1d1*^{+/+} heart transcriptome at ZT12 evidenced, alike the ZT0 vs ZT12 comparison (Fig. 1b,c), strong 280 281 dysregulation of clock-related processes without any significant enrichment in NFκB-driven responses 282 (Supplementary Fig. 7a). Up- and downregulated genes were searched against the EnrichR ChEA 283 database to identify transcription factors binding in the vicinity of their promoters, on the basis of aggregated ChIP-seq studies ³⁴. This analysis did not identify NFKB as a potential regulator of 284 285 dysregulated genes (Supplementary Fig. 7b). Overexpressing inhibitory wild type or super repressor 286 (SR) $I\kappa B\alpha^{35}$ did not protect or enhance digoxin's effect on REV-ERB α stability (Supplementary Fig. 7c). 287 Finally, the pharmacological NFkB modulator betulinic acid did not prevent the effect of digoxin on 288 REV-ERBa protein levels, definitively excluding this pathway as a player in the observed REV-ERBa 289 degradation (Supplementary Fig. 7d).

290Our results thus show that SRC kinase, described as part of the digoxin-controlled "NKA291signalosome", contributes to the observed decrease in REV-ERBα protein levels in AC16 and U2OS cells.

Bay 11-7082, which inhibits $I\kappa B\alpha$, E2 ligases Ubc13 and UbcH7, the E3 ligase LUBAC and the proteasome exerts a potent activity in our system, the effect of which being unrelated to the inhibition of linear ubiquitinylation by the LUBAC complex. These results rather pointed to the involvement of UPS in REV-ERB α degradation.

296

297 Digoxin induces the proteasomal degradation of REV-ERBα.

298 The UPS and autophagy are 2 main branches of the protein quality control pathway. 299 Preliminary investigations ruled out other branches involving protein neddylation (using the NEDD8 300 inhibitor MLN4924/pevonedistat) or sumoylation (using the SUMO inhibitor 2-D08) as controlling REV-301 ERBα stability (Supplementary Fig. 8). The autophagy pathway was not modulated by digoxin 302 (Supplementary Fig. 9a), contrasting with reports in cancerous cells 28557 ³⁶. Accordingly, 303 pharmacological modulation of autophagy by either chloroquine, bafilomycin or rapamycin did not 304 impact digoxin-induced effects (Supplementary Fig. 9b-d). Knocking down P62/SQSTM1 expression, 305 which was the sole component of the autophagy pathway upregulated by digoxin (Supplementary Fig. 306 9a), had no effect (Supplementary Fig. 9e).

307 We then explored if the UPS is implicated in digoxin-induced REV-ERB α degradation. The 308 proteasome catalytic subunit beta 5 (PSBM5) inhibitors bortezomib (BTZ) and clasto-lactacystin β-309 lactone, albeit to a lesser extent, protected REV-ERBα from digoxin-induced degradation (Fig. 6a,b and 310 Extended data Fig. 8a,b). Protection by BTZ was also observed in U2OS cells (Extended data Fig. 8c), 311 suggesting that UPS is a conserved component of the REV-ERB α degradation pathway. As 312 exogenously-expressed wild type REV-ERBa is equally sensitive to digoxin treatment (Fig. 6d and 313 Extended data Fig. 8d), we assessed whether REV-ERBa undergoes ubiquitinylation and is modified 314 upon digoxin treatment using overexpressed Flag-REV-ERBα in AC16 cells. Mono-, multi- and poly-315 ubiquitinylated proteins were isolated from whole cell extracts and REV-ERBa content was analyzed 316 by western blot analysis. REV-ERBa was eluted along with other mono- and poly-ubiquitinated 317 proteins, and its concentration decreased in digoxin-exposed cellular extracts, and increased in 318 BTZ+digoxin-treated cells (Fig. 6e), indicative of a digoxin-controlled REV-ERB α ubiquitinylation. The 319 H602F REV-ERBa mutant, which is unable to bind its natural ligand heme ³⁷, showed higher expression in basal conditions as reported ³⁸ and was digoxin-resistant (Fig. 6d and Extended data Fig. 8d), 320 321 suggesting that heme binding to REV-ERBa promotes a conformation required for proteasomal 322 degradation.

To determine which component(s) of the UPS pathway could be involved in REV-ERBα degradation, we isolated REV-ERBα interacting partners by RIME (rapid immunoprecipitation followed

by mass spectrometry identification of endogenous proteins)³⁹ using the digoxin-sensitive U2OS and 325 326 HepG2 cells. We then filtered the generated list of interactors against the Gene Ontology "cellular 327 component" or "molecular functions" databases using "proteasome" or "ubiquitin" as biological terms 328 and interacting significantly with REV-ERB α (Supplementary Dataset 6). We identified 16 UPS-related 329 proteins interacting with REV-ERBα, including several E2 (UBE2L3) and E3 (PRPF19, TRIM21) ubiquitin 330 ligases, the NCoR complex component TBL1XR1, and 4 subunits of the proteasome (PSMA4, PSMA5, 331 PSME3, PSMD2) (Fig. 7a). To this list of proteins potentially funneling REV-ERBα towards proteasomal 332 degradation, we added a number of E2 and E3/E4 ubiguitin ligases previously reported to regulate 333 REV-ERBa protein stability (SIAH2 ⁴⁰, FBWX7 ⁴¹, HUWE1 ⁴²) or predicted to act on REV-ERBa using the 334 proteome-wide E3 ligases-substrate interaction network (UbiBrowser⁴³) such as BRCA1, CBL, MDM2, 335 STUB1, TRIM33 and the ubiquitination factors UBE4A and UBE4B. The final list totaled 28 proteins (Fig. 336 7b), from which 19 were tested for their involvement in REV-ERB α degradation. AC16 cells were thus 337 transfected with siRNAs or treated with pharmacological inhibitors when available. Results are 338 summarized (Fig. 7) from the quantification (Extended data Fig. 9) of raw data (Extended data Fig. 10). NEDD8, PSME3, the FBXW7-dependent destabilizing kinase CDK1 41 , the REV-ERB α stabilizing kinase 339 GSK3^β⁴⁴, BRCA1, UBE4A, MDM2, STUB1 and TRIM33 were inactive in our assay (Extended data Fig. 9). 340 341 Six entities significantly contributed to REV-ERBa stability in basal conditions including the REV-ERBa-342 interacting, NCoR complex component TBL1XR1 and the E3 ligases SIAH2, HUWE1, FBXW7, CBL and 343 UBE4B. Quite unexpectedly, impaired expression of the E2 ligase-encoding gene UBE2L3/UBCH7 344 decreased basal REV-ERBa expression (Extended data Fig. 9d). However, none of these E3 ligases 345 modulated the digoxin-mediated REV-ERBa degradation, with the exception of SIAH2, whose 346 knockdown partially maintained REV-ERBα level (Extended data Fig. 9a). Thus, digoxin-mediated 347 activation of UPS triggers REV-ERB α degradation through a complex chain of events yet to be 348 identified, but including the E3 ligase SIAH2 and the proteasome subunit PSMB5 (Fig. 8).

DISCUSSION

In the current era of precision medicine wherein therapies are formulated and administered to attain maximal benefit through increased efficiency and reduced side effects, chronotherapy is an important step further towards this goal. As an edifying example, chrono-chemotherapy is beneficial in combating cancer by using a circadian window of higher drug tolerability and efficiency ⁴⁵. Chronotherapy has been recently extended to hypertension treatment at bedtime, and proved more efficient at decreasing CVD events ⁴⁶.

357 The unevenly distributed rate of occurrence of cardiac events suggest a differential sensitivity of the heart to injury along the 24h day-night period ⁸. ToD dependent functional variations occur in 358 359 heart physiology, which are partly reflected by the significantly oscillating expression of approximately 360 13% of cardiac genes, which are involved in multiple functions from signaling and growth to 361 transcription and cardiac remodeling ^{3,47}. Similarly, proteomic analysis established striking differences in night versus day protein levels in heart ⁴⁸, suggesting that timed cardiac proteostasis is crucial to 362 363 proper heart function. Along these lines, we reported a strong correlation between the time of surgery 364 and perioperative myocardial tolerance to injury in patients undergoing aortic valve replacement. This 365 ToD-dependent differential myocardial sensitivity to IRI was attributed to the rhythmic expression of the nuclear receptor REV-ERBα and of its anti-apoptotic target gene P21¹⁰. We also suggested the 366 367 therapeutic potential of pharmacological inhibition of REV-ERBa for prophylactic cardioprotection. 368 This provided an alternative approach to physical ischemic PC in which multiple distal, brief ischemic 369 episodes induce cardioprotective signaling pathways that bestow protection in a future event of 370 prolonged ischemia.

