

1 **Small molecule inhibition of IRE1 α kinase/RNase has anti-fibrotic effects in the lung**

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18

19 **Abstract**

20

21 Endoplasmic reticulum stress (ER stress) has been implicated in the pathogenesis of
22 idiopathic pulmonary fibrosis (IPF), a disease of progressive fibrosis and respiratory failure. ER
23 stress activates a signaling pathway called the unfolded protein response (UPR) that either
24 restores homeostasis or promotes apoptosis. The bifunctional kinase/RNase IRE1 α is a UPR
25 sensor that promotes apoptosis if ER stress remains high (i.e., a “terminal” UPR). Using multiple
26 small molecule inhibitors against IRE1 α , we show that ER stress-induced apoptosis of murine

27 alveolar epithelial cells can be mitigated in vitro. In vivo, we show that bleomycin exposure to
28 murine lungs causes early ER stress to activate IRE1 α and the terminal UPR prior to
29 development of pulmonary fibrosis. Small-molecule IRE1 α kinase-inhibiting RNase attenuators
30 (KIRAs) that we developed were used to evaluate the importance of IRE1 α activation in
31 bleomycin-induced pulmonary fibrosis. One such KIRA—KIRA7—provided systemically to mice
32 at the time of bleomycin exposure decreases terminal UPR signaling and prevents lung fibrosis.
33 Administration of KIRA7 14 days after bleomycin exposure even promoted the reversal of
34 established fibrosis. Finally, we show that KIRA8, a nanomolar-potent, monoselective KIRA
35 compound derived from a completely different scaffold than KIRA7, likewise promoted reversal
36 of established fibrosis. These results demonstrate that IRE1 α may be a promising target in
37 pulmonary fibrosis and that kinase inhibitors of IRE1 α may eventually be developed into
38 efficacious anti-fibrotic drugs.

39

40 **Introduction**

41 The unfolded protein response (UPR) is a conserved signaling pathway that is activated
42 when eukaryotic cells experience protein folding stress in the endoplasmic reticulum (i.e., ER
43 stress). The mammalian UPR is mediated by three sensors of unfolded protein situated in the
44 ER membrane: IRE1 α , ATF6, and PERK; of these, IRE1 α is the most ancient member as IRE1
45 orthologs are present in all eukaryotes [1].

46 Depending on the severity of ER stress, the activity of IRE1 α determines cell fate
47 outcomes. When ER stress is remediable, IRE1 α homodimerizes in the ER membrane, causing
48 autophosphorylation and selective activation of its C-terminal RNase domain to catalyze the
49 frame-shift splicing of the mRNA encoding the transcription factor XBP1 into its active form (i.e.,
50 XBP1s transcription factor) [2–4]. XBP1s in turn promotes homeostasis by upregulating
51 transcription of components of the protein folding machinery including chaperones and post-
52 translational modification enzymes [5,6]. If these adaptive outputs fail to resolve ER stress,

53 IRE1 α continues to self-associate into higher-order oligomers on the ER membrane, resulting in
54 kinase hyperphosphorylation and RNase hyperactivation [7,8]. Hyperactivated IRE1 α catalyzes
55 endonucleolytic degradation of many messenger RNAs that localize to the ER membrane [7,9].
56 Hyperactivated IRE1 α also cleaves and degrades the precursor of the microRNA miR-17, which
57 in turn relieves the repression of thioredoxin-interacting protein (TXNIP). This pathway
58 culminates in apoptosis and is thus called the “terminal UPR” [10]; terminal UPR signaling
59 underlies several diseases of premature cell loss [11].

