1 **Model-based identification of the crosstalks and feedbacks that determine the**
2 **doxorubicin response dynamics of the JNK-p38-p53 network.**

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7

8 **Abstract**

9 Cellular responses to perturbations and drugs are determined by interconnected
10 networks, rather than linear pathways. Individually, the JNK, p38 and p53 stress and
11 DNA-damage response networks are well understood and regulate critical cell-fate
12 decisions, such as apoptosis, in response to many chemotherapeutical agents, such as
13 doxorubicin. To better understand how interactions between these pathways
14 determine the dynamic behaviour of the entire network, we constructed a data-driven
15 mathematical model. This model contains mechanistic details about the kinase
16 cascades that activate JNK, p38, AKT and p53, and free parameters that describe
17 possible interactions between these pathways. Fitting this model to experimental
18 time-course perturbation data (five time-courses with six time-points under five
19 different conditions), identified specific network interactions that can explain the
20 observed network responses. JNK emerged as an important control node. JNK
21 exhibited a positive feedback loop, was tightly controlled by negative feedback and
22 crosstalk from p38 and AKT, respectively, and was the strongest activator of p53.
23 Compared to static network reconstruction methods, such as modular response
24 analysis, the model-based approach identifies biochemical mechanisms and explains
25 the dynamic control of cell signalling.

26 Keywords: cancer signalling, DNA damage response, dynamic modelling, ordinary
27 differential equations, parameter estimation.

28

29 Introduction

30 Cellular responses to perturbations or drugs are determined by interconnected
 31 networks, rather than linear pathways¹. For example, even within a single pathway
 32 such as the ERK pathway, negative feedback can induce a transient response to a
 33 sustained stimulus thereby promoting cell proliferation, whereas positive feedback
 34 can ensure a sustained response and cell differentiation, even for pulsatile, transient
 35 inputs². Further, the response behaviour of one pathway may also be controlled by
 36 crosstalks from other pathways. For example, crosstalk from AKT signalling shifts the
 37 activation curve of the JNK stress response towards higher tolerable stress inputs, and
 38 may even completely disable the JNK bi-stable switch^{3,4}.

39 The JNK, p38 and p53 stress and DNA-damage response networks are of particular
 40 interest, because they regulate vital cell fate decisions in response to many
 41 chemotherapeutic agents used in cancer therapy^{4,5}. Firstly, the stress-activated
 42 kinases JNK and p38 can induce programmed cell-death and initiate the cell-cycle
 43 arrest by phosphorylating their respective substrates⁶⁻⁹. Secondly, the kinase AKT
 44 promotes cell-survival and can for example suppress the stress response by
 45 phosphorylating and inhibiting kinases upstream of JNK and p38^{3,10,11}. Thirdly, the
 46 transcription factor p53 maintains genomic stability and suppresses cancer formation
 47 by activating DNA repair proteins, arresting cell growth by pausing the cell cycle,
 48 initiating cell-death, and promoting senescence by shortening telomeres^{5,7}. Most of
 49 these p53 functions require the activation of p53, which is usually achieved by
 50 phosphorylation of p53 at sites in its transcriptional domain. Kinases known to target
 51 these phosphorylations are amongst others JNK, p38 and AKT. The resulting activation
 52 inhibits the p53-MDM2 interaction and MDM2 mediated degradation. In response, the
 53 increasing concentration of active p53 induces the expression of p53 target genes, that
 54 by driving the mentioned cell fates.

55 The described p53 activation is induced by many stresses and drugs, including
 56 Doxorubicin, which is one of the most frequently used anti-cancer drugs in
 57 combination with surgery^{12,13}. Doxorubicin is a chemotherapeutic agent from the
 58 anthracycline class, acting as a DNA damaging agent. Doxorubicin is part of standard
 59 chemotherapy for many cancers, such as breast and ovarian cancer, but is often
 60 ineffective. Doxorubicin not only activates p53 but also JNK and p38. But how the
 61 dynamic interplay between these pathways controls the Doxorubicin response is not
 62 understood.

63 We hypothesised that Doxorubicin activates JNK, p38 and p53, and that the dynamic
 64 behaviour of this response is controlled by specific network interactions between
 65 these pathways. To test this hypothesis, we developed a model-based systems
 66 identification approach and built a data-driven mathematical model of the JNK, p38,
 67 AKT and p53 network. This model contains mechanistic details about the kinase
 68 cascades that activate JNK, p38, AKT and p53, and free parameters that describe
 69 possible interactions between these pathways. Fitting this model to experimental
 70 time-course perturbation data obtained from MCF10A mammary epithelial cells (a
 71 cell-line model for non-mutated, non-malignant breast cells) identified the specific
 72 network interactions that could explain the observed network responses. JNK
 73 signalling activating p53 emerged as a master regulator boosted by positive feedback

74 to amplify the JNK response and tightly controlled by indirect negative feedback and
75 crosstalk from p38 and AKT, respectively.

76 **Results**

77 **A mathematical model of the JNK, p38, AKT and p53 response** 78 **dynamics**

79 To investigate the dynamic crosstalks between JNK, p38, AKT and p53 pathways in
80 relation to Doxorubicin treatments, we constructed a mathematical model of ordinary
81 differential equations. A reaction kinetic graph was defined summarising the current
82 knowledge of the network from the literature (Fig 1). In this model, ATM activates two
83 stress-response cascades and p53. Firstly, ATM phosphorylates MKK4 and MKK7,
84 which in turn phosphorylate and activate JNK. Secondly, ATM phosphorylates MKK3
85 and MKK6, which in turn phosphorylate and activate p38. Other possible
86 intermediates, such as the MAP3Ks upstream of MKK4/7 and MKK3/6 are not
87 included in the model¹⁴. Further, ATM mediates the phosphorylation, of p53, which
88 happens directly and indirectly^{5,15}, but for simplicity, ATM's effect on p53 is modelled
89 in a single reaction. Both p53 and phospho-p53 induce the expression of MDM2,
90 leading to a negative feedback loop¹⁶. p53 phosphorylation stabilises p53 by inhibiting
91 MDM2-binding and MDM2-mediated degradation¹⁷.

92 From this reaction kinetic graph a dynamic model in terms of ordinary differential
93 equations (ODEs) can be derived¹⁸. Table 1 list all the reactions and their rate laws,
94 Table 2 the resulting system of ODEs and their initial conditions.

95 To limit the complexity of the model and keep the model calibration feasible, the
96 model contains a few simplifying assumptions^{4,18}. Firstly, the activation of JNK, p38
97 and their upstream kinases requires the phosphorylation at two conserved residues.
98 The two phosphorylations were modelled as a single
99 phosphorylation/dephosphorylation cycle. This keeps the model simple and focusses
100 on the active, double-phosphorylated JNK and p38 forms for which we generated
101 experimental data. Secondly, MKK4 and MKK7, and also MKK3 and MKK6, were
102 lumped into two combined model components called MKK4/7 and MKK3/6,
103 respectively. The justification is that the only function in the context of this model is
104 the activation of JNK and p38, respectively. Thirdly, albeit there are many phospho-
105 sites on p53, most these phosphorylation sites share a common function, which is the
106 activation and stabilisation of p53⁵. Thus, we only modelled a phosphorylated and an
107 unphosphorylated form of p53.

108 **Modelling inhibitors**

109 Inhibitors were modelled as additional factors in the activating phosphorylation
110 reactions (Table 1) assuming a non-competitive mechanism¹⁹⁻²¹. For example, the
111 inhibition of JNK is described by the SP term in v9, which is the reaction for
112 phosphorylating and activating JNK. Admissible values for these inhibition factors
113 range from zero (complete inhibition) to one (no inhibition). We adopted the following
114 notation: SP, SB and LY in reactions v9, ap38 and v13, denote the effectiveness of the
115 JNK (SP6001250), p38 (SB203580) and PI3K (LY294002) inhibitors, accordingly. The
116 values of these inhibition factors were estimated from the experimental data as the
117 ratio between the phosphorylated target, for example pJNK, in inhibited versus
118 uninhibited conditions at t=2h (Methods). This yielded the following values SP = 0.27,

119 LY = 0.65, SB = 0.40. The SB203580 inhibitor does not directly inhibit the
120 phosphorylation of p38 but only blocks its catalytic activity. Thus, to verify the
121 effectiveness of the inhibition, we measured the phosphorylation of the p38 substrate
122 MAPKAPK2 (Fig S1), and estimated SB = 0.4.

123 Because the p38 inhibitor does not directly affect the phosphorylation of p38, the
124 phosphorylation level of p38 does not correspond to p38 activity²². In the presence of
125 the inhibitor the phosphorylated p38 is not active. To differentiate phosphorylated
126 form of p38 from the active form, we introduced an additional model variable called
127 ap38 that describes the active form of p38 as $ap38 = SB \cdot pp38$, where $pp38$ denotes
128 phosphorylated p38.