With the realization that NKA, the primary target of digoxin and other CTS, controls the activation of multiple signaling pathways including part of the RISK and SAFE pathways such as AKT, PKC and tyrosine kinases (Fig. 4), the use of CTS as PC agents has been proposed ¹⁶. Cardioprotection is observed in rats dosed with a sub-ionotropic dose of ouabain followed by washout similarly to IPC ^{25,49}. However, this strategy has not yet gained momentum, as digoxin use in heart failure and atrial fibrillation has stirred a 200-year long history of controversies, leading to clinical hesitancy to widen digoxin and other CTS use in PC and other pathologies ⁵⁰⁻⁵².

Our study however demonstrates a ToD-dependent action of digoxin on myocardial integrity.
The decreased susceptibility to IRI in mice dosed with digoxin when REV-ERBα expression reaches its
maximum (ZT9) could be potentially attributed to 2 principal effects: (1) circadian rhythm alteration
and/or (2) pharmacological cardiac preconditioning through the rapid and potent activation of many
components of the RISK and SAFE pathway including PI3K, AKT, ERK1/2, GSK3β, and NFκB (Fig. 3).

383 Genetic ablation of REV-ERBa showed that its expression is mandatory to observe digoxin protective 384 effects. Digoxin treatment in mice relieved REV-ERBa repression on several genes including the 385 cardioprotective Bmal1 and cdkn1a (Extended data Fig. 1 and Supplementary Dataset 4). The anti-386 apoptotic P21 is not required for the cardioprotective effect of digoxin. Cardioprotective activities of 387 BMAL1 have been elegantly documented through the use of genetic models ⁵³. The ZT12-digoxin 388 phenotype is reminiscent of that of CCM mice, whose clock is locked at the wake-to-sleep transition 389 (ZTO), display improved tolerance to MIRI at ZT12 and in which *Bmal1* is similarly upregulated with 390 dampened cyclicity⁸. Identified BMAL1 target genes include Nampt or Cog10b which replenish cellular 391 stores in cardioprotective NAD⁺ and ubiquinone respectively ⁵⁴. However, digoxin-exposed hearts 392 displayed no altered expression of these and other BMAL1 target genes (Supplementary Dataset 4), an 393 outcome likely due to the short time frame (4 hours) of the acute digoxin treatment. Thus, BMAL1 is 394 unlikely to play a role in the observed phenomenon.

395 In contrast to these cardioprotective effects at ZT9, digoxin treatment of REV-ERB α -depleted hearts (ZTO) resulted in, albeit non-significant, trend to increased susceptibility to MIRI. As our 396 397 transcriptomic studies did not point to obvious mechanisms, the molecular basis for this differential 398 sensitivity to digoxin at ZTO is unclear at this stage. Multiple parameters not assessible by gene 399 expression studies may indeed control heart sensitivity to MIRI. Circadian variations of heart 400 metabolism and temporal gating of signaling pathways may contribute to this time-specific response 401 ⁵³, while NKA α 1 expression is constant throughout the day-night cycle (Extended data Fig. 2 and ⁵⁵). 402 Alternatively, the subcellular localization of NKA subunits, which is exquisitely regulated and 403 dependent on protein-protein interactions, may vary and affect it signaling properties.

404 An unexpected, yet one of the most important findings in our study is the impact of digoxin on 405 REV-ERBa protein levels in vivo and in human primary and immortalized cardiomyocytes. Exogenously 406 overexpressed REV-ERB α was found to be equally sensitive to digoxin and a target for the 407 ubiquitination machinery. . In contrast, the overexpressed ligand binding-crippled H602F REV-ERBa 408 mutant was resistant to digoxin-induced degradation. Thus, heme-binding to REV-ERBα, which may 409 induce specific conformational changes in the aporeceptor structure, seems to be an important 410 prerequisite for physiological (^{56,57} and Fig. 6d) and digoxin-triggered degradation through the 411 proteasomal pathway. Interestingly, heme regulatory motif (HRM)- or PAS (PER-ARNT-Sim)-containing 412 transcription factors and clock components such as NPAS2, CLOCK, PER1 and PER2 are also subjected to heme-dependent protein degradation ^{58,59,60}, linking heme synthesis and circadian rhythm 413 414 regulation.

Our data thus clearly point to a beneficial effect of acute REV-ERBα antagonism in cardioprotection. This observation seems at odds with investigations reporting improvement of failing hearts 1-day post-MI following treatment with SR9009, a REV-ERB agonist ⁶¹, which induces cardiac transcriptomic alterations ^{62,63}, and blockade of cardiac remodeling through an anti-inflammatory mechanism ⁶⁴. However, prolonged agonist treatment decreases, through a negative auto-feedback loop, REV-ERBα protein level ⁶¹. Alternatively, it also triggers systemic immune cell mobilization ⁶⁵, a process unlikely to be at play in our acute system.

422 CTS-controlled protein degradation is not unprecedented. Ouabain and digoxin triggers the 423 degradation of the nuclear estrogen receptor $ER\alpha$ in primary and metastatic breast cancer cells, 424 providing an additional mechanism for the reported CTS cytotoxicity against cancer cells ⁶⁶. Bufalin, 425 which also induces REV-ERB α degradation (Extended data Fig. 3f), strongly impacts on p160 steroid 426 receptor coactivator SRC1 and SRC3 protein stability ⁶⁷. Knockdown experiments however ruled out a 427 contribution of SRC1 and 3 in our system (Supplementary Fig. 10). The pleotropic effects of CTS thus 428 call for a careful examination of their side-effects. However, their use in cardiac PC as a timed, single, 429 low dose administration is an appealing therapeutic strategy to improve patient outcome, as 430 unwanted effects of these FDA-approved drugs will be predictably limited. On the contrary, prolonged 431 exposure to CTS in either CVD or cancer therapy might impinge on the molecular clock in several organs 432 including the liver (Supplementary Fig. 2), inducing metabolic alterations with unpredictable 433 outcomes. Alternative approaches may be considered to target REV-ERB α for elimination. Combining 434 REV-ERBα ligands to an E3 recognition motif to generate a PROteolysis TArgeting Chimera (PROTAC) 435 may be an innovative strategy to lessen heart susceptibility to MIRI.

METHODS

438 *Reagents*

All information about reagents, chemicals, siRNAs, Taqman probes, antibodies, plasmids, transfection
 reagents, kits and softwares are provided in the accompanying Supplementary Information file.

441

442 Animal Experimentation

443 All experiments were approved by the Comité d'Ethique en Expérimentation Animale du Nord-444 Pas de Calais CEEA75. To eliminate sex as a confounder, male mice were used throughout this study. 445 C57BL6/J wild-type male mice (10-14 weeks) were purchased from Charles River Laboratories and housed in a temperature-controlled environment (23-25°C, 40-60% humidity) with a 12h/12h light-446 447 dark cycle, ZTO being lights-on. Mice had free access to water and were fed ad libitum a standard chow 448 diet (Safe Diet A04). Cdkn1a/p21^{-/-}mice and their wild-type littermates were obtained from Charles 449 River (France, B6. 129.S6(Cg)-Cdkn1atm1Led/J - SN 16565), Nr1d1^{-/-} mice (SV129.Nr1d1^{-/- 68}) were bred, 450 housed and fed as above.

451 To study the impact of digoxin in vivo, animals were injected intraperitoneally with digoxin or 452 vehicle (10% ethanol, 40% propylene glycol, 0.08% citric acid, 0.3% sodium phosphate) at ZT5 or ZT20 453 at indicated concentrations (in most cases 1 mpk). The investigator was blinded to the treatment. 454 Mice were sacrificed by cervical dislocation at ZT9 or ZT0. Organs were harvested and snap-frozen for 455 protein and RNA analysis. Heart sensitivity to MIRI was tested using the ex vivo Langendorff perfused 456 mouse heart model ⁶⁹. Isolated hearts were retrogradely perfused with a 5% CO₂-95% O₂-saturated 457 modified Krebs-Henseleit (118.5 mM NaCl, 25 mM NaHCO₃, 4.7 mM KCl, 1.2 mM MgSO₄, 1.2 mM 458 KH₂PO₄) buffer (1.4 mM Ca⁺⁺; 11 mM glucose) at a constant flow rate of 2.5 mL/min (Masterflex L/S 459 peristaltic pump) at 37°C with an imposed pacing (9 Hz, 540 bpm). Hearts were stabilized for 20 min 460 in these conditions then subjected to a normothermic, 30 min global ischemia by switching off the 461 coronary perfusion. Hearts were then re-perfused for 30 min. Heart linear contractions were 462 measured by passing a hook through the heart apex with a pre-load of 1.5 g and recorded. Perfusion 463 pressure was recorded with a Labtrax 4/24T (World Precision Instruments) device and integrated using 464 the LabScribe data acquisition and analysis software (v 2.0). Ex vivo preconditioning was achieved by 465 submitting hearts to an iterative (4 times) ischemia-reperfusion sequence (2x 5 min)(see Fig. 1a).

