

1 **Cell autonomous role of iASPP deficiency in causing**
2 **cardiocutaneous disorders**

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36 The authors declare no conflict of interest

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38 **Running title:** Intrinsic roles of iASPP in heart and skin

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40 **Key words:** iASPP, Heart Failure, Cutaneous Disorder, Desmosomes, Cell

41 Adhesion

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Abstract

Desmosome components are frequently mutated in cardiac and cutaneous disorders in animals and humans and enhanced inflammation is a common feature of these diseases. Previous studies showed that inhibitor of Apoptosis Stimulating p53 Protein (iASPP) regulates desmosome integrity at cell-cell junctions and transcription in the nucleus, and its deficiency causes cardiocutaneous disorder in mice, cattle and humans. As iASPP is a ubiquitously expressed shuttling protein with multiple functions, a key question is whether the observed cardiocutaneous phenotypes are caused by loss of a cell autonomous role of iASPP in cardiomyocytes and keratinocytes specifically or by a loss of iASPP in other cell types such as immune cells. To address this, we developed cardiomyocyte- and keratinocyte-specific iASPP-deficient mouse models and show that the cell-type specific loss of iASPP in cardiomyocytes or keratinocytes is sufficient to induce cardiac or cutaneous disorders, respectively. Additionally, keratinocyte-specific iASPP-deficient mice have delayed eyelid development and wound healing. In keratinocytes, junctional iASPP is critical for stabilizing desmosomes and iASPP deficiency results in increased and disorganized cell migration, as well as impaired cell adhesion, consistent with delayed wound healing. The identification of a cell autonomous role of iASPP deficiency in causing cardiocutaneous syndrome, impaired eyelid development and wound healing suggests that variants in the iASPP gene also may contribute to polygenic heart and skin diseases.

Introduction

Thousands of human diseases are caused by single gene defects and these diseases affect >25-30 million people in the US alone¹. Detailed mechanistic insights into how single gene defects can cause rare genetic disorders advance our understanding of the illness, and may also provide important insights for polygenic diseases, such as cancer and cardiovascular diseases, and physiological processes like development and wound healing. Cardiocutaneous syndromes are examples of disorders that can be caused by single gene defects, and patients with cardiocutaneous syndromes often present with cardiac and epidermal abnormalities that primarily originate from defects in cell junction proteins. For example, Naxos and Carvajal syndromes are cardiocutaneous syndromes caused by mutations in desmosomal intercellular junction genes such as γ -catenin and desmoplakin, respectively. Patients with Naxos or Carvajal syndrome have Arrhythmogenic Right Ventricular Cardiomyopathy (ARVC), a type of dilated cardiomyopathy (DCM), along with epidermal abnormalities that include palmoplantar keratoderma and woolly hair^{2,3}.

Mutations in components of desmosomes are involved in many cardiocutaneous syndromes⁴. Desmosomes are anchoring junctions predominantly found in cells that endure physical stress, such as cardiomyocytes and epidermal keratinocytes. Desmosomes link intermediate filaments (IFs) to intercellular junctions, forming cellular scaffolding that distributes mechanical forces throughout the tissue⁵. The three classes of desmosome components are: the cadherin family, (i.e. desmoglein (*DSG*) and desmocollin (*DSC*)); the armadillo family, (i.e. γ -

106 catenin (*JUP*) and plakophilin (*PKP*); and the plakin family (i.e.
107 desmoplakin (*DSP*)), responsible for anchoring IFs such as desmin and
108 keratin to desmosomes in cardiomyocytes and keratinocytes,
109 respectively⁶. For understanding the mechanisms underlying
110 cardiocutaneous syndromes, an important question is whether the DCM
111 and cutaneous phenotypes are caused by cell intrinsic mechanisms in
112 specific cell types (i.e. compromised desmosome integrity in
113 cardiomyocytes and keratinocytes, respectively) or if the phenotypes are
114 due to extrinsic mechanisms (i.e. paracrine signaling from other
115 cells/tissues). For example, inflammation is an extrinsic mechanism and
116 almost all cardiocutaneous patients exhibit elevated levels of
117 inflammatory cytokines regardless of their gene mutation status⁷.
118 Inflammation is also a key process in several models of DCM⁸. For
119 example, gamma-delta T cells have been reported to induce and/or
120 mediate DCM⁹, in part by initiating cardiomyocyte apoptosis¹⁰.
121 Therefore, it is plausible that the inflammatory response could be
122 involved in cardiac dysfunction in cardiocutaneous patients.

123 A recently identified single gene mutation that causes
124 cardiocutaneous syndrome (and cardiocutaneous disorder in cattle and
125 mice) is *PPP1R13L*¹¹⁻¹⁵, which encodes inhibitor of Apoptosis
126 Stimulating p53 Protein (iASPP), an evolutionarily conserved inhibitor
127 of p53 and p63^{16,17}. iASPP is a regulator of desmosomes¹³, but is also
128 implicated in inflammatory pathways^{11,18}. For example, iASPP was
129 originally identified as a binding partner for NF-κB in a yeast two-hybrid
130 assay¹⁸ and, consistent with this, iASPP-deficiency in patients and mice
131 results in elevated expression of inflammation-related genes, some of

132 which are NF- κ B targets^{11,12}. Given that iASPP is ubiquitously expressed
133 and germline mutations in the iASPP gene could affect the function of all
134 iASPP-expressing cells as well as the multiple pathways it regulates, a
135 key challenge is to identify the molecular mechanisms by which the
136 faulty gene causes the observed phenotype.

137 In addition, the function of iASPP in different cell types seems to
138 depend on its localization. iASPP can localize at cell-cell junctions, the
139 cytoplasm and the nucleus, and can shuttle between different cellular
140 compartments depending on intracellular signals¹⁹⁻²¹. For example, in
141 basal keratinocytes of the skin iASPP is in the nucleus where it regulates
142 p63, a master transcription factor of stratified squamous epithelium¹⁷; in
143 differentiating keratinocytes and in cardiomyocytes iASPP localizes at
144 cell-cell junctions, where it interacts with desmoplakin and intermediate
145 filaments to stabilize desmosomes¹³. Therefore, shuttling of iASPP
146 between cell-cell junctions and the nucleus could have profound effects
147 on desmosome stability and nuclear gene expression²⁰, and loss of both
148 junctional and nuclear iASPP could be important in cardiocutaneous
149 syndrome. For example, loss of nuclear iASPP could result in activation
150 of NF- κ B in certain cell types leading to enhanced secretion and activity
151 of inflammatory mediators¹¹, whereas loss of cell-junctional iASPP could
152 result in desmosome instability¹³.

153 Here we use specific deletion of iASPP in either cardiomyocytes
154 or keratinocytes to address three main questions: 1) are the cardiac and
155 cutaneous phenotypes due to loss of iASPP in cardiomyocytes and
156 keratinocytes, respectively; 2) what is the potential role of iASPP loss in

157 keratinocytes in development and wound healing; and 3)
158 mechanistically, how can iASPP regulate keratinocyte function? This
159 study provides evidence for an important cell autonomous role of iASPP
160 *in vivo*.

161

162 **Results**

163

164 **Cardiomyocyte- and keratinocyte-specific iASPP deficiency** 165 **recapitulates the main features of cardiocutaneous syndrome**

166 We previously showed that germline iASPP deficiency in mice
167 results in cardiac¹³ and cutaneous disorders¹⁷. To examine if the observed
168 cardiocutaneous phenotype can be induced by iASPP deficiency in
169 cardiomyocytes or keratinocytes only, we crossed mice with a floxed
170 iASPP gene to mice with Cre recombinase controlled either by a cardiac-
171 specific α -myosin heavy chain (α MyHC) promoter or an epidermis-
172 specific keratin 14 (K14) promoter, respectively (**Supplementary**
173 **Figure 1A**; see schematic)^{22,23}. The reported effectiveness of these Cre
174 promoters is quite high, as >90% recombination and gene deletion in
175 cardiac muscle cells has been achieved using the α MyHC promoter^{22,24}
176 and only a small number of cells with incomplete gene deletion in
177 epidermis and hair bulge have been reported using the K14 promoter²⁵.
178 We observed comparable deletion of iASPP in our tissue-specific models
179 (**Supplementary Figure 1B** for keratinocytes; see below for
180 cardiomyocytes).