60 Recent studies have implicated the UPR in the pathophysiology of human idiopathic
61 pulmonary fibrosis (IPF), a disease of progressive interstitial fibrosis which leads to severe
62 debilitation and eventually respiratory failure and death [12]. Upregulation of the UPR occurs in
63 both familial IPF and sporadic (non-familial) IPF. In one family, a missense mutation in
64 Surfactant Protein C (SFTPC) causes misfolding of the protein and consequent ER stress in
65 alveolar epithelial cells [13,14], which is recapitulated in a mouse model bearing the same
66 mutation [15]. In types 1 and 4 of the Hermansky-Pudlak syndrome, patients have homozygous
67 loss-of-function at the *HPS1* or *HPS4* loci and develop a devastating pulmonary fibrosis virtually
68 indistinguishable from IPF by early adulthood [16]. *HPS1* or *HPS4* loss-of-function causes
69 accumulation of immature surfactant protein in alveolar epithelial cells and consequently ER
70 stress and apoptosis [17]. Activation of the UPR has also been demonstrated in patients with
71 non-familial (sporadic) IPF [18,19], although the trigger for UPR activation in these patients is
72 unclear. It has been suggested that environmental toxins (cigarette smoke) and infectious
73 agents (e.g. herpesviruses) may increase the secretory workload of alveolar epithelial cells,
74 which may exhaust homeostatic UPR signaling, and ultimately trigger the terminal UPR and cell
75 death [19–21]. In mice, the chemical ER stress inducer tunicamycin is not sufficient to induce
76 fibrosis, but exacerbates fibrosis in the presence of another injury in the form of bleomycin [15].
77 Administration of the nonspecific chaperones 4-PBA or TUDCA mitigated fibrosis after
78 bleomycin injury [22].

79 Together, these findings suggest that dampening UPR signaling, perhaps through
80 inhibiting IRE1 α , might provide therapeutic benefit in IPF. To these ends, our groups have
81 developed small-molecule compounds that inhibit the IRE1 α kinase and thereby allosterically
82 regulate its RNase activity [23–25]. These kinase-inhibiting RNase attenuating (KIRA)
83 compounds dose-dependently reduce IRE1 α 's destructive UPR signaling and thereby spare
84 cells experiencing unrelieved ER stress [8,26].

85 Here we show that ER stress induces apoptosis in a mouse alveolar epithelial cell line
86 and mouse primary type II alveolar epithelial cells, and that inhibiting the IRE1 α RNase
87 mitigates apoptosis in vitro. We further show that ER stress and stereotypic terminal UPR
88 signature changes occur in mouse models of bleomycin-induced pulmonary fibrosis, and that
89 administration of a KIRA compound at the time of bleomycin exposure prevents the full fibrosis
90 phenotype. Importantly, we show that KIRA compounds are efficacious even when
91 administered *after* the onset of fibrosis. These studies suggest the possible benefit of the KIRA
92 class of compounds in human IPF.

93

94 **Results**

95 *ER stress-induced apoptosis in alveolar epithelial cells depends on IRE1 α activity*

96 Severe or persistent ER stress has been shown to induce apoptosis in diverse cell types
97 [11]. Since chronic epithelial injury is thought to underlie both familial and sporadic IPF, we
98 assessed whether ER stress induces apoptosis in alveolar epithelial cells. The mouse epithelial
99 cell line MLE12 was exposed to tunicamycin, which inhibits N-glycosylation and induces protein
100 misfolding in the endoplasmic reticulum. Tunicamycin induced robust splicing of XBP1 in MLE12
101 cells, which was dose-dependently inhibited by provision of the covalent IRE1 α RNase inhibitor
102 STF-083010 [27] (Fig 1A). Low doses of tunicamycin (30 ng/ml) were sufficient to induce
103 apoptosis in MLE12 cells as measured by annexin V expression (Fig 1B). Tunicamycin-induced
104 apoptosis depended on IRE1 α RNase activity, since STF-083010 promoted cell survival. Next,

105 we isolated primary type II alveolar epithelial cells from mouse lungs by dissociation and cell
106 sorting as previously described [28,29]. As in the MLE12 cell lines, tunicamycin induced
107 apoptosis in primary type II alveolar epithelial cells in an IRE1 α -dependent manner (Fig 1C).

108

109 **Fig 1. ER stress activates IRE1 α to induce apoptosis in alveolar epithelial cells. (A)**

110 Ethidium bromide-stained agarose gel of XBP1 cDNA amplicons. Mouse Lung Epithelial
111 (MLE12) cells were exposed to Tunicamycin (Tun) 2.5 μ g/ml and indicated
112 concentrations of STF-083010 for 4 hrs. The cDNA amplicon of unspliced XBP1 mRNA
113 is cleaved by a PstI site within a 26 nucleotide intron to give 2U and 3U. IRE1 α -mediated
114 cleavage of the intron and re-ligation *in vivo* removes the PstI site to give the 1S
115 (spliced) amplicon. * denotes a spliced/unspliced XBP1 hybrid amplicon. The ratio of
116 spliced over (spliced + unspliced) amplicons—1S/(1S+2U+3U)—is reported as %XBP1
117 splicing under respective lanes. (B) Percent MLE12 cells staining positive for Annexin V
118 after treatment with Tm (30 ng/ml) and STF-083010 (50 μ M) for 72 hrs. (C) Percent
119 primary mouse alveolar epithelial cells (AEC) staining positive for Annexin V after
120 treatment with Tm (100 ng/ml) and STF-083010 (62.5 μ M) for 72 hrs. Three independent
121 biological samples were used for Annexin V staining experiments. Data are means +/-
122 SD. P value: * <0.05.