Infarct sizes were measured using the 2,3,5-triphenyltetrazolium chloride (TTC) staining
method. TTC (1%) was injected through the aortic canulae and incubated for 10 min at 37°C. Infarcted
(white) and viable (red) areas were measured by computerized planimetry (Adobe Photoshop).
Exclusion criteria for perfused hearts were a time to perfusion >3 min, a coronary perfusion pressure
> 140 mm Hg or < 40 mm Hg or raising transiently above 180 mm Hg.

472 Cell lines and human primary ventricular cardiomyocytes

The AC16 human cardiomyocyte cell line ²¹ was from Millipore (SCC109). Cells were
maintained in DMEM/F12 Ham (Sigma-Aldrich) supplemented with 2mM L-glutamine (Thermo Fisher
Scientific), 12.5% of fetal bovine serum (FBS) and 1% penicillin-streptomycin (Thermo Fisher Scientific).
The U2OS cell line was from ATCC (HTB-96) and maintained in McCoy 5A medium (Thermo

477 Fisher Scientific) supplemented with 10% FBS and 1% penicillin–streptomycin.

478 Authenticated (STR-profiled) HepG2 cells were from ATCC (HB-8095) and grown in DMEM 479 containing 25 mM Glc supplemented with 10% FBS, 1 mM nonessential amino acids, 1 mM sodium 480 pyruvate, and 1% penicillin/streptomycin.

Human primary ventricular cardiomyocytes were from PromoCell (C-12811). These cells are from ventricles of an explanted heart of a male donor who underwent heart transplantation. Cells were tested and validated for cell specific markers, morphology, viability and adherence rate. Cells were maintained in Myocyte Growth Medium (C-22070, PromoCell) supplemented with Myocyte growth medium supplement mix (C-39270, PromoCell).

For all experiments, confluent cells were synchronized using 100nM dexamethasone (Sigma)
for 2 hours, then washed in 1x PBS. This defined T₀, after which cells were maintained in their
respective growth media for further experimentation. Cell sampling and treatment were performed
at indicated times. Cell viability was determined at indicated concentrations (see Fig. legends) using
CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega, G7570). DMSO was used at concentrations
below 0.02% to avoid unwanted effects on REV-ERBα protein stability. Luciferase real-time recording
was as described ³³, with a stabilization period (T₀-T₁₂).

493

All cell lines were routinely (once a month) monitored for mycoplasma.

494

495 RNA extraction and RT-qPCR analysis

496 RNA extraction from cultured cells was carried out using the NucleoSpin® RNA Midi kit 497 (Macherey-Nagel). Total RNA from mouse heart was extracted from samples pulverized in liquid 498 nitrogen using TRIzol™ Reagent (Thermo Fisher Scientific). Prior to cDNA preparation with the High 499 Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific), total RNA extracts were subjected 500 to DNase I treatment. cDNAs were quantified using TaqMan Gene Expression Assays. Gene expression 501 was normalized to 18S rRNA expression and relative gene expressions were calculated using the 2^{-ΔΔCt} 502 method ⁷⁰.

503

504 Protein extraction, Western blotting (WB) and Simple Western immunoassays

505 Proteins were extracted using M-PER cell lysis buffer (Pierce) supplemented with Halt Protein 506 Phosphatase and Protease cocktail (1:100 dilution, Pierce). Following a brief vortexing and 507 centrifugation (30 min., 16,000 G), the supernatant protein concentration was determined using the 508 BCA protein assay kit (Pierce).

Frotein expression level was studied either by western blotting or by WES Simple Western analysis
 (Simple Western[™])⁷¹.

- Western blotting: 20µg of protein were resolved by 12% SDS-PAGE under reducing conditions and blotted onto a nitrocellulose membrane. The membrane was blocked in 5% BSA in 1x
 TRIS-buffered Saline-Tween (TBS-T) and incubated overnight with the indicated antibody.
 Membranes were washed 3 times in 1x TBS-T and incubated with an anti-mouse HRP-labelled secondary antibody (1:10,000) for 1 hour. SuperSignal[™] West Dura Extended Duration substrate (Thermo Fisher Scientific) was used to detect antigen-antibody complexes.
- WES analysis: 3.8 µg proteins were resolved by size by capillary electrophoresis. After
 immobilization onto the capillary by photoactivated capture chemistry, target proteins were
 identified using specific primary antibodies (see Supplemental Information for references) and
 immunoprobed using an HRP-conjugated secondary antibody and a chemiluminescent
 substrate (ProteinSimple). Chemiluminescent signals were detected and quantified using the
 Compass for SW software (ProteinSimple).

523 Protein levels were normalized to relative changes in HSP90AA1, β -actin or vinculin as indicated, 524 which were run in parallel on different gels/WES plates at the same time using the same biological 525 samples.

526

527 Cloning and transfection

528 Transfections were performed 24 hours (T-₂₄) prior to dexamethasone-induced 529 synchronization. The pEZ-M11-hREV-ERB α (plasmid Genecopoeia) (referred to as Flag-REV-ERB α) was 530 used to overexpress flagged human REV-ERB α or its derivatives. Human flagged REV-ERB α mutant 531 (hREV-ERB α -H602F) deficient in binding heme was generated using the Flag-REV-ERB α plasmid by 532 substituting H602 with F⁷².

The human *Bmal1*-luciferase construct (Bmal-Luc) used for real-time bioluminescence studies was obtained by cloning the *Bmal1* promoter region (-350 to + 100) relative to the gene transcriptional start site into the SV40-driven, red-emitting luciferase pCBR reporter vector (Promega). Overexpression of wild type and constitutively active HIF1 α was achieved by using HA-HIF1 α -pcDNA3 (WT-HIF1 α) and HA-HIF1 α P402A/P564A-pcDNA3 (CA-HIF1 α), respectively ⁷³. These plasmids were provided by W. Kaelin (Addgene plasmid # 18949, RRID: Addgene #18955Fugene HD® (Promega) was used to transfect AC16 cells. Transfection of U2OS was achieved using JetPEI® (Polyplus Transfection).

540 For immunoprecipitation experiments, 10µg pEZ-M11-REV-ERBα were transfected into 1.1x10⁷ AC16

541 or U2OS cells. Transfection efficiency was controlled by RT-qPCR, western blotting or WES analysis.

542

543 Small interfering RNA

Transfections were performed 24 hours (T₋₂₄) prior to dexamethasone-induced synchronization. AC16 cells were transfected for 24 hours with 10nM siRNAs (Control: On-target plus non-targeting pool or On-target plus Smart pool siRNAs; see details in the Appendix) using Lipofectamine RNAiMAX (Thermo Fisher Scientific). Silencing experiments in U2OS cells were carried out by transfecting 10nM siRNAs using INTERFERin[®] (Polyplus Transfection). Silencing efficiency was monitored by RT-qPCR or western blotting.