181 Similar to germline iASPP-deficient animals¹³, α MyHC-Cre
182 iASPP-deficient mice presented with complete penetrance of the
183 myocardial phenotype, including scarring of the myocardium (**Figure**
184 **1A**). Furthermore, we observed dilation of the left ventricle (LV) with
185 prominent scarring of the septum and LV free wall in adult mice
186 (**Supplementary Figure 1C**). In contrast, K14-Cre iASPP-deficient
187 mice had no apparent myocardial abnormalities (**Figure 1B**), but had
188 corneal opacity, woolly hair and palmoplantar phenotype, similar to the
189 germline iASPP-deficient mice (**Figure 1C-D**). Progressive focal
190 palmoplantar skin lesions (**Figure 1D**) did not have full penetrance and
191 were observed from 10 weeks of age in less than 50% of both germline
192 and K14-Cre iASPP-deficient mice. Hematoxylin and eosin staining of
193 sections from the paws of adult mice showed focal thickening of the
194 *stratum corneum* (SC) and *stratum spinosum* (SS) in both germline
195 iASPP-deficient and K14-Cre iASPP-deficient mice compared to wild-
196 type controls (**Supplementary Figure 1D**), similar to that seen in
197 patients with palmoplantar keratodermas. In addition, incomplete
198 maturation of keratinocytes in the cornified layer was observed
199 (**Supplementary Figure 1D**). These results show that intrinsic loss of
200 iASPP in cardiomyocytes or keratinocytes is sufficient to recapitulate
201 heart and skin phenotypes observed with germline iASPP deficiency,
202 supporting the cell autonomous role of iASPP deficiency in causing
203 gross abnormalities of cardiocutaneous disorder. Lack of the myocardial
204 phenotype in the K14-Cre iASPP-deficient mice, and absence of the
205 woolly hair and eye phenotype in the α MyHC-Cre iASPP-deficient mice

206 (Figure 1A-C), confirm the fidelity of our Cre promoters in generating
207 epidermal- and myocardial-specific iASPP-deficient mice.

208

209 **Cardiomyocyte-specific iASPP deficiency causes defects in**
210 **intercalated discs and cardiac dysfunction**

211 Defects in the integrity of desmosomes and intercalated discs are
212 known to cause DCM. Our previous studies showed that iASPP locates
213 at intercalated discs in cardiomyocytes and controls desmosome
214 integrity, and germline iASPP-deficient mice suffer from DCM¹³. If
215 iASPP has a cell autonomous role in controlling desmosome integrity in
216 cardiomyocytes, a cardiomyocyte-specific deficiency of iASPP should
217 cause similar phenotypes to those in germline iASPP-deficient mice¹³.
218 To test this hypothesis we studied desmosome integrity in
219 cardiomyocytes of α MyHC-Cre iASPP-deficient mice. iASPP has
220 previously been shown to bind desmoplakin and anchor the
221 cardiomyocyte-specific intermediate filament desmin at the intercalated
222 discs, but not with other intercalated disc structural proteins such as α , β
223 and γ -catenins, connexin 43, p120 and N-cadherin under the same
224 conditions¹³. Consistent with a cell autonomous role of iASPP, we
225 identified a failure in desmin anchoring at the myocyte-myocyte
226 junctions in the myocardium of α MyHC-Cre iASPP-deficient mice
227 compared to wild-type mice (Figure 2A, yellow arrowheads denote
228 myocyte-myocyte junctions), similar to results previously observed in
229 germline iASPP-deficient mice¹³. The lack of iASPP expression in
230 cardiomyocytes at the intercalated discs from α MyHC-Cre iASPP-

231 deficient mice (**Figure 2A**) also confirms the efficacy of this Cre. As
232 expected, we did not observe loss of desmin at the myocyte-myocyte
233 junctions of myocardium in K14-Cre iASPP-deficient mice (**Figure 2A**).

234 Hematoxylin and eosin or Masson's trichrome staining of heart
235 sections from α MyHC-Cre iASPP-deficient and wild type animals
236 showed that widespread fibrosis is detected in α MyHC-Cre iASPP-
237 deficient hearts, but not in hearts from K14-Cre iASPP-deficient mice
238 (**Supplementary Figure 2A**). We measured various parameters of
239 ventricular function using magnetic resonance imaging (MRI), which
240 showed that α MyHC-Cre iASPP-deficient mice at 12 weeks have a
241 dilated RV and abnormal LV (**Figure 2B**, top; white arrowhead denotes
242 RV free wall and yellow arrowhead denotes LV free wall). MRI analysis
243 of LV function showed that α MyHC-Cre iASPP-deficient mice have a
244 significant increase in LV mass, along with a decrease in LV function (as
245 assessed by an increase in LV end-diastolic volume, LV end-systolic
246 volume and decreased LV ejection fraction) compared to wild-type
247 controls (**Figure 2B**, bottom; also see **Supplementary Videos 1 and 2**).
248 As expected, the α MyHC-Cre iASPP-deficient mice had decreased
249 survival compared to wild-type control mice, with a median survival of
250 17.3 weeks (121 days) (**Supplementary Figure 2B**). Therefore,
251 α MyHC-Cre iASPP-deficient mice progress to ventricular failure in a
252 similar manner to germline iASPP-deficient mice. These data support a
253 cell intrinsic role of iASPP in controlling the integrity of desmosomes
254 and intercalated discs in cardiomyocytes and in preventing DCM.

255

256 **iASPP in keratin 14 keratinocytes is required for mouse eyelid**
257 **closure independently of p53**

258 We next examined the basis of the eye phenotype in iASPP-
259 deficient mice more closely. In mammals, eyelid closure protects
260 developing ocular structures, particularly the cornea, from environmental
261 insults until the eye becomes functionally mature^{26,27}. Impaired closure
262 causes inflammation and severe corneal opacity; defects in the anterior
263 eye segment and blindness are observed shortly after birth²⁸⁻³¹. In
264 humans, eyelid closure occurs *in utero*²⁹. In mice it involves coordinated
265 migration of keratinocytes across the cornea from E14.5 with eyelids
266 fusing by E16.5, and remaining closed until postnatal day 12^{26,27}. Defects
267 in eyelid closure during development can lead to corneal opacity³².

268 Eyelid closure defects at birth and corneal opacity in adult mice
269 are two of the main features of germline iASPP deficiency that occur
270 with full penetrance^{12,33}. Importantly, cloudy cornea has also been
271 reported in 1 of 3 examined patients with an iASPP-null mutation, and
272 eye abnormalities have been found in all 4 examined aborted fetuses with
273 iASPP mutations¹¹.

274 As iASPP is an inhibitor of p53, we investigated whether the
275 eyelid closure defect was due to elevated p53 activity. We created
276 germline double knockout mice lacking iASPP and p53 (see Materials
277 and Methods). Consistent with previous findings^{17,33}, iASPP deficient
278 E16.6 embryos exhibited open eyelids whereas p53-deficient embryos
279 did not. Intriguingly, p53 and iASPP double knockout embryos retained
280 the open eyelid phenotype (**Figure 3A**). This suggests that the open

281 eyelid phenotype in iASPP-deficient embryos occurs independently of
282 p53 activity.

283 We then further investigated the intrinsic role of iASPP in
284 keratin 14 expressing keratinocytes in controlling eyelid closure and
285 preventing corneal opacity. We observed impaired extension of eyelid
286 epithelial sheets across the cornea of both K14-Cre and germline iASPP-
287 deficient E16.5 embryos compared to wild-type embryos (**Figure 3B**),
288 albeit with lower penetrance of 71% in K14-Cre (10 out of 14 embryos
289 examined) compared to 100% penetrance in germline iASPP-deficient
290 embryos (n=16). Consistent with this, around 68% of K14-Cre iASPP-
291 deficient mice (53 out of 78 mice examined) exhibited detectable corneal
292 opacity. This suggests that iASPP deficiency in keratin 14 expressing
293 keratinocytes is an important contributing factor to the eyelid closure
294 defects and corneal opacity in germline iASPP-deficient embryos and
295 adult mice.