129 **Modelling crosstalks**

130 The interactions between the canonical pathways were modelled using crosstalk
131 terms (Fig 1, Table 1). The model assumes that all the crosstalks between the
132 pathways are possible and pJNK, pp38, pAKT mediate these crosstalks. It further
133 assumes that the crosstalks and feedbacks act on the phosphorylation reactions of
134 MKK4/7 upstream of JNK (v3), MKK3/6 upstream of p38 (v5), PI3K upstream of AKT
135 (v7) and p53 (v15). These reactions were chosen for mediating the crosstalks because
136 many known crosstalks act on the level of these MAP2Ks and because these are the
137 first reactions in the model where more upstream crosstalks would take effect in the
138 model^{3,4,23}. Mathematically, these crosstalks are described by Hill-type equations that
139 modify the nominal phosphorylation rate laws

$$v_i = \tilde{v}_i(y) \frac{K_{ji}^{n_{ji}} + a_{ji} p x^{n_{ji}}}{K_{ji}^{n_{ji}} + p x^{n_{ji}}},$$

140 where v_i denotes the modified reaction rate taking account of the crosstalks, $\tilde{v}_i(y)$ the
141 nominal, unmodified rate law of the phosphorylation reaction,
142 $y \in \left(\frac{MKK4}{7}, \frac{MKK3}{6}, PI3K, p53\right)$ the substrate to be phosphorylated,
143 $p x \in (pJNK, pp38, pAKT)$ the kinase mediating the crosstalk, $K_{ji} > 0$ the half-
144 activation constant of the crosstalk, $a_{ji} \in [0, \infty)$ the strength of the crosstalk, n_{ji} its Hill
145 exponent, and where the indices $j \in (j, p, a)$ indicate the source of the crosstalk (j for
146 pJNK, p for pp38, a for AKT), and $i \in (3, 5, 7, 15)$ the number of the reaction they modify
147 (Table 1).

148 The model allows for crosstalks from each kinase pathway to each other kinase
149 pathway and p53. The aim is to estimate the activation threshold and strength of these
150 crosstalks, whereby $a_{ji} < 1$ describe negative crosstalks, $a_{ji} \approx 1$ no crosstalk, and
151 $a_{ji} > 1$ positive crosstalks.

152

153 **Perturbation experiments generated a rich time-course data set for** 154 **model calibration**

155 To characterise the network dynamics and calibrate the model, we generated time-
156 course data in response to Doxorubicin +/- inhibitors (Fig S2). MCF10A cells were
157 used as a non-malignant breast cell line model that does not harbour mutations in the
158 modelled pathways. Western blots were used to measure the responses of pJNK, pp38,

159 pAKT and p53 in the presence and absence of the following inhibitors JNK, p38 and
160 PI3K. The data were quantified and normalised as described in the Methods. The
161 resulting dataset comprised at least three biological replicates of five time-courses at
162 six timepoint in four different conditions. This totalled 120 data-points per replicate
163 and build the basis for estimating the parameters.

164 The perturbation data exhibited the following salient features. Firstly, JNK inhibition
165 strongly inhibited the pJNK response, slightly delayed and reduced the pp38 response,
166 and strongly decreased the p53 response (Fig 2). Secondly, p38 inhibition markedly
167 increased the pJNK and pp38 responses, and slightly reduced the p53 response (Fig 2).
168 That p38 inhibition increased the pp38 response can be explained as follows. The p38
169 inhibitor SB203580 inhibits p38 catalytic activity by binding to the ATP binding
170 pocket, but does not inhibit phosphorylation of p38 by upstream kinases²². The
171 increase of p38 phosphorylation the presence of the inhibitor can therefore be
172 explained by a negative feedback loop and the release of this negative feedback in
173 response to the inhibitor. Thirdly, PI3K inhibition strongly increased the pJNK and
174 pp38 responses and markedly decreased the p53 response (Fig 2). Last but not least,
175 pAKT exhibited very minor changes in response to doxorubicin (Fig 2), albeit pAKT
176 levels were affected by the inhibitors, with the most prominent reductions observed
177 with PI3K and p38 inhibition (Fig 2).

178 No direct conclusions should be drawn from these perturbation experiments
179 regarding the network structure²⁴. The measured responses constitute so-called global
180 responses that are the consequences of direct and indirect network interactions. For
181 example, the observed impairment of the p53 response in response to the JNK
182 inhibitor could be due to a direct JNK-p53 interaction or an indirect JNK-p38-p53
183 interaction loop. To detangle direct from indirect effects, formal network
184 reconstruction techniques have to be applied²⁴⁻²⁸. Here, we use the constructed model
185 and parameter estimation to identify the interactions parameters.

186 **Parameter estimation lead to reliable model simulations fitting the** 187 **experimental data**

188 The aim of the parameter estimation is to fit the experimental data to the model and
189 estimate all unknown parameters, in particular the crosstalk parameters. To fit the
190 experimental data, we used PEPSSBI with the GSLDC global optimisation algorithm²⁹.
191 Biologically feasible parameter bounds were used (*Methods*, Suppl. File 1). Because we
192 had many parameters to estimate, the parameter estimation was performed in a
193 modular fashion divided into four steps. For each step, we performed 100-rounds of
194 independent parameter estimation runs and selected the estimate with the best
195 goodness-of-fit value for the next step. First, we obtained an estimate of the
196 feedforward parameters of the upstream pathways (k_1 to k_{14} and K_{m1} to K_{m14}) by
197 fitting to the measured pJNK, pp38 and pAKT time-courses in response to Doxorubicin
198 in control conditions without any inhibitors. In this step, the inhibitor data were not
199 used, and the crosstalks were neglected by fixing $a_{ji} = 1$. Second, we estimated the
200 crosstalks parameters of the upstream kinase pathways (a_{j3} , a_{j5} , a_{j7} , a_{p3} , a_{p5} , a_{p7} ,
201 a_{a3} , a_{a5} , a_{a7} , K_{j3} , K_{j5} , K_{j7} , K_{p3} , K_{p5} , K_{p7} , K_{a3} , K_{a5} , K_{a7}) by fitting the measured pJNK,
202 pp38 and pAKT time-courses for all four conditions. Third, to get a parameter estimate
203 of the downstream MDM2-p53 module (k_{15} , k_{16} , k_{19} , k_{20} , K_{m15} , K_{m16} , K_{m18} and
204 K_{m19}), we used the best fitting estimates from the previous step, kept these fixed and

205 fitted the measured p53 time-course. Finally, we estimated all the crosstalks
206 parameters (aj3, aj5, aj7, ap3, ap5, ap7, aa3, aa5, aa7, Kj3, Kj5, Kj7, Kp3, Kp5, Kp7, Ka3,
207 Ka5, Ka7, aj15, ap15, aa15, Kj15, Kp15, Ka15) by fitting all the data. Supplementary
208 File 1 contains all parameter estimates.

209 Figure 2 shows a good correspondence of the model simulations with the experimental
210 data. The simulations from all 100 parameter estimation runs fitted the data well.

211 **Model based systems identification revealed critical feedback and** 212 **crosstalk structures**

213 The parameter estimation yielded estimates for the crosstalk parameters of the
214 network. First, we describe the identified crosstalk structure of the best fitting
215 estimate. Then we judge the reliability of the identified structure based on the
216 distribution of all 100 estimated parameter sets.

217 The best fitting model exhibited a direct positive feedback loop from JNK onto itself, a
218 indirect negative feedback loop in which JNK facilitated the activation of p38, which in
219 turn inhibited the activation of JNK, and negative crosstalk from AKT to JNK (Fig. 3a).
220 All these interactions activated at low concentrations (small K_{ji} values). p38 activity
221 was controlled by the same indirect negative feedback loop (p38 $\rightarrow$ JNK $\rightarrow$ p38), a direct
222 positive feedback that only activated at high pp38 concentrations (high value for K_{p5}),
223 and negative crosstalk from AKT activating at low pAKT concentrations. All crosstalks
224 to AKT were very weak (a_{j7} values close to 1). The p53 response was controlled by
225 strong positive crosstalk from JNK and AKT activating at low pJNK and high pAKT
226 concentrations, respectively, and negative crosstalk from p38 activating at low pp38
227 concentrations. Figure 3a depicts the inferred network structure of the best fitting
228 model.

229 To judge the reliability of the identified network structure we can look at the
230 distribution of the estimated interaction parameters for all 100 estimation runs (Fig.
231 3b). Some estimates are more dispersed the others. The most consistent estimates are

- 232 • Positive crosstalk from JNK to p38 (aj5) that activates at a range of pJNK
233 concentrations (Kj5)
- 234 • Negative crosstalk from p38 to JNK (ap3) that activates at low pp38
235 concentrations (Kp3)
- 236 • Negative crosstalk from AKT to JNK (aa3) and p38 (aa5) that activates at
237 intermediate pAKT concentrations (Ka3, Ka5)
- 238 • Strong positive crosstalk from JNK to p53 (aj15) that activates at low pJNK
239 concentrations (Kj15)
- 240 • Negative crosstalk from p38 to p53 (ap15) that activates at low p38
241 concentrations (Kp15).
- 242 • Strong positive crosstalk from AKT to p53 (aa15) that activates at high pAKT
243 concentrations (Ka15).