550

551 *Immunoprecipitation and ubiquitination assay*

552 Using the Crosslink Magnetic IP/Co-IP kit (Pierce), immunoprecipitation was carried out as 553 described earlier ³³. For ubiquitination assays, 1.7x10⁷ cells were transfected with 10µg pEZ-M11-REV-ERBa plasmid. On the following day, cells were treated with 0.5µM digoxin (Sigma-Aldrich) with or 554 555 without 1 μ M clasto-lactacystin β -lactone (Sigma-Aldrich) or 10 μ M bortezomib (Sigma-Aldrich) for 24 556 hours. Ubiquitinylated proteins were captured using, the UBI-CAPTURE-Q® kit (ENZO Life Sciences). 557 Ubiquitinylated proteins were resolved by 10% SDS-PAGE and were probed with anti-REV-ERBa 558 antibody (Cell Signaling Tech.) or the anti-mono/polyubiguitinylated conjugates monoclonal antibody FK2 (ENZO Life Sciences). 559

560

561 PamGene Kinase Array

Sample preparation: AC16 cells were plated in 12-well fibronectin-gelatin coated plates (Sigma). Cells were synchronized using 100nM dexamethasone as described above. Twenty hours later, cells were treated with 0.5 or 5μM digoxin. After 4 hours of incubation, cells were washed twice with 250 μL 1x PBS and lysed in 250 μL M-PER cell lysis buffer (Pierce) supplemented with Halt Protein Phosphatase and protease inhibitors (Pierce). Lysates were vortexed for 30 seconds and centrifugated (30 min., 16,000 G, 4°C). Protein concentration was determined with the BCA protein assay kit (Pierce).

 PamStation Kinomic analysis: Kinomic profiling of lysates was performed using a PamStation®12 (PamGene Intl) and Serine/Threonine Kinase (STK) PamChip® Arrays. Each STK
 PamChip® Array contains 144 peptides: 4 positive control phosphorylated peptides and 140 consensus phospho-peptide sequences representing the Ser/Thr kinome. Briefly, PamChip arrays were blocked with 2% BSA (1-30 cycles). After the initial blocking step, 2 µg proteins from cell lysates premixed with 4µl of 10x PK buffer (PamGene), 0.4 µl of 4mM ATP, 0.4 µl of 575 100x BSA, and 0.5 μl anti-STK primary antibody adjusted to a total volume of 40 μl in distilled
576 water were added to individual PamChip arrays. The mix was then pumped (30 cycles) through
577 the PamChip® then arrays were washed with 1x PBS/0.01% Tween buffer followed by injection
578 of 30 μl of PamChip detection mix [0.4 μl of FITC-labelled anti-STK antibody (1 μg/μl in TRIS
579 buffer, PamgGene), 3μl of 10x Ab buffer (PamGene) and 26.6 μl H₂O]. Real-time fluorescence
580 signal was quantified by EVOLVE (PamGene.

581 Image and data analysis of kinomic profiles: The BIONAVIGATOR software Suite (PamGene) 582 was used to analyze images and to (log_2) transform signal intensities. The kinase upstream 583 analysis algorithm within the BIONAVIGATOR Software Suite was employed to identify 584 putatively active kinases. A final score is calculated by the software based on the scores 585 obtained for specificity and significance. Kinases are ranked based on the mean final score 586 (MFS). A heat map was plotted using MFS scores. To visualize kinase activity changes in 587 digoxin-treated versus untreated cells/tissue, kinase trees were generated using the KinHub 588 platform (http://www.kinhub.org/kinmap/).

589

590 Microarray analysis, term enrichment analysis and network analysis

591 Gene expression from mouse hearts or AC16 cells was analyzed with Affymetrix GeneChip Hu 592 (or Mo)Gene 2.0 ST arrays after RNA amplification, sscDNA labeling and purification. RNA was 593 amplified using the GeneChip[™] WT PLUS Reagent Kit (Thermo Fisher Scientific), retrotranscribed to 594 sscDNA and labeled using GeneChip[™] WT Terminal Labeling Kit (Thermo Fisher Scientific), followed by 595 hybridization on the GeneChip Mouse or Human Gene 2.0 ST Array (Affymetrix). Raw data were processed using GIANT (Galaxy-based interactive tools for Analysis of Transcriptomic data ⁷⁴. Using 596 597 the GSEA module, normalized data were converted toward a GSEA compatible format for further 598 analysis.

599

600 Real-time measurement of bioluminescence

601 Real-time monitoring of bioluminescence was performed using a Kronos AB-2550 602 luminometer (ATTO). AC16 or U2OS cells (1.1x10⁷) were plated on P150 cell culture plates. When 603 reaching 80% confluency, cells were transfected with the *Bmal*-Luc plasmid (10µg) using either Fugene 604 HD[®] (Promega) or JetPEI[®] (Polyplus Transfection) for AC16 or U2OS cells, respectively. Twenty-four 605 hours after transfection, cells were split into 35mm cell culture dishes. Cells were synchronized 606 (100nM dexamethasone,2 hours) and the medium was replaced with AC16 or U2OS phenol red-free 607 growth media. Cells were treated as indicated (see Fig. legends). Prior to starting light emission 608 monitoring, 200 µM beetle luciferin (Promega) was added to growth medium. Bioluminescence was 609 measured for 1 min. at intervals of 10 min. under 5% CO₂ at 37°C for 4 days. Signals were quantified,

610 analyzed and detrended using the ATTO KRONOS software.

611

612

12 Rapid immunoprecipitation and mass spectrometry of endogenous proteins (RIME)

613 RIME assays were performed using U2OS overexpressing Flagged-hREV-ERBα or HepG2 cells. 614 To overexpress REV-ERBa, cells were transfected with the Flag-REV-ERBa expression vector using 615 JetPEI (Polyplus transfection). Twenty-four hours after transfection, the medium was replaced by fresh 616 medium and cells were further incubated for 24h. Cells were synchronized (2h, 100 nM 617 dexamethasone), washed with 1x PBS and incubated for 20h with fresh media. Cells were cross-linked 618 for 10 min with 1% formaldehyde at 37°C in 1x DMEM. After quenching with 1.25 M glycine, cells were 619 washed twice with ice-cold 1x PBS. Fixed cells were scraped in ice-cold 1x PBS supplemented with 620 protease and phosphatase inhibitors (PIC, Roche). After centrifugation (1,000g, 10 min, 4°C), cells were 621 lysed in 50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP40, 0.25% Triton 622 X-100 buffer supplemented with PIC. After centrifugation (1,000g, 10 min, 4°C), supernatants 623 (cytosolic fraction) and pellets (nuclear fraction) were collected. Pellets were washed in 10 mM TRIS-624 HCl pH8.0, 200 mM NaCl, 1 mM EDTA and 0.5 mM EGTA buffer containing PIC , then in 10 mM TRIS-625 HCl pH8.0, 200 mM NaCl, 1 mM EDTA and 0.5 mM EGTA, 0.1% sodium deoxycholate and 0.5% N-626 laurylsarcosine buffer (with PIC). Nuclei were suspended into 50 mM HEPES-KOH pH 7.5, 1 mM EDTA, 627 0.5 M LiCl₂, 0.7% sodium deoxycholate, 1% NP40 and 1x PIC. After a 10 min sonication, Triton X-100 628 (1% v:v final) was added and samples were centrifuged at 20,000g for 10 min, yielding the nuclear 629 extract. In parallel, SureBeads[™] Protein A or G magnetic beads (Bio-Rad) were saturated with 5 mg/mL 630 BSA. Four μg of rabbit monoclonal anti-REV-ERBα antibody (#13418, Cell Signalling Technology), 10 μg 631 of mouse monoclonal anti-Flag antibody (#F1804, Sigma-Aldrich), 10 µg of control rabbit IgG (sc-2027, 632 Santa Cruz Biotechnology) or mouse IgG (sc-2025, Santa Cruz Biotechnology) were immobilized on SureBeads[™] Protein A (Rabbit) or G (Mouse) magnetic beads. 633

After centrifugation, cytosolic or nuclear extracts were incubated overnight with immobilized
antibodies at 4°C. Beads were washed with 1x RIPA buffer [50 mM HEPES-KOH, pH 7.6, 1 mM EDTA,
0.7% (v:v) sodium deoxycholate, 1% (v:v) NP-40 and 0.5 M LiCl] then with 0.1 M ammonium hydrogen
carbonate. Beads were dried and flash-frozen in liquid nitrogen.LC-MS/MS analysis was performed to
identify REV-ERBα interactants as described ⁷².