296 To investigate the potential cause of eyelid closure defects in
297 germline iASPP-deficient embryos, we assessed cell proliferation and
298 apoptosis in the eyelids. Proliferation was assessed by BrdU
299 incorporation, which labels proliferating S-phase cells, in E14.5, E15.5
300 and E16.5 embryos and Ki67 in E15.5 embryos (**Supplementary Figure**
301 **3A-B**). The numbers of proliferating cells in the examined eyelid regions
302 of iASPP-deficient and wild-type embryos were similar (**Supplementary**
303 **Figure 3A-B**).

304 Apoptosis in the eyelids of iASPP-deficient and wild-type
305 embryos was examined by TUNEL and cleaved caspase 3 staining
306 (**Supplementary Figure 3C-D**). Although we consistently observed a

307 slight increase in the number of TUNEL and cleaved caspase 3 positive
308 cells at the leading edge of the eyelids in germline iASPP-deficient
309 embryos compared to wild-type embryos, the difference failed to reach
310 statistical significance. Future studies are needed to confirm whether the
311 observed subtle difference in cell death is sufficient to explain the
312 phenotype.

313

314 **iASPP is required for desmosome and keratin intermediate filament** 315 **integrity and epithelial cell adhesion**

316 Patients who present with striate palmoplantar keratoderma
317 (SPKK) associated with desmoplakin mutations have perinuclear
318 aggregation of keratin filaments in their cells³⁴. In addition, epidermis-
319 specific deletion of desmoplakin results in morphologically normal but
320 malfunctioning desmosomes as a result of disrupted keratin filaments³⁵.
321 Given the observed histological features of palmoplantar keratoderma in
322 iASPP-deficient mice (**Figure 1D, Supplementary Figure 1D**) and our
323 recent study describing iASPP as a regulator of desmosomal integrity¹³,
324 we examined if iASPP deficiency in mice could lead to disruption of
325 keratin filaments and desmosome adhesion in the epidermis and in
326 differentiating keratinocytes. If iASPP's role in regulation of
327 desmosomes is cell autonomous, we expect to observe keratin
328 aggregation and desmosome weakness in keratinocytes and other
329 epithelial cell lines lacking iASPP.

330 High-resolution electron microscopy (EM) of epidermis revealed
331 an increase in keratin aggregates *in vivo* (**Figure 4A, Supplementary**
332 **Figure 4A, red arrowheads**) and a defect in desmosome and intermediate

333 filament interactions in germline iASPP-deficient mice compared to
334 wild-type mice (**Figure 4A**). These findings were confirmed *in vitro* with
335 immunostaining of Keratin 5 (K5) intermediate filaments in
336 differentiating primary keratinocytes grown in high calcium media for 24
337 hours. K5 intermediate filaments were clearly visible in wild-type
338 primary mouse keratinocytes, while K5 aggregates accumulated in the
339 cytoplasm of iASPP-deficient keratinocytes (**Figure 4B**, white
340 arrowheads indicating red foci in cytoplasm). A propensity for keratin
341 aggregation in iASPP-deficient epidermis and keratinocytes agrees with
342 a mechanical role of iASPP in controlling desmosome integrity. We
343 further examined *in vitro* if the integrity and function of desmosomal
344 adhesion is compromised in the absence of iASPP. In a disperse
345 mechanical dissociation assay, iASPP-deficient keratinocytes or
346 epithelial UT-SCC74a cells had increased fragmentation compared to
347 wild-type or control siRNA-treated cell monolayers (**Figure 4C** and
348 **Supplementary Figure 4B**), which suggests impaired adhesion and
349 decreased resistance to mechanical stress. In a calcium-chelation assay,
350 keratinocytes lacking iASPP lost cell-cell adhesion when calcium was
351 withdrawn, as demonstrated by scattering of cells and lack of
352 desmoplakin at cell-cell contacts (**Figure 4D**). This suggests that iASPP
353 has an important role in controlling keratinocyte adhesion, as a loss of
354 iASPP renders cells unable to form hyperadhesive, calcium-independent
355 desmosomes (**Figure 4D**).

356 Defects in desmosomes will affect cell migration *in vitro*^{25,36,37},
357 so we assessed migration in the presence or absence of iASPP in the non-
358 tumorigenic desmosome-rich epithelial cell line MCF10A and primary

359 mouse skin keratinocytes, (as eyelid epithelium is composed of
360 keratinocytes equivalent to that of skin that are constituted of K14-
361 positive cells). A scratch wound assay using MCF10A cells treated with
362 control or iASPP siRNA showed increased cell migration in iASPP-
363 deficient compared to control cells (**Figure 4E, Supplementary Figure**
364 **4C, right panel, and Supplementary Video 3 and 4**). Similar results
365 were observed in primary mouse keratinocytes from iASPP-deficient
366 mice compared to wild-type mice (**Supplementary Figure 4D**).
367 Furthermore, we observed a higher degree of disorganized migration in
368 iASPP-deficient MCF10A cells compared to control cells
369 (**Supplementary Figure 4C, left panel**). Migratory behavior of iASPP-
370 deficient and wild-type keratinocytes within cell clusters of semi-
371 confluent cultures was assessed by pre-treatment with mitomycin C, an
372 agent that blocks cell proliferation. The migration distance was increased
373 and the pattern of migration was more scattered in iASPP-deficient
374 keratinocytes than wild-type cells (**Figure 4F, Supplementary Figure**
375 **4E and Supplementary Video 5 and 6**). This suggests that keratinocyte
376 adhesion requires iASPP to withstand mechanical stress and to migrate in
377 a coordinated manner. These data also show that the importance of
378 iASPP in mediating cell-cell adhesion is not limited to keratinocytes, as
379 another iASPP-deficient epithelial cell type also migrated in a more
380 disorganized manner.

381

382 **Mice with iASPP-deficient K14-expressing keratinocytes have**
383 **delayed cutaneous wound healing *in vivo***

384 Defects in desmosome dynamics and coordinated cell migration
385 are known to disrupt wound healing *in vivo*^{25,38}. Given our results
386 supporting iASPP's role in regulating keratinocyte migration and
387 desmosomal adhesion dynamics, we subjected wild-type, K14-Cre and
388 germline iASPP-deficient mice to full skin thickness punch biopsies and
389 measured the size of the wounds every other day for 14 days. Both
390 germline and K14-Cre iASPP-deficient mice exhibited delayed wound
391 healing up to day 8 compared to control mice (**Figure 5A-B**), but by
392 days 10-14 both cohorts of mice had fully healed wounds
393 (**Supplementary Figure 5A-B**). Wound healing in K14-Cre iASPP-
394 deficient mice was significantly delayed between days 4 and 8, while in
395 germline iASPP-deficient mice the healing delay was between days 2
396 and 6 (**Figure 5A-B**).

397 To assess the basis of the delay in wound healing, we performed
398 a BrdU proliferation assay *in vivo* and detected no differences between
399 keratinocytes at the wound site of iASPP-deficient and wild-type mice
400 (**Supplementary Figure 5C**). Furthermore, we did not detect any
401 significant differences in apoptosis of keratinocytes at the wound site
402 between iASPP-deficient and wild-type mice as assessed by cleaved
403 caspase 3 staining (**Supplementary Figure 5D**) and TUNEL assay
404 (**Supplementary Figure 5E**). We examined the histology of the wound
405 edge epidermis and observed an increase in intercellular spaces between
406 keratinocytes of iASPP-deficient compared to wild-type mice (**Figure**
407 **5C**). The area of an average intercellular space at the wound edge, as
408 quantified using Image J, was significantly higher in the absence of
409 iASPP (**Supplementary Figure 5F**). Cell-cell dissociation was also

410 observed at the wound edge of iASPP-deficient mice using
411 immunofluorescence staining for desmosome component Desmoglein I
412 and II (**Supplementary Figure 5G**). This suggests that in the absence of
413 iASPP, proliferation and apoptosis are unaffected during wound healing
414 but there is disrupted cell adhesion *in vivo*. This could contribute to a
415 lack of coordinated cell migration, impairing efficient wound re-
416 epithelization *in vivo*, and this might also explain the faster and
417 disorganized migration observed *in vitro* (**Figure 4E-F**).