244 The estimated crosstalk strengths from p38 and JNK to AKT (aj7, ap7) were
245 concentrated near zero on the log scale indicating that these crosstalks are very weak.

246 The most dispersed estimates were the direct JNK feedback (aj3), the direct p38
247 feedback (ap5), which depending on the parameter estimation run, could take both
248 positive and negative values. Therefore, we analysed these estimates in more detail.

Specific correlations within the estimated crosstalk parameters revealed mutually exclusive crosstalk patterns between JNK and p38

The large dispersion of the direct JNK and p38 feedback parameters (a_{j3} and a_{p5}) indicates the practical non-identifiability of these parameters³⁰. In situations where unique parameter values cannot be identified, they are often correlated with other parameters. When such correlations exist, relations between different parameters could still be identifiable³⁰. For example, the identified value of the JNK feedback strength (a_{j3}) could depend on the crosstalk strength from p38 (a_{p3}) as both crosstalks act on the same reaction (v_3). To confirm this hypothesis, we performed a correlation analysis and looked at the relationships between the estimated parameter values.

To better understand the results of the parameter estimation, we investigated the relationships between the parameters in a correlation analysis. We observed a strong negative correlation between the direct JNK feedback parameter a_{j3} and the p38-to-JNK crosstalk parameter a_{p3} (Fig 4a). This anti-correlation is consistent with models that feature either a strong JNK feedback or a strong crosstalk from p38, but not both. Indeed, a scatter plot of the estimates for a_{j3} versus a_{p3} (Fig 4b) confirms that a unique value for each parameter was not practically identifiable, but the relationship between these two parameters was. The scatter plot shows a mutually exclusive pattern where JNK features either a strong positive crosstalk from p38 (top-left corner) or positive feedback onto itself (bottom right corner).

A similar, albeit weaker, pattern was observed between the direct p38 feedback a_{p5} and the JNK-to-p38 crosstalk a_{j5} (Fig 4c). Most parameter estimates featured a negative p38 feedback accompanied by a positive JNK-to-p38 crosstalk (top right), whereas negative JNK-to-p38 crosstalk was predominantly accompanied by positive p38 feedback.

Modular Response Analysis identified a similar network structure

To benchmark the inferred network, we compared our model-based results to a well-established network inference method Modular Response Analysis (MRA). The strength of MRA is that it can identify the exact network structure between all measured nodes given that each node has been perturbed 1-by-1 in experiments and measured. Because not all measured nodes were perturbed in our network (p53 was not perturbed), we applied a recent extension of MRA (Bayesian MRA) that can deal with incomplete perturbation data. Unlike our model-based systems identification, MRA cannot identify the reaction-kinetic mechanistic details of the crosstalks. Nevertheless, MRA allows us to reconstruct the connectivity of the network and compare the result to our model-based inference.

We applied Bayesian MRA (BMRA) on the measured nodes (see Methods for details) and compared the results to the estimated crosstalk parameters a_{ji} , Figure 5. BMRA uses the so-called global response coefficients, that is the measured relative changes of the steady-state responses to a set of perturbations, to infer the connectivity of the network in terms of so-called local response coefficient r_{ji} . A non-zero r_{ji} means node j is directly regulated node i . For technical reasons, BMRA cannot identify direct feedbacks, because the diagonal elements of the local response coefficients have to be normalised $r_{ii} = 1$. Therefore, only off-diagonal interactions (crosstalks) can be compared.

295 The BMRA results were largely concordant with the model-based results; 8 out of 9
 296 crosstalk interactions were estimated to have the same sign for both methods (Fig. 5).
 297 For both methods, p38 was positively regulated by JNK and negatively by AKT, p53
 298 was positively regulated by JNK and AKT and negatively by p38, AKT was positively
 299 regulated by JNK and negatively regulated by p38. The only discrepancy was observed
 300 for the p38-to-JNK crosstalk. JNK was negatively regulated by AKT for both methods,
 301 but in contrast to the model which inferred a positive regulation by p38, BMRA
 302 inferred a negative regulation. This discrepancy for the p38-to-JNK crosstalk might be
 303 explained by the limitations of BMRA, which assumes small perturbations, is sensitive
 304 to measurement noise, and the requires that the responses are measured in steady
 305 state. In contrast, our model can simulate the dynamic time-courses and matches these
 306 experimental data well (Fig. 2). The experimental data show a strong increase of the
 307 dynamic pJNK response under p38 inhibited conditions, which is indicative of a
 308 negative crosstalk. Accordingly, our model-based approach identified a negative
 309 crosstalk from p38 to JNK ($a_{p3} < 0$).

310 **Structural sensitivity analysis established JNK as a master regulator of** 311 **the network response dynamics**

312 Next, we wanted to better understand how the crosstalks and feedbacks control the
 313 dynamic behaviour of system. Therefore, we performed a structural sensitivity
 314 analysis in which each crosstalk and feedback was removed one-by-one, and the such
 315 perturbed model was simulated. In contrast to inhibitor experiments, which block all
 316 outgoing interactions from the inhibited node, this simulation-based approach reveals
 317 the control of each individual crosstalk.

318 Figure 6a shows the simulated responses for each perturbation. Overall, the largest
 319 sensitivities were observed for interactions relating to JNK (Fig 6b). Five of the seven
 320 largest sensitivities were related to JNK (a_{j3} , positive feedback; a_{j5} , a_{p3} ; negative
 321 feedback via p38; a_{a3} , negative crosstalk from AKT; a_{j15} , JNK activating p53). The
 322 remaining two large sensitivities were related to AKT (a_{a5} , negative crosstalk to p38;
 323 a_{j15} , positive crosstalk to p53). These can be interpreted as follows. The JNK positive
 324 feedback amplifies the pJNK response, and also had the greatest impact on p53
 325 activation compared to all other upstream crosstalk parameters. The JNK negative
 326 feedback loop via p38 ($a_{j5} > 1$, $a_{p3} < 0$) serves to attenuate the JNK response while
 327 concomitantly amplifying the p38 response. AKT was not notably controlled by any
 328 crosstalk parameter, while individual AKT-to-JNK and AKT-to-p38 crosstalks served to
 329 attenuate the JNK and p38 responses, respectively. In summary, large sensitivities
 330 were observed for crosstalk parameters regulating JNK or mediating JNK-dependent
 331 effects.

332

333 **Discussion**

334 We have shown that Doxorubicin activates the JNK, p38 and p53 network in MCF10A
 335 cells, and constructed a data-driven mathematical model of these network dynamics.
 336 This model describes the mechanism of the network interactions, and contained free
 337 parameters describing possible interactions between the modelled pathways.
 338 Estimating these parameters explained the experimentally measured time-course
 339 perturbation data and identified the specific crosstalk patterns that control the

340 Doxorubicin response. The identified model shows that that direct JNK positive
341 feedback serves to amplify the JNK doxorubicin response but is tightly controlled by
342 indirect negative feedback via p38 and negative crosstalk from AKT. The role of the
343 indirect negative feedback is to attenuate the JNK response while concomitantly
344 amplifying the p38 response. JNK, not p38, was a major activator of p53, which
345 underlines the importance of controlling the JNK response. AKT also contributed
346 positively to p53 activation. In the following we discuss the relevance of each
347 crosstalk.

348 The most consistent model estimates were positive crosstalk from JNK to p38 and
349 negative crosstalk from p38 to JNK constituting the indirect negative feedback loop,
350 negative crosstalks from AKT to both JNK and p38, and positive crosstalks from JNK
351 and AKT to p53.

352 The negative crosstalks from p38 and AKT to JNK are well supported by the literature
353 and their biochemical mechanisms have been established. Chemical inhibition or
354 knock-out of p38 caused JNK hyperactivation in several contexts, demonstrating that
355 p38 negatively regulates JNK³¹⁻³⁶. Mechanisms concern both posttranslational and
356 transcriptional processes depending on stimulus and include the decreased
357 phosphorylation of MKK4³³ and MKK7 and the upregulation of the JNK dual specificity
358 phosphatase DUSP1³⁶. In single cells, p38 cross inhibition suppresses JNK activation
359 dynamics to ensure the right amount of fractional cell killing³⁷. The inhibition of JNK
360 by AKT is also well established. AKT can phosphorylate and inhibit the JNK upstream
361 kinases MKK4 and MKK7 at S80²³ and T385³, respectively.