639

640 Apoptosis and autophagy antibody arrays

641 To evaluate global changes in the apoptosis and autophagic pathways in digoxin-treated cells, 642 the Human Apoptosis Array C1 (AAH-APO-1-2, Raybiotech) and the Autophagy Array C1 (AAH-ATG-1-643 2, Raybiotech) were employed. These arrays detect 43 and 20 human proteins regulating apoptosis 644 and autophagy, respectively. Briefly, AC16 cells were plated in P150 cell culture plates. At confluence, 645 cells were synchronized as above. Eighteen hours post synchronization, cells were treated either with 646 0.5μ M digoxin or vehicle and lysed 6h later (T₂₄) in lysis buffer (Raybiotech). Antibody arrays were 647 blocked using the blocking buffer (2 mL, Raybiotech) for 30 minutes and 250µg of fresh total protein 648 lysate (1 mL) was added to each antibody array (1 sample/array). Arrays were incubated overnight at 649 4°C, then washed thoroughly in washing buffer (1 mL/wash, Raybiotech) and were further incubated 650 overnight at 4°C with biotinylated antibody cocktail (1 mL, Raybiotech). Arrays were washed twice (1 651 mL/wash) then incubated in 1x HRP-Streptavidin Concentrate for 2h at room temperature. All 652 blocking, washing and incubation steps were performed under gentle rotation (0.5-1 cycle/sec). After 653 a 2h incubation, arrays were washed once followed by chemiluminescence detection using an 654 Invitrogen[™] iBright[™] FL1500 Imaging System (Thermo Fisher Scientific). Spot intensities on each array 655 were quantified using Image Studio[™] Lite (LI-COR Biosciences). The RayBiotech Microsoft® Excel-656 based analysis software tool was used to average, normalize and subtract the background signal. 657 Heatmaps were generated using GraphPad Prism (v. 9.4).

658

659 Statistics and reproducibility

Statistical analysis was performed using GraphPad Prism (v. 9.4). Data are plotted as the mean 660 661 ± SEM. At least 3 independent experimental replicates were obtained. In vitro data were determined 662 to have equal variances using the F test. For 2-group comparisons, an unpaired 2-tailed t-test with 663 Welch correction was used. For multiple comparisons with one variable, a 1-way ANOVA followed by 664 the Tukey multiple comparison test (each group compared to every other group) was used. Multiple 665 comparisons with more than one variable were carried out using a 2-way ANOVA followed by a Tukey's 666 multiple comparison test. Cyclical patterns of gene expression or of protein levels were determined 667 using JTK_Cycle 75. Individual expression/level data were plotted in Prism as a time series and non-668 linearly fitted using the "sine wave with nonzero baseline" function, using a wavelength constraint 669 previously determined by JTK cycle (in the 20-24 hours range). For infarct size measurements, based 670 on an observed standard deviation of 8%, with α set at 0.05, β at 0.20 and a size effect of 10%, the 671 group size was set at n=7. In vivo datasets were considered to have unequal variances and groups 672 were compared using either a one-sided Welch's t-test, or a Welch ANOVA followed by the Dunnett 673 multiple comparison test unless mentioned otherwise. In all instances, P values < 0.05 were 674 considered statistically significant. All samples used in this study were biological replicates, not technical replicates. All experiments were independently performed at least twice to reproduce similarresults.

Differential gene expression was analyzed after normalization of signals to the median of all samples, log₂ transformation and exclusion of the 10th lowest percentile considered as technically unreliable. The Limma package, based on an Empirical Bayes method, is embedded into the Giant suite and was used to identify genes exhibiting an expression fold-change > 1.2 with an FDR < 0.05 (moderated t-test followed by a Benjamini-Hochberg multiple testing correction). Biological term enrichment analysis was performed using the DAVID NCBI portal (v6.8) ⁷⁶.

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684 **Reporting summary**

685 Further information on research design is available in the Nature Research Reporting Summary

686 linked to this article.

| 688 | Data availability |
|-----|---|
| 689 | All data are available in the main text or the supplementary materials. Source data are |
| 690 | available for this paper. Microarray data that support the finding of this study has been deposited in |
| 691 | the National Center of Biotechnology Information's Gene Expression Omnibus and are accessible |
| 692 | through accession number GSE183659, GSE183660 and GSE183661. Other transcriptomic datasets |
| 693 | have been described in ¹⁰ and deposited under the accession number GSE62459. Whole mouse heart |
| 694 | circadian gene expression was analyzed using the GSE180108 dataset ⁴⁷ . The mass spectrometry |
| 695 | proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE 77 partner |
| 696 | repository with the dataset identifier PXD03660 |
| 697 | |

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| 710 | Conceptualization: MV, PL, BS; Methodology: MV, JSA, AB, AH, SC, JE, PL; Validation: MV, CG, AH, SC, |
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| 714 | COMPETING INTERESTS STATEMENT |
| 715 | Nothing to report |
| 716 | |

FIGURE LEGENDS

718 Fig. 1. Time of the day- and REV-ERBα-dependent effects of digoxin in mouse heart. (a) Time of the 719 day dependency of mouse heart sensitivity to MIRI and preconditioning (PC). Upper panel: 720 experimental outline. Lower panel: infarct sizes at ZTO and ZT12, with or without prior exposure to an 721 iterative short ischemia/reperfusion sequence. Results are shown as mean+/-SEM (n=8-9) which were 722 compared using a two-way ANOVA test, followed by a Benjamini-Hochberg multiple comparison test. 723 Infarct sizes were calculated based on necrosis area/area at risk. (b) Left panel: overrepresented Gene 724 Ontology Biological Processes (GO BP) terms in differentially expressed genes (ZTO vs ZT12) mouse 725 hearts; right panel: up- or downregulated and/or rhythmically-expressed (period=22-24h) genes. (c) 726 Time-dependent expression of mouse heart genes. Normalized expression data are expressed as mean 727 +/-SEM (n=3). (d) MIRI at ZTO or ZT9 in wild type mouse hearts after injection of vehicle or digoxin (IP: 728 1mg/kg). **Top:** experimental outline and representative transversal sections of TTC-stained mouse 729 hearts post IR (red stain: healthy tissue, unstained: necrotic tissue); Bottom: infarct size 730 measurements. Results are shown as mean+/-SEM (n=7-13) which were compared using two-way 731 ANOVA test, followed by a Benjamini-Hochberg multiple comparison test. (e) REV-ERBa protein level 732 in mouse heart whole extracts. Upper panel: representative Wes analysis of REV-ERBα protein levels 733 in mouse whole hearts. Bottom panel: quantification of REV-ERB α protein levels as a function of time. 734 Results are shown as mean+/-SEM (n=3-4, see also Supplementary Fig. 2a) which were and compared 735 using Brown-Forsythe & Welch ANOVA test, followed by Dunnett's multiple comparison test. (f) In 736 vivo digoxin ED₅₀. REV-ERBα levels were assessed at ZT9 in whole heart extracts. Results are shown 737 as mean+/-SEM (n=9-16, see also Supplementary Fig. 2b) which were analyzed using nonlinear fit 738 regression (least square method). (g) MIRI at ZT9. Myocardial IR tolerance was evaluated at ZT9 in wild type vs Nr1d1^{-/-} or Cdkn1a^{-/-} mouse hearts treated either with vehicle or digoxin (ZT5, IP: 1mg/kg). 739 740 Results are shown as mean+/-SEM (n=6-7) which were compared using a two-tailed Welch's t-test. All 741 measurements were from distinct samples.

742 Fig. 2. Digoxin affects REV-ERBa protein stability in a cell-autonomous manner. (a) Cyclic REV-743 ERBa protein levels in AC16 cells. Top panel: experimental outline. REV-ERBa levels were determined 744 in synchronized human AC16 cells treated with vehicle or digoxin (0.5μ M) at T₀ and harvested at T₀, T₆, 745 T_{12} , T_{18} and T_{24} . Dex: dexamethasone. Bottom panel: Representative image from WES analysis. 746 HSP90 α was used as a protein loading control. Numbers indicate mean fold-changes [relative to 747 maximal REV-ERBa expression (24h, untreated cells)]. Results are shown as mean+/-SEM (n=3) which 748 were compared using a one-way ANOVA test, followed by a Tukey's multiple comparison test (see Extended data Fig. 3a). *: **: P<0.01, ***: P<0.005. (b) NR1D1 and BMAL1 mRNA expression in 749 750 synchronized AC16 cells. Cells were treated with vehicle or digoxin (0.5μ M) at T₀ and harvested at T₂₄

751 (n=5). Results are shown as mean+/-SEM (n=5) which were compared using an unpaired two-sided t-752 test. (c) Real-time monitoring of the BMAL1 promoter activity. Top panel: experimental outline. 753 Bottom panel: bioluminescence signal measured using a KRONOS (Atto) luminometer in AC16 cells 754 transfected with the *Bmal1*-Luc(iferase) plasmid and treated with vehicle or digoxin (0.5 μ M) (n=3). 755 (d) CDKN1A/P21 protein level in AC16 cells. P21 level was assessed using the ProteinSimple Wes 756 system in AC16 cells treated with vehicle or digoxin (0.5 µM). Results are shown as mean+/-SEM (n=5) 757 which were compared using a unpaired two-sided t-test. ***: P<0.005. HSP90 α was used as a protein 758 loading control. All measurements were from distinct samples.