418 **Discussion**

419 Here we show that loss of iASPP specifically in cardiomyocytes
420 or keratinocytes is sufficient to induce phenotypes of cardiocutaneous
421 disorder including cardiac dysfunction or palmoplantar keratoderma,
422 delayed eyelid development and wound healing, respectively. These
423 findings are consistent with previous studies showing that cardiac- or
424 epidermis-specific deletion of desmoplakin, a binding partner of iASPP
425 and an important desmosome component, leads to cardiac dysfunction or
426 keratin abnormalities, respectively^{35,39}.

427 Importantly, we show that tissue-specific deletion of iASPP in
428 cardiomyocytes is sufficient to induce cardiomyopathy with full
429 penetrance, similar to germline deletion of iASPP¹³. We previously
430 showed that RV dilation in germline iASPP-deficient mice can occur as
431 early as day 16.5 *in utero* with no obvious structural abnormalities in the
432 LV, but that both ventricles were dilated in 12 week-old mice¹³. We
433 predict that RV failure would precede LV failure, as the RV is the
434 predominant ventricle *in utero*. In this study we analysed the ventricles
435 of α MyHC-Cre iASPP-deficient postnatal pups and adult mice, which

436 may explain why we observed dilation of both ventricles. The phenotype
437 caused by iASPP deficiency is consistent with that caused by loss of
438 desmin, a component of desmosomes, which also results in biventricular
439 dilation⁴⁰ and ARVC^{41,42}. These data suggest that iASPP needs to be
440 added to the list of candidate genes involved in general DCM.

441 Compared to germline iASPP-deficient embryos, in K14-Cre
442 iASPP-deficient embryos we observed lower penetrance of the open-
443 eyelid and corneal opacity phenotypes (70% vs. 100%) and slightly
444 improved wound closure in K14-Cre iASPP deficient mice. The slightly
445 reduced phenotype penetrance and severity could be caused by
446 incomplete deletion of iASPP by K14-Cre. However, we cannot rule out
447 that a non-cell autonomous role of iASPP may contribute to the
448 penetrance and severity of these phenotypes. Additionally, we observed
449 an intriguing difference in the effects of iASPP deficiency on the degree
450 of structural abnormalities of intercalated discs (IDs) and desmosomes in
451 cardiomyocytes of the heart vs. keratinocytes of the skin. In iASPP-
452 deficient cardiac tissue, there was a significant loss of desmin
453 localization at the IDs (**Figure 2A**), whereas desmosome morphology
454 appeared largely unaffected in our electron microscopy analysis of the
455 germline iASPP deficient mouse skin (**Figure 4A**). Nevertheless, iASPP-
456 deficiency caused defective keratin IF network (**Figure 4A-B**,
457 **Supplementary Figure 4A**) in the skin and keratinocytes. Similar
458 results have been observed in mice with epidermis-specific deletion of
459 desmoplakin, with the desmosomes appearing morphologically normal
460 but dysfunctional, as shown by the collapse of keratin filaments³⁵. It is
461 important to note that the dorsal skin rather than palmoplantar epidermis

462 was used for electron microscopy; the latter may have a stronger
463 phenotype due to increased exposure to mechanical stress. A systemic
464 analysis of both palmoplantar and dorsal skin epidermis is needed to
465 examine the full extent of abnormalities in desmosome morphology and
466 keratin aggregation.

467 An interesting finding was that the eye phenotypes in both
468 germline and K14-Cre iASPP-deficient mice are similar to those
469 observed in patients and in fetuses aborted between 21 and 31 weeks of
470 gestation with iASPP mutations¹¹. The morphogenetic process of eyelid
471 closure in humans parallels that in mice, though in humans this process
472 occurs *in utero* between weeks 7-25 weeks of gestation. Our results
473 indicate that iASPP expression in K14-expressing cells is important for
474 efficient embryonic eyelid closure and prevention of eye defects.

475 iASPP is a shuttling multifunctional protein and in the nucleus it
476 is critical for inhibiting p63 function, for example during differentiation
477 of keratinocytes¹⁷ and cardiomyocyte development¹³. p63 regulates target
478 genes involved in adhesion (such as Perp, a desmosome component⁴³),
479 and the defects in cell migration *in vitro* in the absence of a significant
480 change in proliferation or apoptosis and delayed wound healing *in vivo*
481 that we see with iASPP deficiency are reminiscent of the phenotype
482 observed in Perp deficient cells and animals²⁵. Although we have
483 preliminary data that iASPP deficiency does not affect Perp expression
484 (data not shown), it could be argued that iASPP might control
485 keratinocyte function through its ability to regulate p63 transcription.
486 iASPP is also likely to affect keratinocyte function through its
487 cytoplasmic and junctional roles. We have shown previously that iASPP

488 becomes more cytoplasmic/junctional in differentiated keratinocytes or
489 post-mitotic cardiomyocytes¹³. In this study, our data suggest that iASPP
490 can directly influence keratinocyte cell adhesion via stabilizing
491 desmosomes. Therefore, the skin phenotype could be due to junctional
492 iASPP controlling the integrity of desmosome and keratin intermediate
493 filaments, as demonstrated in this study. Notably, iASPP can shuttle
494 between the cytoplasm and nucleus and so may also have a novel role
495 connecting desmosomes and transcriptional programs through its ability
496 to bind transcription factors such as p53, p63 and NF-κB (**Figure 6**).
497 Further elucidating how junctional proteins might influence
498 transcriptional programs and cell fate determination is an interesting area
499 for future study. In summary, our results support a cell autonomous role
500 of iASPP in cardiomyocytes and keratinocytes in the regulation of
501 desmosome integrity. This newly identified cell intrinsic role of iASPP
502 adds to our understanding of why iASPP deficiency causes
503 cardiocutaneous disorders in *wa3* mice¹², CWH Poll Hereford calves¹⁴
504 and patients¹¹.

505 **Acknowledgments**

506 We would like to thank Indrika Ratnayaka for tissue sectioning, Mark
507 Shipman for technical assistance with microscopy, Leonie Diffley
508 (University of Manchester) for assisting with the MRI experiments,
509 Guillermina Lozano (MD Anderson Cancer Center) for the p53 knockout
510 mice, and Mary Muers for critical reading of the manuscript. This work
511 was predominantly funded by the Ludwig Institute for Cancer Research
512 (LICR) Ltd. J.E.S. is a BHF senior Basic Science Research Fellow
513 (FS/11/50/29038), and acknowledges a Wellcome Trust Core Award

514 (090532/Z/09/Z). G.S. acknowledges support from Alberta Innovates
515 Health Solutions.

516

517 **Author Contributions**

518 **Z.D., G.S.** and **X.L.** designed the experiments, analyzed data and wrote
519 the paper. **Y.H.** initiated junctional iASPP study in keratinocytes. The
520 myocardial-specific iASPP-deficient mice were generated and
521 maintained by **E.A.S.** and **G.S.**, and the epidermis-specific iASPP-
522 deficient mice by **K. C.** and **Z.D.** Figure contributions: **Z.D.**- 1A,B,D;
523 3; 4B,C,D,F; 5; S1A, S1D; S3; S4B-E; S5; **G.S.**- 1A; 2B; S1C; S2B
524 **Y.H.**- 4B, 4E; **K. C.** -1C; 5A-B; S1B; S5A-B; **E.A.S.** – 1C; 2A; S2A;
525 **M.J.W.**-S1C; **F.Y.Z.**-S4C; S5F **R.D.G.**- S1C; **D.J.P.F.**- 4A; S4A;
526 **D.M.**-2B; **J.E.S.**-2B. **D.M.** and **J.E.S.** performed and analyzed the
527 experiments in 2B. **X.L.** supervised the work and provided the funding
528 for the study. All authors edited and approved the manuscript.