362 The activation of p53 by JNK and AKT is also well supported by the literature. JNK can
363 phosphorylate and stabilise p53 at S20³⁸ and T81^{39,40}, and doxorubicin induced JNK
364 mediated p53 activation and cell death in SY5Y cells³. Although p53 can also be
365 phosphorylated by p38 at S33⁴¹⁻⁴³ and S46⁴¹, our p38 inhibition had only minor effects
366 on the p53 response. Accordingly, the identified p38-p53 crosstalk was weak in our
367 model. In contrast, the identified AKT-p53 crosstalk was strong. Literature suggests
368 that this positive crosstalk from AKT to p53 is likely not direct; p53 stabilization in
369 response to DNA damage depends on AKT mediated phosphorylation and inactivation
370 of GSK3 that normally phosphorylates and inactivates MDM2⁴⁴. Thus, AKT activity
371 inactivates GSK3 and MDM2 thereby preventing p53 protein degradation and
372 facilitating p53 accumulation. Summarizing, these observations suggest that a direct
373 p38-p53 crosstalk plays a minor role in the doxorubicin response, and that p53
374 activation is mainly controlled by crosstalk from JNK and AKT.

375 The identified positive crosstalk from JNK to p38 positive is novel, and no potential
376 mechanisms have been reported. However, JNK can exhibit a positive feedback by
377 phosphorylating MKK7 at T66 and T83, thereby enhancing its own activation³.
378 Considering that the p38 upstream kinases MKK3 and MKK6 are structurally very
379 similar to MKK7, is conceivable that JNK might phosphorylate p38 upstream kinases
380 MKK3 and MKK6, thereby enhancing the activation of these kinases. Another possible
381 mechanism for mediating crosstalks are dual specificity phosphatases (DUSPs) that
382 are often induced by JNK and/or p38 signalling and that can act as negative and
383 positive regulators⁴. An example for the latter is DUSP22 which has been shown to
384 enhance JNK activation⁴⁵.

385 In contrast to the network interactions discussed above, the estimates of the JNK
386 feedback were more variable and could take positive or negative values. However, all
387 six best fitting model exhibited positive feedback with low half activation constants. In
388 contrast, estimates that featured a negative feedback strength exhibited large values
389 for the half activation, thus diminishing the negative effect. Further, JNK positive
390 feedback under stress conditions is consistent with the literature. JNK positive
391 feedback was observed under many stress conditions and cell types^{4,14,46-50}.
392 Concerning the mechanism, experiments in SY5Y cells have shown that JNK
393 phosphorylates its upstream activator MKK7 at the T66 site (that flanks the D domain
394 responsible for substrate docking), which increased JNK phosphorylation at the
395 catalytic site (probably by relieving a N-terminal auto-inhibitory domain). These
396 literature data corroborate the results of best fitting models and the identification a
397 JNK positive feedback.

398 Controlling the JNK activation dynamics seems particularly important considering that
399 JNK activation can exhibit two phases; an early, transient phase that promotes cell
400 survival and a late, sustained phase that promotes apoptosis^{4,51,52}. In response to cell
401 stress, such as doxorubicin, the proapoptotic activation of JNK represent a critical
402 signalling mode. The emerging picture is that proapoptotic JNK signalling is controlled
403 by many loops. Once activated, the JNK positive feedback promotes apoptosis. It makes
404 sense that this positive loop must be tightly controlled and kept in check by negative
405 feedback loops and crosstalks. In this context the function of p38 and AKT signalling is
406 attenuate the JNK response and control the levels of cell death^{3,37}. For example, in
407 single cells, cross-inhibition of JNK by p38 lead to right amount of fractional cell killing,
408 while blockade of p38 signalling lead to hyperactivation of JNK in all cells and
409 eradication of the entire cell population³⁷.

410 The developed model-based approach for systems identification has several
411 advantages over Modular Response Analysis and similar approaches^{24-26,53-55}. In
412 contrast to standard network inference method, the model-based approach describes
413 the biochemical mechanisms of the identified network interactions and can be used to
414 analyse the dynamics of the system and its control. Applying BMRA to our data yielded
415 very similar results in terms of the identified network structure. However, BMRA
416 infers these interactions from steady-state data, assumes small perturbations, does not
417 yield a mechanistic dynamic model, and can thus not explain the system's activation
418 dynamics. In contrast our model-based approach focusses on gaining mechanistic
419 insight and explaining the system dynamics. That the shape of the JNK response
420 dynamics is important was observed previously, where the steepness of the pJNK
421 dose-response was prognostic of patient survival³. Similar observations were made for
422 the p53 activation dynamics^{1,5,56-58}. Rapid activation of p53 leading to higher p53
423 amplitudes promotes cell death⁵⁷. In those time-dependent, highly dynamic situations
424 a model-based approach is more appropriate.

425 The model also suggests optimal designs for follow up experiments. The estimates of
426 the JNK feedback were particularly varied, which could be resolved by measuring the
427 upstream kinases MKK4 and MKK7 to estimate the exact feedback strength. The JNK
428 and p38 crosstalks could activate at either low or high pJNK and pp38 concentrations,
429 which could be resolved by recording a dose response to precisely estimate the half-
430 activation constants. These model-based experimental designs could be used in follow
431 up-experiments to remove some of the observed parameter uncertainties. The model

could also be used to identify and explain crosstalks for other drugs and cell types. An interesting open question is how context specific these crosstalks are and whether they change depending on cell type and drug stimulus^{59,60}. Generally, the developed model-based systems identification approach could also be applied to other models and crosstalk systems. Considering that a dynamic model pJNK responses was prognostic of patient survival³, it would also be interesting to test whether our model can predict patient-specific response to doxorubicin containing chemotherapy, for example in breast cancer. An interesting question is whether the pJNK response alone or in combination with p38 and p53, is predictive of clinical drug-responses and/or prognostic of relapse and patient survival.

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Author Contributions

Conceptualisation: LT and DF; Methodology: LT, MH and DF; Investigation: LT and DF; Formal Analysis: LT and DF; Data curation: DF; Resources: MH and DF; Writing – original draft: LT; Writing – review and editing: MH and DF; Funding Acquisition: LT and DF; Supervision: MH and DF.

Competing interest

The authors declare no competing financial or non-financial interests.

Fig 1: Scheme of the stress and DNA-damage response model. The black arrows

460 indicate biochemical reactions (phosphorylation, dephosphorylation, synthesis and
461 degradation). The grey arrows indicate known canonical pathway interactions from
462 the literature. Coloured lines indicate potential crosstalks (activation or inhibition)
463 from phosphorylated JNK (pJNK), phosphorylated p38 (pp38), and phosphorylated
464 AKT (pAKT) onto the other pathways as indicated in green, red, and orange,
465 accordingly. Numbers on the reactions correspond to the rate laws in Table 1. Red
466 arrows indicate inhibitors for JNK, p38 and PI3K/AKT.

467
468
469 **Fig 2: Model fit to the experimental data after the final round of parameter**
470 **estimation.** Lines represent simulations as indicated, circles and error-bars
471 experimental data (mean and standard error of the mean) from three independent
472 experiments. Colours indicate experimental conditions as follows: Doxorubicin
473 treatment alone (control, blue), or in combination with JNK inhibitor (SP600125 at
474 20 μ M, red), p38 inhibitor (SB203580 at 100 μ M, yellow) or PI3K inhibitor (LY294002
475 at 10nM, purple). Shown are the trajectories over time after Doxorubicin treatment at
476 $t=0$ h for phosphorylated JNK (pJNK), phosphorylated p38 (pp38), phosphorylated AKT
477 (pAKT), and total p53 (p53t).

Fig 3: Identified interactions (a) Interaction map of based on the best fitting estimate. Black arrows represent phosphorylation and dephosphorylation of the measured model components. The coloured lines indicate the estimated crosstalks, with the type of the arrowhead indicating the sign of the interaction; $\rightarrow$ for activation $\log_{10} a_{ji} > 0$, $\rightarrow$ for inhibition $\log_{10} a_{ji} < 0$. Line thickness is proportional to the strength of the estimated crosstalks $|a_{ji}|$. Large arrowheads indicate $K_{ji} < 0.1$, small arrowheads $K_{ji} > 0.1$. (b) Distribution of the crosstalk parameter estimates. Each blue dot represents a parameter estimate from n=100 estimation runs. The red dot indicates the parameter estimate with the best goodness-of-fit value. x-axis is on the log10 scale.

Fig 4: Parameter correlations based on n=100 estimates. (a) Heatmap of the Pearson correlation coefficients for parameter estimates of the crosstalk parameters from the last round of parameter estimation. (b) Scatterplot of a_{j3} versus a_{p3} , and (c) a_{j5} versus a_{p5} . C denotes the Pearson correlation coefficient.

Fig 5: Comparison of the model-based systems identification result with Bayesian Modular response analysis. Each circle represents a crosstalk interaction from the node on the x-axis to the node on the y-axis. (a) Model-based result. The color of the circle represent the sign and strength of the interaction a_{ji} as indicated in the colorbar legend, the size the activation threshold K_{ji} . (b) BMRA result. The color represent the sign and size of the estimated local response coefficient r_{ji} , the size the associated estimated probability of a nonzero interaction.