123

124 *ER stress precedes fibrosis in the bleomycin model pulmonary fibrosis*

125 To evaluate the role of IRE1 α *in vivo*, we turned to the bleomycin model of pulmonary
126 fibrosis. C57/BL6 mice were exposed to a single dose of intranasal bleomycin (1.5 units/kg),
127 and whole lung RNA was collected at intervals after exposure. Total XBP mRNA levels rise
128 within 2 days of bleomycin exposure (p<0.01), followed by markers of terminal UPR activation:
129 the chaperone BiP (p<0.001), and the transcription factors ATF4 (p<0.01) and CHOP (p<0.05)

130 (Fig 2A). This wave of ER stress and terminal UPR activation peaks at day 8 and remains
131 elevated through day 26 (Fig 2A). This initial, early wave of terminal UPR signaling is followed
132 by overt fibrosis; mRNA levels of collagen 1A1 and fibronectin rise at day 8 and peak on day 14,
133 followed by waning levels over the following two weeks (Fig 2B).

134

135 **Fig 2. ER stress and terminal UPR signaling precede fibrosis in bleomycin-**
136 **exposed mice.** (A) Quantitative PCR of terminal UPR markers XBP1, BiP, ATF4, and
137 CHOP from mice at 0, 2, 8, 14, 20, or 26 days after a single exposure to bleomycin.
138 Each mouse is represented by a dot, and whiskers denote group mean +/- SEM. (B)
139 Quantitative PCR for collagen 1A1 mRNA and fibronectin mRNA from at 0, 2, 8, 14, 20,
140 or 26 days after a single exposure to bleomycin. Each mouse is represented by a dot,
141 and whiskers denote group mean +/- SEM. P values: * <0.05 , ** <0.01 , *** <0.001 .

142

143 *KIRA7 modulation of IRE1 α prevents bleomycin-induced fibrosis*

144 The timing of ER stress and terminal UPR activation compared to fibrosis onset
145 suggested that administration of an IRE1 α inhibitor during the period shortly after bleomycin
146 exposure might mitigate bleomycin-induced fibrosis. The RNase inhibitor STF-083010 is a poor
147 tool compound for in vivo use in part due to its high IC₅₀ of 9.9 μ M and because of poor
148 solubility (Selleckchem manufacturer datasheet). Therefore, for in vivo experiments we used
149 KIRA7, an imidazopyrazine compound that binds the IRE1 α kinase to allosterically inhibit its
150 RNase activity (Fig 3A). KIRA7, also referred to a Compound 13 [25], is a modification of our
151 previously published compound KIRA6 [8] with comparable potency and pharmacokinetic
152 properties. Mice were exposed to a single dose of intranasal bleomycin (1.5 units/kg), then
153 treated with either KIRA7 (5 mg/kg/day i.p.) or an equivalent volume of vehicle, starting on the

154 day of bleomycin exposure and continuing daily for 14 days. Whole lung protein and RNA were
155 collected on day 14 for analysis (Fig 3B).

156

157 **Fig 3. KIRA7 prevents bleomycin-induced fibrosis.** (A) Chemical structure of KIRA7.
158 (B) Schematic of the KIRA7 prevention regimen. Mice were exposed to saline or
159 bleomycin once, then treated with KIRA7 or vehicle beginning from the time of bleomycin
160 exposure and daily for two weeks after exposure. (C) Western blot of terminal UPR
161 transcription factors XBP1s and ATF4 from mice treated according to the prevention
162 regimen. (D) Quantitative PCR of terminal UPR markers BiP and CHOP from mice
163 treated according to the prevention regimen. Each mouse is represented by a dot, and
164 whiskers denote group mean +/- SEM. (E) Hematoxylin and eosin stained sections from
165 mice treated according to the prevention regimen, at low magnification (top) and high
166 magnification (bottom). (F) Markers of fibrosis (lung weight, hydroxyproline content,
167 collagen 1A1 mRNA, and fibronectin mRNA) from mice treated according to the
168 prevention regimen. Each mouse is represented by a dot, and whiskers denote group
169 mean +/- SEM. P values: * <0.05 , ** <0.01 , *** <0.001 .