529

530 **Materials and Methods**

531 All animals were handled according to IACUC guidelines and all animal
532 work was approved by the Oxford University ethical review and licenced
533 by the UK home office. Procedures were carried out following the Home
534 Office Animal Scientific Procedures Act 1986 guidelines.

535

536 **Mouse Colonies.** Generation of iASPP transgenic mouse in which the
537 loxP-flanked exon8 of iASPP gene, PPP1R13L, is deleted globally by
538 CMV-Cre or tamoxifen inducible R26Cre⁺ER^T, resulting in a frameshift
539 mutation, has been previously described¹⁷. α MyHC-iASPP ^{Δ 8/ Δ 8} and K14-

540 iASPP^{Δ8/Δ8} mice were generated in a pure C57BL/6 background. Briefly
 541 iASPP^{LoxP/LoxP} mice were backcrossed into a C57BL/6 background strain
 542 prior to being crossed with αMyHC-Cre and R26R-EYFP mice (Jackson
 543 Laboratory) to generate αMyHC-iASPP^{Δ8/Δ8} mice, or with K14-Cre mice
 544 to generate K14-iASPP^{Δ8/Δ8} mice. Mice heterozygous for the iASPP
 545 LoxP allele were used to generate littermate control and knockout
 546 animals for all experiments. Genotyping was done using PCR primers for
 547 iASPP wildtype allele I8 (5'CCGAATTGGAGAAGTGAAGC-3') and
 548 E8 (5'-AGAGCAGCCTCAGAGCATGG-3'), loxP allele FLP2 (5'-
 549 CCGAATTGGAGAAGTGAAGC-3') and FRNT9 (5'-
 550 GGGTAGGAAAAAGGGCTGAG-3'), and deletion allele I8 and FLP2.
 551 Cre transgene was genotyped using Cre-F (5'-
 552 CATTTGGGCCAGCTAAACAT-3') and Cre-R (5'-
 553 ATTCTCCCACCGTCAGTACG-3'), and K14 Cre with K14-Cre-F (5'-
 554 GCTCTCTGTCACCCTGGCTA-3') and Cre-R. Balb/C p53^{+/-} mice, as
 555 previously described⁴⁴, were kindly provided by Guillermina Lozano
 556 (MD Anderson Cancer Center) and were crossed with iASPP^{+Δ8} mice to
 557 generate double heterozygotes on a mixed background, which were
 558 further crossed to generate compound genotypes presented in this study.
 559 Primers: 5'-CACAGCGTGGTGGTACCTTA-3' (exon 6), 5'-
 560 TAAGGATAGGTCGGCGGTTC-3' (exon 7), and 5'-
 561 CATCGCCTTCTATCGCCTTC-3' (neomycin resistance gene) were
 562 used to genotype p53 transgenic mice.
 563
 564 **Magnetic Resonance Imaging (MRI) Acquisition and Analysis.**

565 MRI for LV function assessment was carried out on a 9.4 T (400 MHz)
566 MRI system (Agilent, Santa Clara, CA) as described previously^{45,46} using
567 a four-channel cardiac array (Rapid Biomedical, Germany). In brief, a
568 double-gated⁴⁷, TGRAPPA-accelerated⁴⁸, multi-frame gradient echo
569 sequence was applied (TE/TR=1.79/4.6 ms, field-of-view 30 × 30 mm,
570 slice thickness 1 mm, matrix size 256 × 258, acceleration factor R = 3) in
571 short-axis orientation. Eight to ten slices were acquired covering the
572 heart from apex to base. Image analysis was performed off-line using
573 Amira 5.3.3 (Hillsboro, Oregon, US), and left-ventricular volumes and
574 mass were determined. End-diastolic and end-systolic frames were
575 selected on a slice by slice basis according to maximal and minimal
576 volume of the left ventricle. Epicardial border was manually outlined
577 first. The left-ventricular cavity was then segmented semi-automatically
578 by thresholding, using the Magic Wand tool, built into Amira. Left-
579 ventricular mass, end-diastolic and end-systolic volume were obtained,
580 from which stroke volume volume (LVSV = LVEDV - LVESV) and
581 ejection fraction (LVEF = LVSV / LVEDV * 100%) were calculated (as
582 described in⁴⁹).

583

584 **Histology and Masson's trichrome staining.** Histology and Masson's
585 trichrome staining was performed as previously described¹³. Briefly,
586 isolated hearts were processed and cryo- or paraffin-sectioned in the mid-
587 orthogonal plane to allow for identification of RV, septum and LV prior
588 to staining. RV, septum and LV were then analysed in detail for
589 structural defects and fibrosis.

590

591 **Cell culture.** Isolation and culture of primary keratinocytes from
592 iASPP^{+/+}, iASPP^{Δ8/Δ8} and iASPP^{LoxP/LoxP}; Cre⁺ER^T mice was performed
593 by dissecting skin from postnatal day 2 pups and floating it over 0.25%
594 trypsin-EDTA (Gibco) overnight at 4°C. This allowed the epidermis to
595 be peeled off and cut up in calcium-free EMEM with 8% calcium
596 stripped FBS, 0.05mM CaCl₂ and 50 μg/ml Gentamicin (low calcium
597 media; LCM). After being passed through a 100 μm cell strainer, the
598 cells were plated on a rat collagen type I (BD Bioscience) coated plastic
599 dishes or cover slips. iASPP deletion was induced by adding 1μM 4-
600 OHT to the cells for 4 days. Induction of cell-cell adhesion of cells was
601 stimulated by supplementing the media with calcium to 1.5 mM final
602 concentration. MCF10a cells were maintained in Dulbecco's modified
603 Eagle's medium/F12 containing 5% horse serum, 20ng/ml EGF,
604 0.5mg/ml hydrocortisone, 100ng/ml cholera toxin, 10μg/ml insulin and
605 100U/ml penicillin/streptomycin solution.

606

607 **siRNA-induced gene silencing.** For siRNA-induced gene silencing, cell
608 lines were transfected with either a SMART-pool of four siRNAs
609 targeting iASPP (LU-003815-00-002 GE Dharmacon) or control
610 siGENOME RISC-free siRNA (D-001220-01-05 GE Dharmacon).
611 Transfection was carried out using DharmaFECT I transfection reagent
612 (T-2001-02 GE Dharmacon) following manufacturer's instructions. Cell
613 assays were performed 72 hours post-transfection.

614

615 ***In vivo* wound healing experiment.** Six to eight weeks old wild-type,
616 iASPP^{Δ8/Δ8} and K14-Cre iASPP^{Δ8/Δ8} female mice in anagen hair phase
617 were anaesthetized with isoflurane inhalation and subcutaneously
618 injected with 1mg/kg Metacam. Their dorsal side was shaved and
619 sterilized with Chloraprep. A full thickness excisional wound was made
620 on the middle paravertebral region of the mouse using a biopsy punch of
621 6mm diameter (Stiefel). Wound closure process was monitored by
622 measuring the wound area with calipers every other day and was
623 represented as the percentage of the initial wound area. Data is presented
624 as mean value \pm 2 standard errors of the mean (SEM). Statistical analysis
625 between two groups was performed using the Mann-Whitney's U-test
626 and $p < 0.05$ was considered statistically significant.

627

628 ***In vitro* wound healing assay**

629 MCF10a treated with iASPP or control siRNA and keratinocytes from
630 iASPP^{LoxP/LoxP} Cre⁺ER^T mice treated with vehicle or 4-OHT to induce
631 iASPP deletion were grown to confluence. The confluent monolayers
632 were wounded by scratching with a disposable pipette tip. The cells were
633 washed and incubated in appropriate media. The wound closure was
634 followed, and images were taken every 20 minutes for up to 72 hours
635 using the timelapse video microscopy. The wound healing rate was
636 calculated as the percentage of the remaining wound area versus the
637 original wound area using ImageJ.

638

639 ***In vitro* motility behavior assay**

640 Keratinocytes from iASPP^{LoxP/LoxP} Cre⁺ER^T mice were grown in
641 keratinocyte defined medium (Gibco) in presence of 1μM 4-OHT or 4-
642 OHT solvent ethanol. Under these conditions cells formed cell-cell
643 contacts and cell clusters. The motility behavior of cells was followed for
644 48 hours using timelapse video microscopy. The motility of individual
645 cells was followed using ImageJ cell tracker. To avoid the effect of cell
646 proliferation on cell motility, keratinocytes were pre-treated with 1ng/ml
647 mitomycin C for 1 hour.