Fig 6: Structural sensitivity analysis of the model. Each interaction in the model was removed one by one and the model was simulated for all parameter estimates. (a) The simulated trajectories for the unperturbed and perturbed models as indicated for all 100 parameter estimates. For the unperturbed model, the experimental data are shown with dot and errorbars representing mean and standard error. (b) Calculated sensitivity coefficients for a subset of the perturbations as indicated for the six best fitting parameter estimates. Red dots represent the best fitting model.

515

516 **STAR Methods**

517 **Lead Contact and Materials Availability**

518 Further information and requests for resources and reagents should be directed to and
519 will be fulfilled by the Lead Contact, Dirk Fey (dirk.fey@ucd.ie). This study did not
520 generate any new unique reagents.

521 **Experimental Model and subject details**

522 **Cell culture**

523 The MCF10A non-tumorigenic mammary epithelial cell line was cultured in DMEM F-
524 12 (Gibco, Cat. No. 11330032) supplemented with 5% heat inactivated horse serum
525 (Gibco, Thermo Fisher Scientific, Cat. No. 16050122), 20µg/L of EGF (PeproTech, Cat.
526 No. 100-47), 500ng/L of hydrocortisone (Sigma-Aldrich, Cat. No. H4001), 100µg of
527 cholera toxin (Sigma-Aldrich, Cat. No. C8052 SIGMA) and 10mg/mL of insulin (Sigma-
528 Aldrich, Cat. No. 91077C). The inhibitors used were: JNK inhibitor (20µM SP00125;
529 SelleckChem, Cat. No. S1460), p38 inhibitor (10nM SB203580; Calbiochem, Cat. No.
530 S1105) and PI3K inhibitor (10µM LY294002; SelleckChem, Cat. No. S2929).

531 **Method Details**

532 **Western blotting**

533 Western blots were prepared from cell lysates after using the following lysis buffer:
534 20mM tris-HCl (pH 7.5), 150 mM NaCl, and 1% Triton X-100 and 1mM MgCl₂. The lysis
535 buffer was complemented with cOMplete Mini Protease Inhibitor Cocktail (Roche, Cat.
536 No. 11 836153 001) and PhosSTOP Phosphatase Inhibitor Cocktail (Roche, Cat. No.
537 4906837001). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western
538 blotting were performed using 10% acrylamide gels with the Mini-PROTEAN® Tetra
539 Cell Systems from Bio-Rad. SuperSignal Femto Chemiluminescent Substrate (Thermo
540 Scientific, Cat. No. 34095) was used to develop Western blots, which were imaged
541 using the Advanced Molecular Vision Chemiluminescence Imaging System.
542 Quantitative Western blotting was performed using multi-strip Western blotting⁶¹. The
543 antibodies used for Western blotting were from Cell Signalling Technology: p-JNK
544 (Thr183/Tyr185, Cat. No. 9251), total JNK (Cat. No. 9252), p-P38 (Thr180/Tyr182,
545 Cat. No. 9211), total p38 (Cat. No. 9212), total p53 (Cat. No. 9282), p-AKT (Ser473, Cat.
546 No. 9271), total AKT (Cat. No. 9272), p-MAPKAPK2 (Thr334, Cat. No. 3041), GAPDH
547 (Cat. No. 2118).

548 **Quantification and statistical analysis**

549 **Quantification and normalisation of time-course data**

550 Western blots were quantified using the ImageJ software (version 1.51j8)⁶². Three
551 pAKT datapoints diverged substantially from the others and have been excluded as
552 outliers and have not been quantified. These are: Replicate 3, 3h control (Fig S2C);
553 Replicate 4, 4h control and 4h JNK inhibitor (Fig S2D). Data normalisation: First, the
554 quantified Western blot data were normalised by dividing the measured values with
555 the GAPDH loading control. Second, to have comparable values between the different
556 inhibitor experiments the phosphorylation time courses were aligned using sum-
557 normalisation as explained previously⁶³. The reference time-course for this sum-
558 normalisation was the measured Doxorubicin time course in the absence of inhibitors
559 (hereafter referred to as Doxorubicin control), which was recorded in all experiments

560 and therefore a suitable reference. Consequently, for each experiment, all recoded
561 values were divided by the sum of the Doxorubicin control time course⁶³. We also
562 assumed that the phosphorylation levels before Doxorubicin treatment are negligible
563 and subtracted the value of the zero time point from all the other values of the
564 measured time course. Next, to obtain values between zero and one for each species,
565 we normalised by dividing with the maximum value of the species over all the
566 experiments and conditions. These zero-one normalised datasets have been used for
567 the parameter estimation of the model. The complete set of numerical values for these
568 data can be found in the PEPSSBI file (Suppl. File 1)

569 Calculating the steady state initial conditions

570 The steady-state initial condition prior to Doxorubicin treatment was calculated as
571 follows. From $doxo = 0$ follows that all phosphorylated protein concentrations are
572 zero and therewith that all phosphorylation and dephosphorylation rates are zero. In
573 particular, with $v16 = v17 = 0$ and $pp53 = 0$ the ODEs for p53 and MDM2 become

$$\begin{aligned}\frac{d}{dt}p53 &= k17 - k19 p53 \frac{Km19^2}{Km19^2 + MDM2^2}, \\ \frac{d}{dt}MDM2 &= k18 \frac{p53^2}{p53^2 + Km18} - k21 MDM2,\end{aligned}$$

574 see Table 1. Solving for the steady-state $\frac{d}{dt}p53 = 0, \frac{d}{dt}MDM2 = 0$ yields

$$\begin{aligned}k17 &= k19 p53 \frac{Km19^2}{Km19^2 + \overline{MDM2}^2}, \\ k18 &= k21 \frac{\overline{MDM2}(p53^2 + Km18)}{p53^2}\end{aligned}$$

575 where we assume $\overline{MDM2} = 0.1$ for the MDM2 steady-state and $\overline{p53} = 0.05$ for the p53
576 steady-state based on our measured data for $doxo = 0$.

577 Similarly, we calculated the non-zero PI3K and AKT steady states by setting
578 $v7 - v8 = 0$ and $v13 - v14 = 0$ and using the conserved moieties for the
579 phosphorylation levels ($AKT + pAKT = 1, PI3K + pPI3K = 1$), yielding

$$\begin{aligned}k8 &= \frac{(\overline{pPI3K0} + Km8) * k7 * \overline{PI3K0}}{(\overline{PI3K0} + Km7) * \overline{pPI3K0}} \\ k14 &= \frac{LY * k13 * \overline{pPI3K0} * \overline{AKT0} * (\overline{pAKT0} + Km14)}{(\overline{AKT0} + Km13) * \overline{pAKT0}}\end{aligned}$$

580 with

$$\begin{aligned}\overline{PI3K0} &= 1 - \overline{pPI3K0}, \\ \overline{AKT0} &= 1 - \overline{pAKT0}\end{aligned}$$

582 where $\overline{pPI3K0}$, $\overline{PI3K0}$, $\overline{pAKT0}$ and $\overline{AKT0}$ denote the initial steady-state values of
583 pPI3K, PI3K, pAKT and AKT, correspondingly. All other parameters as defined in Table
584 1. $\overline{pPI3K0}$ was set to 0.5 and $\overline{pAKT}$ to 0.4, 0.4, 0.28 and 0.23 for the control, JNK
585 inhibited, p38 inhibited and AKT inhibited conditions, correspondingly, according to
586 our measured pAKT data.

Parameter estimation and parameter bounds

Parameters were estimated using the *Parameter Estimation Pipeline for Systems and Synthetic Biology* (PEPSSBI) minimising the sum-of-squares objective function with the GSLDC global optimisation algorithm²⁹. Lower and upper parameter bounds were specified based on biologically reasonable ranges and information from the literature as follows. For phosphorylation reactions we estimated the rate constant in the range $k \in [0.001, 10]$, and for dephosphorylation in the range $k \in [0.0001, 10000]$. All K_m 's were estimated in the range $K_m \in [0.1, 2]$. The MDM2 half-life is about 30 minutes⁶⁴. Using the half-life equation $k = \frac{\log(2)}{T_{1/2}}$, where $T_{1/2}$ denotes the half-life and k the degradation rate constant, we fixed the MDM2 degradation rate constant at $k_{21} = 1.38 \text{ 1/h}$. The p53 half-life in unstimulated, unphosphorylated conditions is about 5 minutes and increases several-fold when stimulated and phosphorylated⁶⁵. Accordingly we allowed for very rapid MDM2 mediated degradation of p53 within the parameter bound $k_{19} \in [8, 40]$, followed by a 10 to 100 fold decrease in degradation rate upon p53 phosphorylation using the parameter bound $k_{deg2} \in [0.01, 0.1]$. For the bounds of the crosstalks parameters we allowed positive or negative effects up to a 100-fold change $a_{ji} \in [0.01, 100]$. The complete parameter estimation setup including experimental data, data normalisation, parameters and parameter bounds, equations and fitting options are specified in the PEPSSBI file (Suppl. File 1).