Fig. 3. Digoxin effect in human primary cardiomyocytes. (a) Rhythmic REV-ERBα protein level. Synchronized primary human cardiomyocyte cells were treated with vehicle or digoxin (0.5 µM) and harvested over a 36-hour period as indicated. (b) *NR1D1* and *BMAL1* mRNA expression in human primary cardiomyocytes. Normalized data are plotted as mean+/-SEM (n=5) and compared using a one-way ANOVA test, followed by a Tukey's multiple comparison test. Vinculin was used as a protein loading control. All measurements were from distinct samples.

765 Fig. 4. Intracellular pathways activation by digoxin. (a) NKA ion pumping vs signalosome activities. 766 Top panel: experimental outline. Synchronized AC16 cells were treated as indicated for 6 hours at T18 767 and harvested at T₂₄. Dex: dexamethasone. Bottom panel: REV-ERB α protein levels in control and 768 treated cells. HSP90 α was used as a protein loading control. Right panel: normalized REV-769 ERB α protein level. Results are shown as mean+/-SEM (n=10, see also Supplementary Fig. 7) which 770 were compared using a one-way ANOVA test, followed by a Tukey's multiple comparison test. (b,c) 771 Kinase activation trees in AC16 cells generated by KinMap using kinase profiling data at low (b) or high 772 (c) digoxin treatment concentration. Circle diameters are proportional to the level of activity of 773 kinases. (d) Heat map quantifying dose-dependent changes in kinase activities (n=3; 1-way ANOVA, *: 774 P<0.05). MFS: median final score. (e) Transcriptomic analysis in vehicle- or digoxin-treated ($0.5\mu M$, 775 5µM) AC16 cells. The heatmap shows the top-ranking 25 genes and indicates dose-dependent changes 776 in mRNA expression. Stars indicate genes whose expression is also altered in mouse heart after digoxin 777 treatment at ZT9. (f) Gene set enrichment analysis of upregulated genes in digoxin-treated AC16 cells.

Fig. 5. Target screening with kinase specific and pathway specific inhibitors. REV-ERBα protein levels
were quantified after a 6 hour-treatment (at T₁₈) with or without digoxin and with or without enzyme
inhibitors: (a) PP2 (20µM, Src kinase inhibitor), (b) ZSTK474 (10µM, PI3 kinase inhibitor), (c) SCH772984
(20µM, ERK 1&2 inhibitor), (d) MK2206 (1µM, AKT1/2/3 kinase inhibitor), (e) KN93 (1µM, CAMK 2&4
inhibitor), (f) PD98059 (20µM, MEK 1&2 inhibitor), (g) RP-8-pCPT-cGMPS (20µM, PKG1&2), (h)
CRT0066101 (5µM, PKD inhibitor), (i) PF-4708671 (10µM, P70S6K1 inhibitor), (j) BAY11-7082 (10µM,

LUBAC and UPS inhibitor), **(k)** HOIPIN8 (10 μ M, LUBAC-HOIP specific inhibitor), **(l)** BMS-345541 (10 μ M, IKK α/β inhibitor), **(m)** TPCA-1 (20nM, IKK β **(n)** Amlexanox (20 μ M, IKK ϵ & TBK1 inhibitor). HSP90 α was used as a protein loading control. Results are shown as mean+/-SEM (n=3-6) which were compared using a one-way ANOVA test, followed by a Tukey's multiple comparison test. All measurements were from distinct samples.

789 Fig. 6. Digoxin triggers REV-ERBa protein degradation through UPS. REV-ERBa protein levels were 790 quantified after a 6 hour-treatment with or without digoxin and with or without the following 791 proteasome inhibitors in synchronized AC16 cells (a) clasto-lactacystin β -lactone (10 μ M, proteasomal 792 inhibitor), (b) bortezomib (BTZ, 200nM, PSMB5 inhibitor) and in synchronized U2OS cells (c) treated 793 with either vehicle, digoxin (0.5µM) and/or bortezomib (200nM, PSMB5 inhibitor. Results are shown 794 as mean+/-SEM (n=3-6) which were compared using a one-way ANOVA test, followed by a Tukey's 795 multiple comparison test. (d) Normalized REV-ERB α protein level was assessed in synchronized AC16 796 cells transfected with Flag-Rev-ERBa or Flag-Rev-ERBa-H602F-encoding plasmids and treated with 797 vehicle or digoxin (0.5 μ M). HSP90 α was used as a protein loading control Results are shown as 798 mean+/-SEM (n=5) which were compared using a one-way ANOVA test, followed by a Tukey's multiple 799 comparison test. (e) Western blot analysis of total ubiquitinylated proteins and of ubiquitinated REV-800 ERBa protein from cell lysates of U20S cells transfected with Flag-Rev-ERBa plasmid and treated with 801 either vehicle, digoxin (0.5µM) and/or bortezomib (BTZ, 200nM, PSMB5 inhibitor) for 2 hours (T18 to 802 T20). Mono- and poly-ubiguitinylated proteins in the lysate were enriched by passing it through a High binding affinity UBI-QAPTURE-Q[®] matrix. All measurements were from distinct samples. *: P<0.05, 803 **: P<0.01, ***: P<0.001, ****: P<0.0001. 804

805 Fig. 7. UPS-related REV-ERBa interactants. (a) REV-ERBa interactants extracted from the RIME 806 dataset filtered against the Gene Ontology "cellular component" or "molecular functions" databases 807 using "proteasome" or "ubiquitin" as biological terms. Green lines indicate known direct interactions 808 as extracted from the String database. Red lines indicate detected direct or indirect interactions with 809 REV-ERB α by RIME. Distances between REV-ERB α and interactants are inversely proportional to the 810 number of identified peptides in the RIME data. Protein structures are from the AlphaFold Protein 811 Structure database. (b) Outcome of REV-ERBa interactant inhibition on digoxin-induced REV-ERBa 812 degradation. The table indicates the list of targets (identified from RIME and literature survey) studied 813 here using siRNA- and/or inhibitor-based strategies, and the outcome of such inhibition on REV-ERBa 814 protein level levels. Upward arrow: increased REV-ERBα protein level; downward arrow: decreased 815 REV-ERBα protein level; equal sign: no change.

- 816 **Fig. 8: Hypothetical scheme for digoxin action in cardiomyocytes.** The main pathways activated by
- 817 digoxin and the impact thereof on REV-ERBα protein stability are shown. More details can be found in
- 818 the text.
- 819

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- 987 77 Perez-Riverol, Y. et al. The PRIDE database resources in 2022: a hub for mass spectrometry988 based proteomics evidences. Nucleic Acids Res 50, D543-d552 (2022).

990 991 992 Extended data Fig. 1. Rhythmic gene and protein level in mouse heart whole tissue extracts. (a) 993 WES analysis of REV-ERBα protein level in mouse hearts collected over a 24-hours period at rest phase 994 (ZT0-ZT12) and active phase (ZT13-ZT24) (n=4). HSP90 α was used as a protein loading control. (b) 995 WES analysis of REV-ERBa protein level in mouse hearts collected at ZT9 after vehicle or digoxin 996 injection (0.1,0.5, and 0.1mpk at ZT5. (c) Cyclic Nr1d1, Nr1d2, Bmal1 and Cdkn1a mRNA expression in 997 mouse hearts collected at rest (ZT0-ZT12) or active (ZT13-ZT24) phases Results are shown as mean+/-SEM (n=3). (d) Nr1d1, Arntl (Bmal1) and Cdkn1a mRNA expression in mouse hearts collected at ZT9 998 999 after vehicle or digoxin (1mpk) injection at ZT5. Results are shown as mean+/-SEM (n=8-9) which were

1000 compared using which were compared using a two-tailed Welch's t-test. (e) P21 protein level in mouse 1001 hearts collected at ZT9 after vehicle or digoxin (1mpk) injection at ZT5. (f) REV-ERBa protein level in 1002 mouse hearts collected at ZTO after vehicle or digoxin (1mpk) injection at ZT20. Basal REV-ERBa 1003 protein level at ZT9 is shown here as reference. Measurements were from distinct samples. 1004 Quantification of data in panels (a), (b) and (c) appears in Fig. 1.