170

171 Although day 14 is after peak expression of markers of the terminal UPR (Fig 2A),
172 protein levels of spliced XBP1 and ATF4 remain significantly elevated in bleomycin-exposed
173 mice, compared to saline-exposed controls (Fig 3C). Treatment of bleomycin-exposed mice with
174 KIRA7 resulted in decreased spliced XBP1 and ATF4, compared to bleomycin exposed mice
175 treated with vehicle (Fig 3C). Likewise, mRNA levels of BiP and CHOP are significantly elevated
176 after bleomycin exposure, and treatment of bleomycin-exposed mice with KIRA7 decreased
177 these levels (Fig 3D, $p<0.05$).

178 These decreases in markers of the terminal UPR were accompanied by significant
179 improvements in bleomycin-induced fibrosis. Histologically, bleomycin exposure induced
180 destruction of alveoli, cell infiltration, and architectural distortion in the lung. By contrast, alveolar
181 architecture was largely preserved in lungs of mice exposed to bleomycin and treated with
182 KIRA7 (Fig 3E). Bleomycin exposure induced fibrosis as measured by lung weight and total
183 hydroxyproline, and both were significantly decreased with KIRA7 treatment ($p<0.05$).
184 Consistent with these decreases, mRNA levels of collagen 1A1 and fibronectin were both
185 significantly decreased by KIRA7 treatment ($p<0.001$ and $p<0.01$, respectively).

186

187 *KIRA7 modulation of IRE1 α mitigates established fibrosis*

188 Administration of KIRA7 protects against fibrosis during the initial injury after bleomycin,
189 which is also when ER stress and terminal UPR markers are at their peak (Fig 2A). We
190 wondered whether KIRA7 might even protect against fibrosis when administered later (i.e., in a
191 therapeutic rather than a prophylactic regimen), two weeks after initial injury, when terminal
192 UPR markers are still elevated albeit to a lesser degree (Fig 2A). In addition, early injury in the
193 first week after bleomycin administration is characterized by neutrophilic and lymphocytic
194 inflammatory infiltration, which is not thought to be characteristic of human IPF [30]. The initial
195 alveolitis clears around day 7, followed by fibroblast proliferation and matrix deposition; fibrosis
196 is typically established by day 14 (Fig 2B) [30]. Thus, administration of KIRA7 after day 14 may
197 have greater relevance to human IPF, where fibrosis may progress for years before the onset of
198 clinical symptoms and diagnosis.

199 As before, mice were exposed to a single dose of intranasal bleomycin (1.5 units/kg).
200 Beginning on day 14, mice were treated with either KIRA7 (5 mg/kg/day i.p.) or an equivalent
201 volume of vehicle; injections were continued daily until day 28. Whole lung protein and RNA
202 were collected on day 28 for analysis (Fig 4A). As expected, bleomycin-exposed mice had
203 increased levels of spliced XBP1, ATF4, and CHOP protein at day 28 (Fig 4B). Treatment of

204 bleomycin-exposed mice was associated with decreases in levels of these proteins (Fig 4B).
205 KIRA7 also blunted bleomycin-induced increases in lung weight ($p<0.001$) and hydroxyproline
206 ($p<0.05$) (Fig 4C). At day 28, mRNA levels of collagen 1A1 and fibronectin remain elevated
207 above baseline, albeit at lower levels compared to day 28 (Fig 4C), consistent with the
208 observation that synthesis of collagen and fibronectin mRNA peaks at day 14 and wanes
209 thereafter (Fig 2B). Nonetheless, administration of KIRA7 decreased mRNA levels of collagen
210 1A1 ($p<0.05$) and fibronectin ($p<0.05$).

211

212 **Fig 4. KIRA7 reverses bleomycin-induced fibrosis when given 2 weeks after**
213 **bleomycin exposure.** (A) Schematic of the KIRA7 reversal regimen. Mice were
214 exposed to saline or bleomycin once, then treated with KIRA7 or vehicle beginning two
215 weeks after bleomycin exposure and continuing daily for two additional weeks. (B)
216 Western blot of terminal UPR transcription factors XBP1s, ATF4, and CHOP from mice
217 treated with KIRA7 according to the reversal regimen. (E) Markers of fibrosis (lung
218 weight, hydroxyproline content, collagen 1A1 mRNA, and fibronectin mRNA) from mice
219 exposed exposed to saline or bleomycin, and treated with KIRA7 according to the
220 reversal regimen. Each mouse is represented by a dot, and whiskers denote group
221 mean \pm SEM. P values: * <0.05 , ** <0.01 , *** <0.001 .