Bayesian modular response analysis

The values of the local response coefficients representing the high-level network structure and their probabilities were estimated using BMRA [REF]. Global response coefficients were calculated according to the

$$R(t)_{ji} = y(t)_{ji} - y(t)_j,$$

where R_{ji} denotes the global response coefficient of node $j \in \{pJNK, pp38, pAKT, p53\}$ to perturbation $i \in \{JNK, p38, AKT\}$ (doxorubicin + inhibition of JNK, p38, or AKT), y_{ij} the measured response of node j to perturbation i , y_j the measured response of node j in control conditions (doxorubicin), and $t \in \{5h, 6h\}$ the timepoint used. Because several replicate measurements were available, the mean was used $y_{ji} = \frac{1}{n} \sum_{k=1}^n y_{ijk}$, which improves the accuracy of the MRA estimates [REF]. Further, it was assumed that both the 5h and 6h time-points were steady state. Both timepoints were used as observations for BRMA, yielding a 4×6 global response matrix $[R(5h)_{ji} \ R(6h)_{ji}]$, and increasing the precision of the BMRA estimates. The following hyper-parameters were used number of Gibbs samples: noit=10,000, burn-in: burnin=5,000, number of repeats: times=10. The code is provided in Suppl. File 1.

Structural sensitivity analysis

Structural sensitivity analysis was performed in Matlab. Each crosstalk was removed one at a time by setting $\log a_{ji} = 0$. The such perturbed system was simulated and compared to the unperturbed simulation. The sensitivity coefficients were calculated at $t = 10h$ approximating steady-state using

$$S(j) = \log_2 \frac{y_{j,perturbed} - y_j}{y_j},$$

where $S(j)$ denotes the sensitivity of node $j \in \{pJNK, pp38, pAKT, p53\}$, $y_{j,perturbed}$ the value of the simulated perturbed trajectory of node j at timepoint $t = 10h$, and y_j the value of the simulated unperturbed trajectory of node j at timepoint $t = 10h$. The code is available in Suppl. File 1.

Data and code availability

The authors declare that the data supporting the findings of this study (including mathematical models, experimental data, and parameter estimates) are available within the paper and its supplementary information files (model code: PEPSSBI files, raw images: png files; quantification: Excel table; parameters: Excel table). In addition, the calibrated best-fitting model will be available from the EMBL-EBI BioModels repository (<http://www.ebi.ac.uk/biomodels/>) under accession number [to be obtained].

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References

- 1 Kolch, W., Halasz, M., Granovskaya, M. & Kholodenko, B. N. The dynamic control of signal transduction networks in cancer cells. *Nature reviews. Cancer* **15**, 515-527, doi:10.1038/nrc3983 (2015).
- 2 Ryu, H. *et al.* Frequency modulation of ERK activation dynamics rewires cell fate. *Molecular systems biology* **12**, 866, doi:10.15252/msb.20166982 (2016).
- 3 Fey, D. *et al.* Signaling pathway models as biomarkers: Patient-specific simulations of JNK activity predict the survival of neuroblastoma patients. *Science signaling* **8**, ra130, doi:10.1126/scisignal.aab0990 (2015).
- 4 Fey, D., Croucher, D. R., Kolch, W. & Kholodenko, B. N. Crosstalk and signaling switches in mitogen-activated protein kinase cascades. *Frontiers in physiology* **3**, 355, doi:10.3389/fphys.2012.00355 (2012).
- 5 Hafner, A., Bulyk, M. L., Jambhekar, A. & Lahav, G. The multiple mechanisms that regulate p53 activity and cell fate. *Nat Rev Mol Cell Biol*, doi:10.1038/s41580-019-0110-x (2019).
- 6 Coffey, E. T. Nuclear and cytosolic JNK signalling in neurons. *Nat Rev Neurosci* **15**, 285-299, doi:10.1038/nrn3729 (2014).
- 7 Stramucci, L., Pranteda, A. & Bossi, G. Insights of Crosstalk between p53 Protein and the MKK3/MKK6/p38 MAPK Signaling Pathway in Cancer. *Cancers (Basel)* **10**, doi:10.3390/cancers10050131 (2018).
- 8 Zeke, A., Misheva, M., Remenyi, A. & Bogoyevitch, M. A. JNK Signaling: Regulation and Functions Based on Complex Protein-Protein Partnerships. *Microbiol Mol Biol Rev* **80**, 793-835, doi:10.1128/MMBR.00043-14 (2016).
- 9 Zou, X. & Blank, M. Targeting p38 MAP kinase signaling in cancer through post-translational modifications. *Cancer Lett* **384**, 19-26, doi:10.1016/j.canlet.2016.10.008 (2017).
- 10 Avan, A., Narayan, R., Giovannetti, E. & Peters, G. J. Role of Akt signaling in resistance to DNA-targeted therapy. *World J Clin Oncol* **7**, 352-369, doi:10.5306/wjco.v7.i5.352 (2016).
- 11 Song, M., Bode, A. M., Dong, Z. & Lee, M. H. AKT as a Therapeutic Target for Cancer. *Cancer Res*, doi:10.1158/0008-5472.CAN-18-2738 (2019).
- 12 Rivankar, S. An overview of doxorubicin formulations in cancer therapy. *J Cancer Res Ther* **10**, 853-858, doi:10.4103/0973-1482.139267 (2014).
- 13 Chen, C. *et al.* Autophagy and doxorubicin resistance in cancer. *Anticancer Drugs* **29**, 1-9, doi:10.1097/CAD.0000000000000572 (2018).

676 14 Fey D, C. D. R., Kolch W, Kholodenko B N. Crosstalk and signaling switches in
677 mitogen-activated protein kinase cascades. *Frontiers in physiology* (2012).

678 15 Zhang, X. P., Liu, F. & Wang, W. Two-phase dynamics of p53 in the DNA damage
679 response. *Proceedings of the National Academy of Sciences of the United States*
680 *of America* **108**, 8990-8995, doi:10.1073/pnas.1100600108 (2011).

681 16 Moll U M, P. O. The MDM2-p53 Interaction. *Molecular Cancer Research* (2003).

682 17 Perry, M. E. The regulation of the p53-mediated stress response by MDM2 and
683 MDM4. *Cold Spring Harb Perspect Biol* **2**, a000968,
684 doi:10.1101/cshperspect.a000968 (2010).

685 18 Fey, D., Dobrzynski, M. & Kholodenko, B. N. in *Quantitative Biology: Theory,*
686 *Computational Methods and Examples of Models* Vol. 1st edition (eds Brian
687 Munsky, William Hlavacek, & Lev Tsimring) 19-47 (MIT Press, 2018).

688 19 Bennett, B. L. *et al.* SP600125, an anthrapyrazolone inhibitor of Jun N-terminal
689 kinase. *Proceedings of the National Academy of Sciences of the United States of*
690 *America* **98**, 13681-13686, doi:10.1073/pnas.251194298 (2001).

691 20 Cuenda, A. *et al.* SB 203580 is a specific inhibitor of a MAP kinase homologue
692 which is stimulated by cellular stresses and interleukin-1. *FEBS Lett* **364**, 229-
693 233, doi:10.1016/0014-5793(95)00357-f (1995).

694 21 Vlahos, C. J., Matter, W. F., Hui, K. Y. & Brown, R. F. A specific inhibitor of
695 phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-
696 4-one (LY294002). *J Biol Chem* **269**, 5241-5248 (1994).

697 22 Kumar, S., Jiang, M. S., Adams, J. L. & Lee, J. C. Pyridinylimidazole compound SB
698 203580 inhibits the activity but not the activation of p38 mitogen-activated
699 protein kinase. *Biochem Biophys Res Commun* **263**, 825-831,
700 doi:10.1006/bbrc.1999.1454 (1999).

701 23 Park, H. S. *et al.* Akt (protein kinase B) negatively regulates SEK1 by means of
702 protein phosphorylation. *J Biol Chem* **277**, 2573-2578,
703 doi:10.1074/jbc.M110299200 (2002).

704 24 Kholodenko, B. N. *et al.* Untangling the wires: a strategy to trace functional
705 interactions in signaling and gene networks. *Proceedings of the National*
706 *Academy of Sciences of the United States of America* **99**, 12841-12846,
707 doi:10.1073/pnas.192442699 (2002).

708 25 Bastiaens, P. *et al.* Silence on the relevant literature and errors in
709 implementation. *Nat Biotechnol* **33**, 336-339, doi:10.1038/nbt.3185 (2015).

710 26 Klinger, B. & Bluthgen, N. Reverse engineering gene regulatory networks by
711 modular response analysis - a benchmark. *Essays Biochem* **62**, 535-547,
712 doi:10.1042/EBC20180012 (2018).

713 27 Lill, D. *et al.* Mapping connections in signaling networks with ambiguous
714 modularity. *NPJ Syst Biol Appl* **5**, 19, doi:10.1038/s41540-019-0096-1 (2019).