1005 Extended data Fig. 2. NKA status in mouse heart and AC16 cells. (a) Left panel: NKA isotype-encoding 1006 mRNA expression in mouse heart. Atp1a1, Atp1a2 and Atp1a3 expression levels are plotted as a 1007 function of time. Per1 mRNA expression is shown as a reference circadian gene. Middle panel: Relative 1008 expression of NKA isotype-encoding mRNA expression in mouse heart at ZT9. Results are shown as 1009 mean+/-SEM (n=8-15) which were compared using Brown-Forsythe & Welch ANOVA test, followed by 1010 Dunnett's multiple comparison test. Right panel: Effect of digoxin treatment on NKA isotype-encoding 1011 mRNA expression in mouse heart at ZT9. Results are shown as mean+/-SEM (n=8-15) which were 1012 compared using Brown-Forsythe & Welch ANOVA test, followed by Dunnett's multiple comparison 1013 test. (b) NKA isotype protein expression level in mouse heart as a function of time. Data quantification 1014 is shown (right panel) as mean+/-SEM (n=3) which were compared using a one-way ANOVA test, followed by a Tukey's multiple comparison test. *: P<0.05, **: P<0.01, ***: P<0.001. (c) Left panel: 1015 1016 Relative expression of NKA isotype-encoding mRNA expression in human AC16 cells (T₂₄). Right panel: 1017 Effect of digoxin treatment on NKA isotype-encoding mRNA expression in human AC16 cells (T_{24}). 1018 Results are shown as mean+/-SEM (n=5-6) which were compared using a one-way ANOVA test, 1019 followed by a Tukey's multiple comparison test. (d) NKA isotype protein expression level in human 1020 AC16 as a function of time. Data quantification is shown (right panel) as mean+/-SEM (n=4) which 1021 were compared using a one-way ANOVA test, followed by a Tukey's multiple comparison test. All 1022 measurements were from distinct samples. *: P<0.05, **: P<0.01

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SUPPLEMENTAL DATA

1023 Extended data Fig. 3. Effects of digoxin and of its structural analogs on REV-ERBa protein stability. 1024 (a) AC16 cellular viability in the presence of increasing concentrations of digoxin. Data are plotted as 1025 mean+/-SEM (n=4) which were compared using a one-way ANOVA test, followed by a Tukey's multiple 1026 comparison test. (b) Protein array analysis of AC16 whole cell protein extract. AC16 cells were treated 1027 for 6hours at T₁₈ as indicated and extracts were probed on membrane spotted with antibodies specific 1028 for components of the apoptotic pathway. Fluorescence signals were quantified and were represented 1029 as a heatmap (n=2). (c) Cyclic REV-ERB α protein levels in human AC16 cells. REV-ERB α levels were 1030 determined in synchronized human AC16 cells treated with vehicle or digoxin (0.5µM) and harvested 1031 at T_0 , T_6 , T_{12} , T_{18} and T_{24} . Quantification of data is shown in Fig. 2A. (d) REV-ERB α protein level was assessed in AC16 cells treated with vehicle, digoxin (0.5 μ M) and/or DigiFab (100 μ g). HSP90 α was used 1032 1033 as a protein loading control. Right panel: normalized data are plotted as mean+/-SEM (n=3) which 1034 were compared using a one-way ANOVA test, followed by a Tukey's multiple comparison test. (e) REV-1035 ERBa protein level was assessed in AC16 cells treated with vehicle or varying concentration of digoxin 1036 as indicated. HSP90 α was used as a protein loading control Right panel: normalized data are plotted 1037 as mean+/-SEM (n=3) and analyzed by nonlinear regression curve fitting. (f) REV-ERB α protein level in 1038 AC16 cells treated with vehicle or varying concentrations of bufalin (0.1, 1.0, 10µM) (n=2). (g) REV-1039 ERB α protein level in AC16 cells treated with vehicle or ouabain (5 μ M) (n=2). HSP90 α was used as a 1040 protein loading control. All measurements were from distinct samples. (h) Time course experiment 1041 in AC16 cells treated at T_{18} with vehicle or digoxin (5µM). REV-ERB α protein level was assessed using 1042 the ProteinSimple Wes system (n=2). HSP90 α was used as a protein loading control. All measurements 1043 were from distinct samples. ***: P<0.001.

1044 Extended data Fig. 4. Digoxin effect in U2OS cells. (a) Rhythmic REV-ERBa and RORa protein level in 1045 synchronized U2OS cells treated with vehicle or digoxin (0.5 μ M) and harvested over a 36-hour period 1046 as indicated. HSP90 α was used as a protein loading control. (b) NR1D1 and BMAL1 mRNA expression 1047 in synchronized U2OS cells treated with vehicle or digoxin (0.5 μ M) and harvested at T₂₄. Normalized 1048 data are plotted as mean+/-SEM (n=6) which were compared using a one-way ANOVA test, followed 1049 by a Tukey's multiple comparison test. (c) Bioluminescent signal in U2OS cells transfected with the 1050 *Bmal1*-Luc plasmid and treated with vehicle or digoxin (0.5 μ M). Detrended data are shown HSP90 α 1051 was used as a protein loading control. All measurements were from distinct samples.

Extended data Fig. 5. Effect of protein kinase inhibition or activation on digoxin-mediated REV-ERBα
 protein level decrease. Representative WES analysis (related to quantified data in Fig. 4) are shown.
 REV-ERBα protein level in synchronized AC16 cells treated with REV-ERBα protein levels were
 quantified after a 6 hour-treatment with or without digoxin and with or without enzyme inhibitors: (a)

1056 PP2 (20μM, Src kinase inhibitor), **(b)** ZSTK474 (10μM, PI3 kinase inhibitor), **(c)** SCH772984 (10μM, ERK 1057 1&2 inhibitor), **(d)** MK2206 (1μM, AKT1/2/3 kinase inhibitor), **(e)** KN93 (10μM, CAMK 2&4 inhibitor), 1058 **(f)** PD98059 (10μM, MEK 1&2 inhibitor), **(g)** RP-8-pCPT-cGMPS (20μM, PKG1&2), **(h)** CRT0066101 1059 (5μM, PKD inhibitor), **(i)** PF-4708671 (10μM, P70S6K1 inhibitor), **(j)** BAY11-7082 (10μM, LUBAC and 1060 UPS inhibitor), **(k)** HOIPIN8 (10μM, LUBAC-HOIP specific inhibitor), **(l)** TPCA-1 (20nM, IKKβinhibitor), 1061 **(m)** TPCA-1 (5μM, IKKβ/α inhibitor) **(n)** amlexanox (1μM, IKKε & TBK1 inhibitor). HSP90α was used as a protein loading control.

1063 Extended data Fig. 6. Effect of protein kinase inhibition or activation on digoxin-mediated REV-ERBa 1064 **protein level decrease.** Quantification of REV-ERBa protein levels in synchronized human AC16 cells 1065 (see Extended data Fig. 7) (a) treated with vehicle, digoxin (0.5µM) and/or SRC inhibitor (saracatinib), 1066 (b) treated with vehicle, digoxin (0.5µM) and/or PI3K inhibitor (LY294002) (c) treated with vehicle, 1067 digoxin $(0.5\mu M)$ and/or PI3K/P70S6K inhibitor (dactolisib), (d) transfected with ERK1 expression vector, 1068 (e) treated with the GSK3 β inhibitor lithium and/or digoxin, (f) transfected with scrambled siRNA (Scr 1069 siRNA) or CAMK4 siRNA and treated with vehicle or digoxin (0.5µM), (g) transfected with scrambled 1070 siRNA (Scr siRNA) or *ERK1&3* siRNA and treated with vehicle or digoxin (0.5µM), (h) transfected with 1071 scrambled siRNA (Scr siRNA) or *PKD1* siRNA and treated with vehicle or digoxin $(0.5\mu M)$, (i) transfected 1072 with scrambled siRNA (Scr siRNA) or PRKG1 siRNA and treated with vehicle or digoxin (0.5µM), (j) 1073 transfected with scrambled siRNA (Scr siRNA) or HOIL siRNA and treated with vehicle or digoxin 1074 (0.5µM) and (k) transfected with an empty (pcDNA3) or OTULIN-expressing (pcDNA3-OTULIN) vector 1075 and treated with vehicle or digoxin (0.5µM). Normalized data are plotted as mean+/-SEM (n=3-6) and 1076 compared using a one-way ANOVA test, followed by a Tukey's multiple comparison test except for (c) 1077 for which groups were compared using an unpaired 2-tailed t-test. All measurements were from 1078 distinct samples.