222

223 *KIRA8 modulation of IRE1 α mitigates established fibrosis*

224 A sulfonamide compound was recently described that is structurally unrelated to KIRA7
225 [31], but possesses the properties of an IRE1 α kinase-inactivating RNase attenuator and we
226 therefore call KIRA8 (Fig 5A). It has exceptional selectivity for IRE1 α in whole-kinome testing,
227 having little activity even against its closely related paralog IRE1 β [26]. KIRA8 is highly potent
228 against the IRE1 α kinase (IC₅₀=5.9 nM, [26]), nearly 10-fold more potent than KIRA7 (IC₅₀=46

229 nM, data not shown). Consistent with this, KIRA8 has higher potency than KIRA7 in inhibiting
230 XBP1 splicing in the alveolar epithelial cell line MLE12 (Fig 5B).

231

232 **Fig 5. KIRA8 reverses bleomycin-induced fibrosis when given 2 weeks after**

233 **bleomycin exposure.** (A) Chemical structure of KIRA8. (B) EtBr-stained agarose gel of
234 XBP1 cDNA amplicons after induction by treating Mouse Lung Epithelial (MLE12) cells
235 with Tunicamycin (Tun) 0.5 μ g/ml and indicated concentrations of KIRA7 or KIRA8 for 8
236 hrs. The ratio of spliced over (spliced + unspliced) amplicons— $1S/(1S+2U+3U)$ —is
237 reported as % XBP1 splicing and reported under respective lanes. (C) Schematic of the
238 KIRA8 reversal regimen. Mice were exposed to saline or bleomycin once, then treated
239 with KIRA8 or vehicle beginning two weeks after bleomycin exposure and continuing
240 daily for two additional weeks. (D) Western blot of terminal UPR transcription factors
241 XBP1s, ATF4, and CHOP from mice treated with KIRA8 according to the reversal
242 regimen. (E) Markers of fibrosis (lung weight, hydroxyproline content, collagen 1A1
243 mRNA, and fibronectin mRNA) from mice exposed exposed to saline or bleomycin, and
244 treated with KIRA8 according to the reversal regimen. Each mouse is represented by a
245 dot, and whiskers denote group mean \pm SEM. P values: * <0.05 , ** <0.01 , *** <0.001 .

246

247 We evaluated the ability of KIRA8 to mitigate established fibrosis. Mice were exposed to
248 a single dose of intranasal bleomycin (1.5 units/kg), and treated with either KIRA8 (50
249 mg/kg/day i.p.) or an equivalent volume of vehicle starting at day 14 and continuing daily until
250 day 28. Whole lung protein and RNA were collected on day 28 for analysis (Fig 5C). KIRA8
251 treated mice had lower levels of spliced XBP1, ATF4, and CHOP protein (Fig 5D). As before,
252 KIRA8 treatment blunted bleomycin-induced increases in lung weight ($p<0.001$) and

253 hydroxyproline ($p < 0.01$), and decreased mRNA expression of collagen 1A1 ($p < 0.001$) and
254 fibronectin ($p < 0.05$) (Fig 5E).

255

256 **Discussion**

257 We have shown that ER stress induces apoptosis in a mouse alveolar epithelial cell line
258 and mouse primary type II alveolar epithelial cells, and that inhibiting the IRE1 α RNase
259 mitigates apoptosis in vitro. *In vivo*, bleomycin exposure to the mouse lung induces ER stress
260 prior to the onset of fibrosis. Highlighting the importance of ER stress and IRE1 α in this model,
261 administration of KIRA7 starting from the time of bleomycin exposure decreased markers of ER
262 stress and prevented fibrosis. Importantly, KIRA7 was efficacious even when administered two
263 weeks after the onset of fibrosis. KIRA8 is a next-generation KIRA compound derived from a
264 completely different scaffold than KIRA7, with nanomolar potency and monoselectivity for the
265 IRE1 α kinase. KIRA8 likewise promoted the reversal of established fibrosis.