715 28 Thomaseth, C. *et al.* Impact of measurement noise, experimental design, and
716 estimation methods on Modular Response Analysis based network
717 reconstruction. *Sci Rep* **8**, 16217, doi:10.1038/s41598-018-34353-3 (2018).

718 29 A, D., D, F. & N, K. B. Performance of objective functions and optimisation
719 procedures for parameter estimation in system biology models. (2017).

720 30 Degasperi, A., Fey, D. & Kholodenko, B. N. Performance of objective functions
721 and optimisation procedures for parameter estimation in system biology
722 models. *NPJ Syst Biol Appl* **3**, 20, doi:10.1038/s41540-017-0023-2 (2017).

723 31 Caballero-Franco, C. *et al.* Tuning of protein kinase circuitry by p38alpha is
724 vital for epithelial tissue homeostasis. *J Biol Chem* **288**, 23788-23797,
725 doi:10.1074/jbc.M113.452029 (2013).

726 32 Cheung, P. C., Campbell, D. G., Nebreda, A. R. & Cohen, P. Feedback control of
727 the protein kinase TAK1 by SAPK2a/p38alpha. *EMBO J* **22**, 5793-5805,
728 doi:10.1093/emboj/cdg552 (2003).

729 33 Heinrichsdorff, J., Luedde, T., Perdiguero, E., Nebreda, A. R. & Pasparakis, M.
730 p38 alpha MAPK inhibits JNK activation and collaborates with IκB kinase
731 2 to prevent endotoxin-induced liver failure. *EMBO Rep* **9**, 1048-1054,
732 doi:10.1038/embo.2008.149 (2008).

733 34 Hui, L. *et al.* p38alpha suppresses normal and cancer cell proliferation by
734 antagonizing the JNK-c-Jun pathway. *Nat Genet* **39**, 741-749,
735 doi:10.1038/ng2033 (2007).

736 35 Pereira, L., Igea, A., Canovas, B., Dolado, I. & Nebreda, A. R. Inhibition of p38
737 MAPK sensitizes tumour cells to cisplatin-induced apoptosis mediated by
738 reactive oxygen species and JNK. *EMBO Mol Med* **5**, 1759-1774,
739 doi:10.1002/emmm.201302732 (2013).

740 36 Staples, C. J., Owens, D. M., Maier, J. V., Cato, A. C. & Keyse, S. M. Cross-talk
741 between the p38alpha and JNK MAPK pathways mediated by MAP kinase
742 phosphatase-1 determines cellular sensitivity to UV radiation. *J Biol Chem* **285**,
743 25928-25940, doi:10.1074/jbc.M110.117911 (2010).

744 37 Miura, H., Kondo, Y., Matsuda, M. & Aoki, K. Cell-to-Cell Heterogeneity in p38-
745 Mediated Cross-Inhibition of JNK Causes Stochastic Cell Death. *Cell Rep* **24**,
746 2658-2668, doi:10.1016/j.celrep.2018.08.020 (2018).

747 38 She, Q. B., Ma, W. Y. & Dong, Z. Role of MAP kinases in UVB-induced
748 phosphorylation of p53 at serine 20. *Oncogene* **21**, 1580-1589,
749 doi:10.1038/sj.onc.1205239 (2002).

750 39 Buschmann, T. *et al.* Jun NH2-terminal kinase phosphorylation of p53 on Thr-
751 81 is important for p53 stabilization and transcriptional activities in response
752 to stress. *Mol Cell Biol* **21**, 2743-2754, doi:10.1128/MCB.21.8.2743-2754.2001
753 (2001).

754 40 Pluquet, O. *et al.* The cytoprotective aminothiol WR1065 activates p53 through
755 a non-genotoxic signaling pathway involving c-Jun N-terminal kinase. *J Biol*
756 *Chem* **278**, 11879-11887, doi:10.1074/jbc.M207396200 (2003).

757 41 Bulavin, D. V. *et al.* Phosphorylation of human p53 by p38 kinase coordinates
758 N-terminal phosphorylation and apoptosis in response to UV radiation. *EMBO J*
759 **18**, 6845-6854, doi:10.1093/emboj/18.23.6845 (1999).

760 42 Kishi, H. *et al.* Osmotic shock induces G1 arrest through p53 phosphorylation at
761 Ser33 by activated p38MAPK without phosphorylation at Ser15 and Ser20. *J*
762 *Biol Chem* **276**, 39115-39122, doi:10.1074/jbc.M105134200 (2001).

763 43 Takekawa, M. *et al.* p53-inducible wip1 phosphatase mediates a negative
764 feedback regulation of p38 MAPK-p53 signaling in response to UV radiation.
765 *EMBO J* **19**, 6517-6526, doi:10.1093/emboj/19.23.6517 (2000).

766 44 Boehme, K. A., Kulikov, R. & Blattner, C. p53 stabilization in response to DNA
767 damage requires Akt/PKB and DNA-PK. *Proceedings of the National Academy of*
768 *Sciences of the United States of America* **105**, 7785-7790,
769 doi:10.1073/pnas.0703423105 (2008).

770 45 Chen, A. J. *et al.* The dual specificity JCAP specifically activates the c-Jun N-
771 terminal kinase pathway. *J Biol Chem* **277**, 36592-36601,
772 doi:10.1074/jbc.M200453200 (2002).

773 46 Bagowski, C. P. & Ferrell, J. E., Jr. Bistability in the JNK cascade. *Current biology :*
774 *CB* **11**, 1176-1182, doi:10.1016/s0960-9822(01)00330-x (2001).

775 47 Furuhashi, M., Takada, E., Noguchi, T., Ichijo, H. & Mizuguchi, J. Apoptosis signal-
776 regulating kinase (ASK)-1 mediates apoptosis through activation of JNK1

777 following engagement of membrane immunoglobulin. *Exp Cell Res* **315**, 3467-
778 3476, doi:10.1016/j.yexcr.2009.09.007 (2009).

779 48 Hirata, Y. *et al.* trans-Fatty acids facilitate DNA damage-induced apoptosis
780 through the mitochondrial JNK-Sab-ROS positive feedback loop. *Sci Rep* **10**,
781 2743, doi:10.1038/s41598-020-59636-6 (2020).

782 49 Schachter, K. A., Du, Y., Lin, A. & Gallo, K. A. Dynamic positive feedback
783 phosphorylation of mixed lineage kinase 3 by JNK reversibly regulates its
784 distribution to Triton-soluble domains. *J Biol Chem* **281**, 19134-19144,
785 doi:10.1074/jbc.M603324200 (2006).

786 50 Shlevkov, E. & Morata, G. A dp53/JNK-dependant feedback amplification loop
787 is essential for the apoptotic response to stress in *Drosophila*. *Cell Death Differ*
788 **19**, 451-460, doi:10.1038/cdd.2011.113 (2012).

789 51 Solinas, G. & Becattini, B. JNK at the crossroad of obesity, insulin resistance, and
790 cell stress response. *Mol Metab* **6**, 174-184, doi:10.1016/j.molmet.2016.12.001
791 (2017).

792 52 Brown, M. *et al.* An initial phase of JNK activation inhibits cell death early in the
793 endoplasmic reticulum stress response. *J Cell Sci* **129**, 2317-2328,
794 doi:10.1242/jcs.179127 (2016).

795 53 Andrec, M., Kholodenko, B. N., Levy, R. M. & Sontag, E. Inference of signaling
796 and gene regulatory networks by steady-state perturbation experiments:
797 structure and accuracy. *Journal of theoretical biology* **232**, 427-441,
798 doi:10.1016/j.jtbi.2004.08.022 (2005).

799 54 Santra, T., Kolch, W. & Kholodenko, B. N. Integrating Bayesian variable
800 selection with Modular Response Analysis to infer biochemical network
801 topology. *BMC Syst Biol* **7**, 57, doi:10.1186/1752-0509-7-57 (2013).

802 55 Dorel, M. *et al.* Modelling signalling networks from perturbation data.
803 *Bioinformatics* **34**, 4079-4086, doi:10.1093/bioinformatics/bty473 (2018).

804 56 Chen, S. H., Forrester, W. & Lahav, G. Schedule-dependent interaction between
805 anticancer treatments. *Science* **351**, 1204-1208, doi:10.1126/science.aac5610
806 (2016).

807 57 Paek, A. L., Liu, J. C., Loewer, A., Forrester, W. C. & Lahav, G. Cell-to-Cell
808 Variation in p53 Dynamics Leads to Fractional Killing. *Cell* **165**, 631-642,
809 doi:10.1016/j.cell.2016.03.025 (2016).

810 58 Stewart-Ornstein, J. & Lahav, G. p53 dynamics in response to DNA damage vary
811 across cell lines and are shaped by efficiency of DNA repair and activity of the
812 kinase ATM. *Science signaling* **10**, doi:10.1126/scisignal.aah6671 (2017).