648

649 ***In vivo* 5-bromo-2'-deoxyuridine (BrdU) incorporation.** The same
650 mice used in the wound healing experiment (see above) were
651 intraperitoneally injected with 50μg/g body weight BrdU 2hrs prior to
652 euthanasia to assess proliferation rate. Immunofluorescence technique
653 described below using anti-BrdU antibody was used to localize BrdU
654 positive cells. The number of cells at the epidermal wound edge that had
655 incorporated BrdU were counted using Image J. Wound edge was
656 identified by immunofluorescence staining for the migration marker
657 Keratin 6.

658

659 **Dispase mechanical dissociation assay.** Confluent iASPP^{+/+} and
660 iASPP^{Δ8/Δ8} keratinocytes were cultured in 1.5 mM calcium for 24 hours,
661 rinsed in PBS and incubated with 2.4U/ml dispase (Roche) for 30
662 minutes at 37 °C. Released keratinocyte monolayers were gently rinsed
663 in PBS, transferred to 15-ml conical tube containing 2 ml PBS and
664 subjected to orbital rotation (20 rpm) for 5 minutes. Fragments were
665 imaged in a 6 well dish using HP Scanjet 5590P, and were counted using

666 Image J cell counter function. The fragments smaller than 4 pixels in
667 diameter (~area 1700 μm^2) were excluded from the quantification.
668 Statistical analysis was done using unpaired *t* test and significance was
669 defined as $p < 0.05$. The same protocol was followed for dispase assay
670 with UT-SCC74A cell line, which was transfected with indicated siRNA
671 for 72 hours.

672

673 **Calcium chelation assay.** Confluent vehicle-treated wildtype or 4-OHT-
674 induced iASPP $\Delta 8/\Delta 8$ primary keratinocytes were maintained in 1.5 mM
675 calcium media for either 2 or 6 days, following which the media was
676 replaced with low calcium media containing 3mM EGTA for 90 minutes.
677 The cells were subsequently subjected to immunocytochemistry protocol
678 with desmoplakin antibody. Calcium chelation protocol has been
679 described before elsewhere⁵⁰.

680

681 **Antibodies.** Antibodies against iASPP—mouse monoclonal LX049.3 and
682 LX128.5—have been described previously¹⁷. Additional antibodies used
683 in this study are: BrdU (ab6326 Abcam), Cleaved Caspase 3 (9661 Cell
684 Signaling), Desmin (ab15200 Abcam), Desmoglein 1&2 (61002 Progen),
685 Desmoplakin 1 & 2 (61003 & 65146 Progen), Keratin 5 (PRB-160P
686 Covance), Keratin 6 (PRB-169P Covance) and Ki67 (ab15589 Abcam).
687 Secondary antibodies carrying a fluorochrome (Alexa Fluor 488, 594 and
688 647) were purchased from Molecular Probes. Secondary anti-mouse,
689 goat or rabbit horseradish peroxidase (HRP)-labelled antibodies for
690 immunoblots were purchased from DakoCytomation.

691

692 **Immunofluorescence.** Confluent cells seeded on coverslips were fixed
693 in 4% paraformaldehyde (PFA) for 15 minutes. After three washes in
694 PBS, cells were permeabilized with 0.5% Triton X-100 for 10 minutes at
695 4°C, blocked with 5% normal goat serum in PBS, and subsequently
696 incubated with appropriate primary antibody for 1 hr at room
697 temperature. After rinsing in PBS, secondary antibodies (1:400) and
698 DAPI were applied to cells for 45 minutes. Images were taken using
699 LSM 710 confocal microscopy. Immunostaining of tissue sections was
700 carried out following protocol described in detail elsewhere¹⁷.

701

702 **TUNEL assay.** Apoptosis in eyelid and wound sections was examined
703 by the TUNEL assay using ApopTag®Red In Situ Apoptosis Detection
704 kit (S7165 Millipore) following manufacturer's instructions.

705

706 **Immunoblotting.** Cell rinsed in ice-cold PBS were lysed in NETN
707 buffer (50mM Tris pH8.0, 150mM NaCl, 1mM EDTA, 1% NP40 +
708 0.1mM Na₃VO₄, protease and phosphatase inhibitors). SDS sample
709 buffer was added to the lysate and samples were boiled for 10 minutes
710 before being subjected to SDS-PAGE on a 6% gel.

711

712 **Statistical analyses.** Sample size and statistical tests used to determine
713 statistical significance between samples are indicated in the appropriate
714 figure legends. Two-sided tests were used for all the analyses. *
715 represents p value < 0.05 , while ** represents $p < 0.01$

716

717

718 **Supplementary Information accompanies this paper on Cell Death**
 719 **and Differentiation website (<http://www.nature.com/cdd>)**

720

721

722 **References**

- 723 1. Brewer, G. J. Drug development for orphan diseases in the
 724 context of personalized medicine. *Transl Res* 2009; **154**: 314-
 725 322.
- 726 2. McKoy, G., Protonotarios, N., Crosby, A., Tsatsopoulou, A.,
 727 Anastasakis, A., Coonar, A. *et al.* Identification of a deletion in
 728 plakoglobin in arrhythmogenic right ventricular cardiomyopathy
 729 with palmoplantar keratoderma and woolly hair (Naxos disease).
 730 *Lancet* 2000; **355**: 2119-2124.
- 731 3. Norgett, E. E., Hatsell, S. J., Carvajal-Huerta, L., Cabezas, J. C.,
 732 Common, J., Purkis, P. E. *et al.* Recessive mutation in
 733 desmoplakin disrupts desmoplakin-intermediate filament
 734 interactions and causes dilated cardiomyopathy, woolly hair and
 735 keratoderma. *Hum Mol Genet* 2000; **9**: 2761-2766.
- 736 4. Bardawil, T., Khalil, S., Bergqvist, C., Abbas, O., Kibbi, A. G.,
 737 Bitar, F. *et al.* Genetics of inherited cardiocutaneous syndromes:
 738 a review. *Open Heart* 2016; **3**: e000442.
- 739 5. Garrod, D. & Chidgey, M. Desmosome structure, composition
 740 and function. *Biochim Biophys Acta* 2008; **1778**: 572-587.
- 741 6. Franke, W. W., Borrmann, C. M., Grund, C. & Pieperhoff, S.
 742 The area composita of adhering junctions connecting heart
 743 muscle cells of vertebrates. I. Molecular definition in intercalated
 744 disks of cardiomyocytes by immunoelectron microscopy of
 745 desmosomal proteins. *Eur J Cell Biol* 2006; **85**: 69-82.
- 746 7. Nitoiu, D., Etheridge, S. L. & Kelsell, D. P. Insights into
 747 desmosome biology from inherited human skin disease and
 748 cardiocutaneous syndromes. *Cell Commun Adhes* 2014; **21**: 129-
 749 140.
- 750 8. Meng, X., Yang, J., Dong, M., Zhang, K., Tu, E., Gao, Q. *et al.*
 751 Regulatory T cells in cardiovascular diseases. *Nat Rev Cardiol*
 752 2016; **13**: 167-179.
- 753 9. Takeda, N., Seko, Y., Oriuchi, N. & Nagai, R. Gamma-delta T-
 754 cell-mediated dilated cardiomyopathy. *Int J Cardiol* 2008; **125**:
 755 130-132.
- 756 10. Huber, S. A. T cells expressing the gamma delta T cell receptor
 757 induce apoptosis in cardiac myocytes. *Cardiovasc Res* 2000; **45**:
 758 579-587.
- 759 11. Falik-Zaccai, T. C., Barsheshet, Y., Mandel, H., Segev, M.,
 760 Lorber, A., Gelberg, S. *et al.* Sequence variation in PPP1R13L
 761 results in a novel form of cardio-cutaneous syndrome. *EMBO*
 762 *Mol Med* 2017; **9**: 319-336.