266 Several mechanisms may account for the effect of KIRA7 and KIRA8 on bleomycin-
267 induced fibrosis. A prevailing view is that IPF is caused by chronic epithelial injury, which
268 induces fibroblast activation and collagen deposition [32]. In some cases, heritable defects in
269 cargo protein folding or post-translational processing leads to unremediated ER stress, IRE1 α
270 activation, terminal UPR signaling, and epithelial cell apoptosis [13,14,16]. Others have
271 proposed that various insults to the alveolar epithelium lead ultimately to terminal UPR signaling
272 [18–21]. In both cases, modulating the activity of IRE1 α would be predicted to blunt terminal
273 UPR signaling, promote alveolar epithelial cell survival, and thus mitigate ongoing fibrosis.

274 Another intriguing possibility is that IRE1 α activity may also contribute directly to
275 pathologic fibroblast behavior. For example, fibroblasts derived from patients with systemic
276 sclerosis, IRE1 α was required for TGF β 1-induced differentiation into activated myofibroblasts
277 [33]. This finding may help explain why late administration of KIRA7 and KIRA8, two weeks after
278 bleomycin injury, can promote the reversal of established fibrosis (Fig 4 and Fig 5). The possible

279 roles of IRE1 α in epithelial cells and fibroblasts are not mutually exclusive, and extensive work
280 outside the scope of this study is needed to elucidate the precise role(s) of IRE1 α in the injured
281 lung.

282 The anti-fibrotic activity of KIRA7 and KIRA8 even when administered late is particularly
283 important when considering potential therapeutic avenues in human disease. In IPF, subclinical
284 fibrosis starts years before patients are symptomatic enough to come to medical attention, and
285 even then diagnosis is often delayed [34,35]. These results suggest that targeting IRE1 α may
286 possibly be effective even when fibrosis is advanced. In addition, severe pathological lung
287 fibrosis, evidenced by the “usual interstitial pneumonia” pattern radiographically and
288 histologically, is the final common endpoint to chronic injury in non-IPF interstitial lung diseases,
289 including connective tissue-associated interstitial lung disease, chronic hypersensitivity
290 pneumonitis, and asbestosis [36]. Targeting IRE1 α holds therapeutic promise to the extent that
291 ER stress is important to the primary pathology and initial injury in these diseases, as has been
292 suggested in some forms of genetic autoimmune-associated interstitial lung disease [37] and
293 asbestosis [38]. To the extent that targeting IRE1 α might mitigate pathological fibroblast activity,
294 targeting IRE1 α may be useful even in cases where ER stress is not part of the primary
295 pathology.

296 In summary, we have shown that intra-airway bleomycin, the most commonly used
297 murine model of pulmonary fibrosis, induces the unfolded protein response and that this
298 response precedes the development of pulmonary fibrosis. Furthermore, two chemically distinct
299 small molecules that inhibit IRE1 α kinase and attenuate its RNase activity (KIRAs) are effective
300 in preventing and reversing bleomycin induced pulmonary fibrosis. This work lays the
301 groundwork for developing KIRAs as novel therapeutics for IPF and other interstitial lung
302 diseases characterized by progressive pulmonary fibrosis.

303

304 **Materials and methods**

305

306 *Tissue Culture*

307 Mouse Lung Epithelial (MLE12) cells were obtained from ATCC and grown in HITES
308 media as formulated by ATCC. Tunicamycin (Tun) was purchased from Millipore. STF-083010,
309 KIRA7 and KIRA8 were synthesized in house. Mouse primary AECII cells were isolated as
310 described [39]. Cells were grown in SAGM media (Lonza CC-3118) on fibronectin coated plates.

311

312 *XBP-1 mRNA splicing*

313 RNA was isolated from whole cells or mouse tissues and reverse transcribed as above
314 to obtain total cDNA. Then, XBP-1 primers were used to amplify an XBP-1 amplicon spanning
315 the 26 nt intron from the cDNA samples in a regular 3-step PCR. Thermal cycles were: 5 min at
316 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 60 °C, and 1 min at 72 °C, followed by 72 °C for 15
317 min, and a 4 °C hold. Mouse XBP1 sense (5'-AGGAAACTGAAAACAGAGTAGCAGC-3') and
318 antisense (5'-TCCTTCTGGGTAGACCTCTGG-3') primers were used. PCR fragments were
319 then digested by PstI, resolved on 3% agarose gels, stained with EtBr and quantified by
320 densitometry using NIH ImageJ.