813 59 Adlung, L. *et al.* Protein abundance of AKT and ERK pathway components
814 governs cell type-specific regulation of proliferation. *Molecular systems biology*
815 **13**, 904, doi:10.15252/msb.20167258 (2017).

816 60 Frohlich, F. *et al.* Efficient Parameter Estimation Enables the Prediction of Drug
817 Response Using a Mechanistic Pan-Cancer Pathway Model. *Cell Syst* **7**, 567-579
818 e566, doi:10.1016/j.cels.2018.10.013 (2018).

819 61 Aksamitiene, E., Hoek, J. B. & Kiyatkin, A. Multistrip Western blotting: a tool for
820 comparative quantitative analysis of multiple proteins. *Methods Mol Biol* **1312**,
821 197-226, doi:10.1007/978-1-4939-2694-7_23 (2015).

822 62 Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years
823 of image analysis. *Nat Methods* **9**, 671-675 (2012).

824 63 Degasper, A. *et al.* Evaluating strategies to normalise biological replicates of
825 Western blot data. *PLoS One* **9**, e87293, doi:10.1371/journal.pone.0087293
826 (2014).

827 64 Peng, Y. *et al.* Stabilization of the MDM2 oncoprotein by mutant p53. *J Biol*
828 *Chem* **276**, 6874-6878, doi:10.1074/jbc.C000781200 (2001).

829 65 Tan, B. X., Khoo, K. H., Lim, T. M. & Lane, D. P. High Mdm4 levels suppress p53
830 activity and enhance its half-life in acute myeloid leukaemia. *Oncotarget* **5**,
831 933-943, doi:10.18632/oncotarget.1559 (2014).
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835 **Supplementary figure S1: Western blot of phospho-MAPKAPK2 to verify the**
836 **effect of p38 inhibitor.** MCF10A have been treated with 1μM Doxorubicin after a 30
837 min pre-treatment of SB203580 at 100μM. Shown are two of the 6 replicates used for
838 calculating the SB203580 inhibition factor.

839 **Supplementary figure S2(a): Western blotting of experiment number 1.** MCF10A
840 cells were plated at 75.000 cell/mL, pre-treated with 20μM JNK inhibitor (SP600125)
841 for 30 minutes and treated with 1μM Doxorubicin for 0-0.25-0.5-0.75-1-2-3-4-5-6-7-8
842 hours. The Western blots were performed on the whole cell lysate using the following
843 antibodies: p-JNK (Thr183/Tyr185), total JNK, p-p38 (Thr180/Thy182), total p38,
844 total p53 and GADPH.

845 **Supplementary figure S2(b): Western blotting of experiment number 2.** MCF10A
846 cells were plated at 75.000 cell/mL, pre-treated with 20μM JNK inhibitor (SP600125),
847 or 100μM p38 inhibitor (SB23580) for 30 minutes, and treated with 1μM Doxorubicin
848 for 0-2-3-4-5-6 hours. The Western blots were performed on the whole cell lysate
849 using the following antibodies: p-JNK (Thr183/Tyr185), total JNK, p-p38
850 (Thr180/Thy182), total p38, total p53, p-AKT (Ser473), total AKT and GADPH.

851 **Supplementary figure S2(c): Western blotting of experiment number 3.** MCF10A
852 cells were plated at 75.000 cell/mL, pre-treated with 20μM JNK inhibitor (SP600125),
853 or 100μM p38 inhibitor (SB23580) for 30 minutes, and treated with 1μM Doxorubicin
854 for 0-2-3-4-5-6 hours. The Western blots were performed on the whole cell lysate
855 using the following antibodies: p-JNK (Thr183/Tyr185), total JNK, p-p38
856 (Thr180/Thy182), total p38, total p53, p-AKT (Ser473), total AKT and GADPH.

857 **Supplementary figure S2(d): Western blotting of experiment number 4.** MCF10A
858 cells were plated at 75.000 cell/mL, pre-treated with 20μM JNK inhibitor (SP600125),
859 or 100μM p38 inhibitor (SB23580) for 30 minutes, and treated with 1μM Doxorubicin
860 for 0-2-3-4-5-6 hours. The Western blots were performed on the whole cell lysate
861 using the following antibodies: p-JNK (Thr183/Tyr185), total JNK, p-p38
862 (Thr180/Thy182), total p38, total p53, p-AKT (Ser473), total AKT and GADPH.

863 **Supplementary figure S2(e): Western blotting of experiment number 5.** MCF10A
864 cells were plated at 75.000 cell/mL, pre-treated with 10nM PI3K inhibitor (LY294002)
865 for 30 minutes, and treated with 1μM Doxorubicin for 0-2-3-4-5-6 hours. The Western
866 blots were performed on the whole cell lysate using the following antibodies: p-JNK
867 (Thr183/Tyr185), total JNK, p-p38 (Thr180/Thy182), total p38, total p53, p-AKT
868 (Ser473), total AKT and GADPH.

869 **Supplementary figure S2(f): Western blotting of experiment number 6.** MCF10A
870 cells were plated at 75.000 cell/mL, pre-treated with 10nM PI3K inhibitor (LY294002)
871 for 30 minutes, and treated with 1μM Doxorubicin for 0-2-3-4-5-6 hours. The Western
872 blots were performed on the whole cell lysate using the following antibodies: p-JNK
873 (Thr183/Tyr185), total JNK, p-p38 (Thr180/Thy182), total p38, total p53, p-AKT
874 (Ser473), total AKT and GADPH.

875 **Supplementary figure S2(g): Western blotting of experiment number 7.** MCF10A
876 cells were plated at 75.000 cell/mL, pre-treated with 100μM p38 inhibitor (SB23580),
877 or 10nM PI3K inhibitor (LY294002) for 30 minutes, and treated with 1μM Doxorubicin

878 for 0-2-3-4-5-6 hours. The Western blots were performed on the whole cell lysate
879 using the following antibodies: p-JNK (Thr183/Tyr185), total JNK, p-p38
880 (Thr180/Thy182), total p38, total p53, p-AKT (Ser473), total AKT and GADPH.

881 **Supplementary figure S3: Identified interaction maps for the best fitting models**
882 **2-5** (parameter estimates in data rows 2-5, Excel file: “Parameter bounds and
883 estimation results.xlsx”, Sheet: “Run 4”). **(a)** 2nd best fitting parameter estimate; **(b)** 3rd
884 best fitting parameter estimate; **(c)** 4th best fitting parameter estimate; **(d)** 5th best
885 fitting parameter estimate. Black arrows represent phosphorylation and
886 dephosphorylation of the measured model components. The coloured lines indicate
887 the estimated crosstalks, with the type of the arrowhead indicating the sign of the
888 interaction; $\blacktriangleright$ for activation $\log_{10} a_{ji} > 0$, $\blacktriangleleft$ for inhibition $\log_{10} a_{ji} < 0$. Line thickness
889 is proportional to the strength of the estimated crosstalks $|a_{ji}|$. Large arrowheads
890 indicate $K_{ji} < 0.1$, small arrowheads $K_{ji} > 0.1$.

891 **Supplementary figure S4: Distribution of the parameter estimates for each**
892 **round of parameter estimation: (a) Round 1; (b) Round 2; (c) Round 3; (d) Round**
893 **4.** Each blue dot represents a parameter estimate from n=100 estimation runs. The red
894 dot indicates the parameter estimate with the best goodness-of-fit value. x-axis is on
895 the log10 scale.

896 **Supplementary figure S5: Distribution of the sensitivities for all n=100 estimates**
897 **from the last round of parameter estimation.** Each interaction in the model was
898 removed one by one and the model was simulated for all parameter estimates (see Fig.
899 6) and the sensitivity coefficients were calculated. The sensitivities were calculated for
900 all parameter estimates, i.e. each data row in Sheet: “Run 4”, Excel file: “Parameter
901 bounds and estimation results.xlsx”. Each blue dot represents the sensitivity of a
902 parameter estimate (n=100 estimation results). Red dots represent the best fitting
903 model.

904 **Supplementary File 1:** zip-file containing the model and parameter estimation code
905 (PEPSSBI), original Western blot images and quantified data, tables specifying all
906 parameters, parameters bounds, and parameter estimates (Excel), model analysis code
907 (Matlab), BMRI code (Matlab). Description of files:
908 Model code (folder): PEBSSPI files (.sbc). One file for each parameter estimation round.
909 Western blot images originals (folder): Bitmap images (.png) of the Western blots.
910 Data (Folder): Text files (.txt). Quantified, normalised Western blot data as text files.
911 BMRI (folder): Matlab code (.m, .mat). Code for Bayesian Modular Response analysis.
912 WB Quantification (Excel file): Tables with the quantified Western blot data.
913 Parameter bounds and estimation results (Excel file): Tables specifying the
914 parameters and their estimation bounds and the results for each parameter estimation
915 round. One sheet for each round.
916 Table model equations (Word file): Table specifying the model equations, including
917 reactions, rate laws, algebraic constraints and ordinary differential equations.

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