- 763 12. Herron, B. J., Rao, C., Liu, S., Laprade, L., Richardson, J. A.,
764 Olivieri, E. *et al.* A mutation in NFkB interacting protein 1
765 results in cardiomyopathy and abnormal skin development in
766 wa3 mice. *Hum Mol Genet* 2005; **14**: 667-677.
- 767 13. Notari, M., Hu, Y., Sutendra, G., Dedeic, Z., Lu, M., Dupays, L.
768 *et al.* iASPP, a previously unidentified regulator of desmosomes,
769 prevents arrhythmogenic right ventricular cardiomyopathy
770 (ARVC)-induced sudden death. *Proc Natl Acad Sci U S A* 2015;
771 **112**: E973-981.
- 772 14. Simpson, M. A., Cook, R. W., Solanki, P., Patton, M. A.,
773 Dennis, J. A. & Crosby, A. H. A mutation in NFkappaB
774 interacting protein 1 causes cardiomyopathy and woolly haircoat
775 syndrome of Poll Hereford cattle. *Anim Genet* 2009; **40**: 42-46.
- 776 15. Whittington, R. J. & Cook, R. W. Cardiomyopathy and woolly
777 haircoat syndrome of Poll Hereford cattle: electrocardiographic
778 findings in affected and unaffected calves. *Aust Vet J* 1988; **65**:
779 341-344.
- 780 16. Bergamaschi, D., Samuels, Y., O'Neil, N. J., Trigiante, G.,
781 Crook, T., Hsieh, J. K. *et al.* iASPP oncoprotein is a key
782 inhibitor of p53 conserved from worm to human. *Nat Genet*
783 2003; **33**: 162-167.
- 784 17. Notari, M., Hu, Y., Koch, S., Lu, M., Ratnayaka, I., Zhong, S. *et*
785 *al.* Inhibitor of apoptosis-stimulating protein of p53 (iASPP)
786 prevents senescence and is required for epithelial stratification.
787 *Proc Natl Acad Sci U S A* 2011; **108**: 16645-16650.
- 788 18. Yang, J. P., Hori, M., Sanda, T. & Okamoto, T. Identification of
789 a novel inhibitor of nuclear factor-kappaB, RelA-associated
790 inhibitor. *J Biol Chem* 1999; **274**: 15662-15670.
- 791 19. Lu, M., Breyssens, H., Salter, V., Zhong, S., Hu, Y., Baer, C. *et*
792 *al.* Restoring p53 function in human melanoma cells by
793 inhibiting MDM2 and cyclin B1/CDK1-phosphorylated nuclear
794 iASPP. *Cancer Cell* 2013; **23**: 618-633.
- 795 20. Lu, M., Muers, M. R. & Lu, X. Introducing STRaNDs: shuttling
796 transcriptional regulators that are non-DNA binding. *Nat Rev*
797 *Mol Cell Biol* 2016; **17**: 523-532.
- 798 21. Slee, E. A., Gillotin, S., Bergamaschi, D., Royer, C., Llanos, S.,
799 Ali, S. *et al.* The N-terminus of a novel isoform of human iASPP
800 is required for its cytoplasmic localization. *Oncogene* 2004; **23**:
801 9007-9016.
- 802 22. Agah, R., Frenkel, P. A., French, B. A., Michael, L. H.,
803 Overbeek, P. A. & Schneider, M. D. Gene recombination in
804 postmitotic cells. Targeted expression of Cre recombinase
805 provokes cardiac-restricted, site-specific rearrangement in adult
806 ventricular muscle in vivo. *J Clin Invest* 1997; **100**: 169-179.
- 807 23. Dassule, H. R., Lewis, P., Bei, M., Maas, R. & McMahon, A. P.
808 Sonic hedgehog regulates growth and morphogenesis of the
809 tooth. *Development* 2000; **127**: 4775-4785.
- 810 24. Chen, J. F., Murchison, E. P., Tang, R., Callis, T. E., Tatsuguchi,
811 M., Deng, Z. *et al.* Targeted deletion of Dicer in the heart leads
812 to dilated cardiomyopathy and heart failure. *Proc Natl Acad Sci*
813 *U S A* 2008; **105**: 2111-2116.

- 814 25. Beaudry, V. G., Ihrle, R. A., Jacobs, S. B., Nguyen, B., Pathak,
815 N., Park, E. *et al.* Loss of the desmosomal component perp
816 impairs wound healing in vivo. *Dermatol Res Pract* 2010; **2010**:
817 759731.
- 818 26. Findlater, G. S., McDougall, R. D. & Kaufman, M. H. Eyelid
819 development, fusion and subsequent reopening in the mouse. *J*
820 *Anat* 1993; **183** (Pt 1): 121-129.
- 821 27. Harris, M. J. & McLeod, M. J. Eyelid growth and fusion in fetal
822 mice. A scanning electron microscope study. *Anat Embryol*
823 *(Berl)* 1982; **164**: 207-220.
- 824 28. Li, G., Gustafson-Brown, C., Hanks, S. K., Nason, K., Arbeit, J.
825 M., Pogliano, K. *et al.* c-Jun is essential for organization of the
826 epidermal leading edge. *Dev Cell* 2003; **4**: 865-877.
- 827 29. Meng, Q., Mongan, M., Carreira, V., Kurita, H., Liu, C. Y., Kao,
828 W. W. *et al.* Eyelid closure in embryogenesis is required for
829 ocular adnexa development. *Invest Ophthalmol Vis Sci* 2014; **55**:
830 7652-7661.
- 831 30. Weng, J., Luo, J., Cheng, X., Jin, C., Zhou, X., Qu, J. *et al.*
832 Deletion of G protein-coupled receptor 48 leads to ocular
833 anterior segment dysgenesis (ASD) through down-regulation of
834 Pitx2. *Proc Natl Acad Sci U S A* 2008; **105**: 6081-6086.
- 835 31. Meng, W., Green, J. & Guest, J. R. FNR-dependent repression of
836 ndh gene expression requires two upstream FNR-binding sites.
837 *Microbiology* 1997; **143** (Pt 5): 1521-1532.
- 838 32. Luetke, N. C., Qiu, T. H., Peiffer, R. L., Oliver, P., Smithies,
839 O. & Lee, D. C. TGF alpha deficiency results in hair follicle and
840 eye abnormalities in targeted and waved-1 mice. *Cell* 1993; **73**:
841 263-278.
- 842 33. Toonen, J., Liang, L. & Sidjanin, D. J. Waved with open eyelids
843 2 (woe2) is a novel spontaneous mouse mutation in the protein
844 phosphatase 1, regulatory (inhibitor) subunit 13 like (Ppp1r13l)
845 gene. *BMC Genet* 2012; **13**: 76.
- 846 34. Wan, H., Dopping-Hepenstal, P. J., Gratian, M. J., Stone, M. G.,
847 Zhu, G., Purkis, P. E. *et al.* Striate palmoplantar keratoderma
848 arising from desmoplakin and desmoglein 1 mutations is
849 associated with contrasting perturbations of desmosomes and the
850 keratin filament network. *Br J Dermatol* 2004; **150**: 878-891.
- 851 35. Vasioukhin, V., Bowers, E., Bauer, C., Degenstein, L. & Fuchs,
852 E. Desmoplakin is essential in epidermal sheet formation. *Nat*
853 *Cell Biol* 2001; **3**: 1076-1085.
- 854 36. South, A. P., Wan, H., Stone, M. G., Dopping-Hepenstal, P. J.,
855 Purkis, P. E., Marshall, J. F. *et al.* Lack of plakophilin 1
856 increases keratinocyte migration and reduces desmosome
857 stability. *J Cell Sci* 2003; **116**: 3303-3314.
- 858 37. Yin, T., Getsios, S., Caldelari, R., Kowalczyk, A. P., Muller, E.
859 J., Jones, J. C. *et al.* Plakoglobin suppresses keratinocyte motility
860 through both cell-cell adhesion-dependent and -independent
861 mechanisms. *Proc Natl Acad Sci U S A* 2005; **102**: 5420-5425.
- 862 38. Thomason, H. A., Cooper, N. H., Ansell, D. M., Chiu, M.,
863 Merrit, A. J., Hardman, M. J. *et al.* Direct evidence that
864 PKCalpha positively regulates wound re-epithelialization:

correlation with changes in desmosomal adhesiveness. *J Pathol* 2012; **227**: 346-356.