321

322 *Annexin V apoptosis*

323 Annexin V staining was used to quantify apoptosis by flow cytometry. Cells were plated
324 in 12-well plates overnight and then treated with various reagents for indicated time periods. On
325 the day of flow cytometry analysis, cells were trypsinized and washed in PBS and resuspended
326 in Annexin V binding buffer with Annexin-V FITC (BD Pharmingen™). Annexin V stained cells
327 were counted using a Becton Dickinson LSRII flow cytometer.

328

329 *Bleomycin-induced pulmonary fibrosis*

330 C57BL6 mice at 12 weeks of age were obtained from Jackson Laboratories. Mice were
331 housed in specific pathogen-free conditions in the Animal Barrier Facility at the University of
332 California, San Francisco. This work was approved by the Institutional Animal Care and Use
333 Committee of the University of California, San Francisco. To induce fibrosis, mice were
334 anesthetized with ketamine and xylazine and exposed to a single dose of intranasal bleomycin
335 (1.5 units/kg). Lungs were harvested at the indicated times. For treatment, KIRA7 or KIRA8 was
336 dissolved in a vehicle consisting of 3% ethanol, 7% Tween-80, and 90% normal saline and
337 injected peritoneally at the indicated dosages and intervals.

338 Lung hydroxyproline content was quantified by reaction with 4-
339 (Dimethylamino)benzaldehyde reaction and colorimetry (Sigma). Messenger RNA levels were
340 measured by reverse transcription and quantitative PCR as described below.

341

342 *RNA isolation and quantitative real-time PCR (qPCR)*

343 A TissueLyser II (Qiagen) was used to homogenize mouse lungs for RNA isolation. RNA
344 was isolated from lung homogenates or cultured cells using either Qiagen RNeasy kits or Trizol
345 (Invitrogen). For cDNA synthesis, 1 µg total RNA was reverse transcribed using the QuantiTect
346 Reverse Transcription Kit (Qiagen). For qPCR, we used SYBR green (Qiagen) and
347 StepOnePlus Real-Time PCR System (Applied Biosystems). Thermal cycles were: 5 min at 95
348 °C, 40 cycles of 15 s at 95 °C, 30 s at 60 °C. Gene expression levels were normalized to 18S
349 rRNA. Primers used for qPCR were as follows:

350 Human/Mouse 18S rRNA: 5'-GTAACCCGTTGAACCCATT-3' and 5'-

351 CCATCCAATCGGTAGTAGCG-3'

352 Mouse XBP1: 5'-CCGTGAGTTTTCTCCCGTAA-3' and 5'-AGAAAGAAAGCCCGGATGAG-3'

353 Mouse BiP: 5'-TCAGCATCAAGCAAGGATTG-3' and 5'-AAGCCGTGGAGAAGATCTGA-3'

354 Mouse ATF4: 5'-GCAAGGAGGATGCCTTTTC-3' and 5'-GTTTCCAGGTCATCCATTCG-3'

355 Mouse CHOP: 5'-CACATCCCAAAGCCCTCGCTCTC-3' and 5'-

356 TCATGCTTGGTGCAGGCTGACCAT-3'

357 Mouse Collagen 1A1: 5'-CCTGGTAAAGATGGTGCC-3' and 5'-

358 CACCAGGTTACCTTTGCGACC-3'

359 Mouse Fibronectin: 5'-ACAGAAATGACCATTGAAGG-3' and 5'-TGTCTGGAGAAAGGTTGATT-

360 3'

361

362 *Western blot*

363 Whole lungs were homogenized using a TissueLyzer II (Qiagen). Nuclear and
364 cytoplasmic fractions were isolated using the NE-PER extraction kit (Thermo Fisher). Western
365 blots were performed using 4%-12% Bis-Tris precast gels (Invitrogen) using MOPS buffer, then
366 transferred onto nitrocellulose membranes. Antibody binding was detected using conjugated
367 secondary antibodies (Li-Cor) on the Li-Cor Odyssey scanner. Antibodies used for Western blot
368 were as follows: XBP1 (Biolegend 9D11A43), HDAC1 (Cell Signaling Technologies 5356), ATF4
369 (Sigma WH0000468M1), TXNIP (MBL International K0205-3), and CHOP (Cell Signaling
370 Technologies 2895).

371

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