1079 Extended data Fig. 7. Effect of protein kinase inhibition or activation on digoxin-mediated REV-ERBa 1080 protein level decrease. WES analysis of REV-ERBα protein levels in synchronized human AC16 cells (a) 1081 treated with vehicle, digoxin (0.5μM) and/or SRC inhibitor (saracatinib, 10μM), (**b**) treated with vehicle, 1082 digoxin (0.5µM) and/or PI3K inhibitor (LY294002, 20µM) (c) treated with vehicle, digoxin (0.5µM) 1083 and/or PI3K/P70S6K inhibitor (dactolisib, 0.1μ M), (d) transfected with ERK1 expression vector, (e) 1084 treated with the GSK3 β inhibitor lithium (20mM) and/or digoxin, (f) transfected with scrambled siRNA 1085 (Scr siRNA) or CAMK4 siRNA and treated with vehicle or digoxin $(0.5\mu M)$, (g) transfected with 1086 scrambled siRNA (Scr siRNA) or ERK1&3 siRNA and treated with vehicle or digoxin (0.5μM), (h) 1087 transfected with scrambled siRNA (Scr siRNA) or PKD1 siRNA and treated with vehicle or digoxin 1088 $(0.5\mu M)$, (i) transfected with scrambled siRNA (Scr siRNA) or *PKG1* siRNA and treated with vehicle or 1089 digoxin (0.5μ M), (j) transfected with scrambled siRNA (Scr siRNA) or HOIL siRNA and treated with vehicle or digoxin (0.5μM) and (k) transfected with an empty (pcDNA3) or OTULIN-expressing
 (pcDNA3-OTULIN) vector and treated with vehicle or digoxin (0.5μM).

1092 Extended data Fig. 8. Proteasome inhibition counteracts digoxin effect on REV-ERBa levels. 1093 Quantification of data appears in Fig. 5. REV-ERBα protein level in synchronized AC16 cells (a) treated 1094 with either vehicle, digoxin (0.5μ M) and/or clasto-lactacystin β -lactone (10μ M, proteasomal Inhibitor), 1095 (b) treated with either vehicle, digoxin $(0.5\mu M)$ and/or bortezomib (200nM, PSMB5 inhibitor), in 1096 synchronized U2OS cells treated (c) with either vehicle, digoxin (0.5µM) and/or bortezomib (200nM, 1097 PSMB5 inhibitor)in synchronized AC16 cells (d) in synchronized AC16 cells transfected with Flag-Rev-ERB α or Flag-Rev-ERB α -H602F then treated with vehicle or digoxin (0.5 μ M). HSP90 α was used as a 1098 1099 protein loading control.

1100 Extended data Fig. 9. Screening E2/E3 ligases potentially involved in digoxin-induced REV-ERBa 1101 degradation. (a) Quantification of REV-ERB α protein level in AC16 cells transfected either with 1102 scrambled siRNA (Scr siRNA) or SIAH2 siRNA and treated with vehicle or digoxin (0.5µM). A strictly 1103 identical protocol was followed to assess the effect of FBXW7 (b), GSK3 β (c), UBE2L3 (d), HUWE1 (e), 1104 PSME3 (f), TBL1XR1 (g), BUB3 (h), CBL, BRCA1, UBE4A (i), UBE4B, MDM2, STUB1 (j), and of TRIM21 and 1105 TRIM33 (k) knockdowns on REV-ERBα stability. Normalized data are plotted as mean+/-SEM (n=3-6) 1106 and compared using a one-way ANOVA test, followed by a Tukey's multiple comparison test. All 1107 measurements were from distinct samples.

1108Extended data Fig. 10. Screening E2/E3 ligases potentially involved in digoxin-induced REV-ERBα1109degradation. (a) REV-ERBα protein level in AC16 cells transfected either with scrambled siRNA (Scr1110siRNA) or SIAH2 siRNA and treated with vehicle or digoxin (0.5µM). A strictly identical protocol was1111followed to assess the effect of FBXW7 (b), GSK3β(c), UBE2L3 (d), HUWE1 (e), PSME3 (f), TBL1XR1 (g),1112BUB3 (h), CBL, BRCA1, UBE4A (i), UBE4B, MDM2, STUB1 (j), and of TRIM21 and TRIM33 (k) knockdowns1113on REV-ERBα stability.

Supplemental Dataset 1. Gene expression profiling in ZTO vs ZT12 mouse hearts. Mouse hearts were
collected at ZTO or ZT12 (n=7 per group) and RNAs were extracted and quantified by Affymetrix arrays.
Results are expressed as fold change and corrected p values are indicated (fold-change > 1.2 with an
FDR < 0.05, after a moderated t-test followed by a Benjamini-Hochberg multiple testing correction).
For the sake of clarity, only protein-encoding genes are indicated.

Supplemental Dataset 2. Gene expression profiling in Nr1d1^{+/+} vs Nr1d1^{-/-} (ZT12) mouse hearts.
Mouse hearts were collected at ZT12 (n=7 per group) from Nr1d1^{+/+} vs Nr1d1^{-/-} mice and RNAs were
extracted and quantified by Affymetrix arrays. Results are expressed as fold change and corrected p

values are indicated (fold-change > 1.2 with an FDR < 0.05, after a moderated t-test followed by a
Benjamini-Hochberg multiple testing correction). For the sake of clarity, only protein-encoding genes
are indicated.

Supplemental Dataset 3. Transcriptomic alterations in digoxin-treated AC16 cells. AC16 cells were treated after synchronization by low (0.5μ M) or high (5μ M) digoxin concentrations (n=3). mRNA levels were quantified by Affymetrix arrays. Results are expressed as fold change and corrected p values are indicated (fold-change > 1.2 with an FDR < 0.05, after a moderated t-test followed by a Benjamini-Hochberg multiple testing correction). For the sake of clarity, only protein-encoding genes are indicated.

Supplemental Dataset 4. Transcriptomic alterations in digoxin-treated mice. Mice were injected at ZT5 and hearts were collected at ZT9 (n=3-5). After RNA extraction, gene expression was assayed and expressed as described in Supplementary Dataset 1 (fold-change > 1.2 with an FDR < 0.05, after a moderated t-test followed by a Benjamini-Hochberg multiple testing correction). For the sake of clarity, only protein-encoding genes are indicated.

- 1136 Supplementary Dataset 5. Transcriptomic alterations common to mouse heart and human AC16
- cells. Data from Supplementary Datasets 1 and 2 were compared and common genes to both lists areindicated.
- Supplementary Dataset 6. REV-ERBα RIME data. REV-ERBα was pulled down from AC16 and HepG2 cells and associated proteins were identified by LC-MS/MS (n=1 for HepG2 and n=2 for U2OS). The list of common REV-ERBα interactants is shown, with selected interactants related to UPS indicated in bold. The threshold for significant interaction was arbitrarily set to 5 detected peptides to select a testable set of interactants.



Vinod et al. - FIGURE



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Vinod et al. - FIGURE 2





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Vinod et al. - FIGURE 4



















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Vinod et al. - FIGURE 6

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| | REV-ERB α | interactants | NEDD8 | PSMD2 | PSMA5 | PSME3 | PSMA4 | UBE2L3 | CUL4A | CAND1 | TBL1XR1 | CDK1 | AIMP2 | TRIM21 | EIF3F | BUB3 | PRPF19 | PCBP2 | SIAH2 | FBXW7 | GSK3B | CBL | BRCA1 | UBE4A | UBE4B | MDM2 | STUB1 | TRIM33 | HUWE1 | PSMB5 |
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Vinod et al. - FIGURE 7

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Vinod et al. - FIGURE 8



Vinod et al. - Ext. Figure 1

С

Mouse heart



Vinod et al. - Ext. Figure 2



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Digoxin duration (min) 0 30 60 90 150 210 kDa Wes: REV-ERBα Wes: HSP90AA1

Vinod et al. - Ext. Figure 3

Human osteosarcoma U2OS cells



Vinod et al. - Ext Figure 4



Vinod et al. - Ext. Figure 5



Vinod et al. - Ext. Figure 6



Vinod et al. - Ext Figure 7



Vinod et al. - Ext. Figure 8



Vinod et al. - Ext Figure 9



Vinod et al. - Ext Figure 10