39. Garcia-Gras, E., Lombardi, R., Giocondo, M. J., Willerson, J. T., Schneider, M. D., Khoury, D. S. *et al.* Suppression of canonical Wnt/beta-catenin signaling by nuclear plakoglobin recapitulates phenotype of arrhythmogenic right ventricular cardiomyopathy. *J Clin Invest* 2006; **116**: 2012-2021.

40. Li, D., Tapscoft, T., Gonzalez, O., Burch, P. E., Quinones, M. A., Zoghbi, W. A. *et al.* Desmin mutation responsible for idiopathic dilated cardiomyopathy. *Circulation* 1999; **100**: 461-464.

41. Klauke, B., Kossmann, S., Gaertner, A., Brand, K., Stork, I., Brodehl, A. *et al.* De novo desmin-mutation N116S is associated with arrhythmogenic right ventricular cardiomyopathy. *Hum Mol Genet* 2010; **19**: 4595-4607.

42. Lorenzon, A., Beffagna, G., Bauce, B., De Bortoli, M., Li Mura, I. E., Calore, M. *et al.* Desmin mutations and arrhythmogenic right ventricular cardiomyopathy. *Am J Cardiol* 2013; **111**: 400-405.

43. Ihrle, R. A., Marques, M. R., Nguyen, B. T., Horner, J. S., Papazoglu, C., Bronson, R. T. *et al.* Perp is a p63-regulated gene essential for epithelial integrity. *Cell* 2005; **120**: 843-856.

44. Tordella, L., Koch, S., Salter, V., Pagotto, A., Doondeea, J. B., Feller, S. M. *et al.* ASPP2 suppresses squamous cell carcinoma via RelA/p65-mediated repression of p63. *Proc Natl Acad Sci U S A* 2013; **110**: 17969-17974.

45. Schneider, J. E., Cassidy, P. J., Lygate, C., Tyler, D. J., Wiesmann, F., Grieve, S. M. *et al.* Fast, high-resolution in vivo cine magnetic resonance imaging in normal and failing mouse hearts on a vertical 11.7 T system. *J Magn Reson Imaging* 2003; **18**: 691-701.

46. Schneider, J. E., Lanz, T., Barnes, H., Stork, L. A., Bohl, S., Lygate, C. A. *et al.* Accelerated cardiac magnetic resonance imaging in the mouse using an eight-channel array at 9.4 Tesla. *Magn Reson Med* 2011; **65**: 60-70.

47. Cassidy, P. J., Schneider, J. E., Grieve, S. M., Lygate, C., Neubauer, S. & Clarke, K. Assessment of motion gating strategies for mouse magnetic resonance at high magnetic fields. *J Magn Reson Imaging* 2004; **19**: 229-237.

48. Breuer, F. A., Kellman, P., Griswold, M. A. & Jakob, P. M. Dynamic autocalibrated parallel imaging using temporal GRAPPA (TGRAPPA). *Magn Reson Med* 2005; **53**: 981-985.

49. Schneider, J. E., Wiesmann, F., Lygate, C. A. & Neubauer, S. How to perform an accurate assessment of cardiac function in mice using high-resolution magnetic resonance imaging. *J Cardiovasc Magn Reson* 2006; **8**: 693-701.

50. Merritt, A. J., Scothern, A. & Bhattacharyya, T. Assays for the calcium sensitivity of desmosomes. *Methods Mol Biol* 2006; **341**: 167-183.

Figure Legends

Figure 1. iASPP deficiency in cardiomyocytes and keratinocytes leads to cardiocutaneous phenotypes

(A) Representative images of hearts isolated from wild-type (+/+), germline (iASPP $\Delta 8/\Delta 8$) and myocardial-specific iASPP-deficient (α MyHC-iASPP $\Delta 8/\Delta 8$) mice (α MyHC indicated as MHC). All images were taken with comparable magnification, showing larger size of hearts from iASPP-deficient mice. White patchy areas are scarring of the myocardium. (B) Representative images at the same magnification, showing hearts isolated from wild-type and epidermis-specific iASPP-deficient (K14-iASPP $\Delta 8/\Delta 8$) mice, with no myocardial abnormalities. (C) Representative images of eyes and fur of wild-type (+/+), germline iASPP $\Delta 8/\Delta 8$, α MyHC-iASPP $\Delta 8/\Delta 8$ and K14-iASPP $\Delta 8/\Delta 8$ adult mice. Sparse wavy coat and eye defects are present in the K14-iASPP $\Delta 8/\Delta 8$ and iASPP $\Delta 8/\Delta 8$ mice. (D) Representative images of the paws of adult wild-type (+/+), germline iASPP $\Delta 8/\Delta 8$ and K14-iASPP $\Delta 8/\Delta 8$ mice. The paws of iASPP $\Delta 8/\Delta 8$ and K14-iASPP $\Delta 8/\Delta 8$ mice have shedding and peeling of the cornified envelope (black arrows), resembling palmoplantar keratoderma.

Figure 2. Myocardial-specific iASPP-deficient mice have loss of desmosomes at cell-cell junctions and compromised cardiac function

(A) Immunofluorescence images of myocardial tissue sections from iASPP (+/+), α MyHC-iASPP $\Delta 8/\Delta 8$ and K14-iASPP $\Delta 8/\Delta 8$ adult mice stained with antibodies against iASPP LX128.5 (green) and desmin (red). Nuclei shown with DAPI on merge panels. Examples of desmosomes are

indicated by yellow arrowheads. **(B)** MRI imaging (top panels) in the orthogonal plane of the hearts of iASPP (+/+) and α MyHC-iASPP $\Delta 8/\Delta 8$ mice (see key) at 12 weeks. Right ventricles indicated by white arrowheads; left ventricles (LV) by yellow arrowheads. MRI analysis in the same mice (lower panels) of LV mass, LV end-diastolic volume (LVEDV), LV end-systolic volume (LVESV) and LV ejection fraction (LVEF). * represents $p < 0.05$ using unpaired *t*-test. Each animal is represented by a coloured symbol (\diamond for males, \circ for females). C is wild-type controls; KO is iASPP-deficient. Black bar indicates the mean.

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Figure 3. iASPP-deficient mice have impaired embryonic eyelid closure

(A) Representative histological sections through the eye from E16.5 p53^{+/+};iASPP^{+/+}, p53^{+/+};iASPP $\Delta 8/\Delta 8$, p53^{-/-};iASPP^{+/+} and p53^{-/-};iASPP $\Delta 8/\Delta 8$ embryos, stained with H&E. Loss of p53 has no effect on eyelid closure in iASPP $\Delta 8/\Delta 8$ embryos. The eyelid is at the top of the image, extending over the eye in the iASPP wild-type embryos. **(B)** Representative sections through the eye from E16.5 iASPP (+/+), iASPP $\Delta 8/\Delta 8$ and K14-iASPP $\Delta 8/\Delta 8$ embryos, stained with H&E as in A.

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Figure 4. iASPP is required for keratin intermediate filament integrity and epithelial cell adhesion

(A) Transmission electron microscopy of epidermal desmosomes from 12 week old iASPP^{+/+} and germline iASPP $\Delta 8/\Delta 8$ littermates. Red arrowheads indicate keratin aggregates. Yellow arrowheads show examples of desmosomes. Black arrows indicate the interaction between

967 the desmosome plaque and intermediate filaments (IF), with IF being
 968 more densely packed at the desmosome interface in the epidermis from
 969 iASPP^{+/+} mice compared to that from iASPP^{Δ8/Δ8} mice. **(B)** (Left panel)
 970 Immunofluorescence staining showing increased aggregation of K5
 971 filaments (red) in iASPP-deficient primary mouse keratinocytes
 972 compared to control cells. The nuclear stain DAPI is shown in blue.
 973 Arrowheads point to dense cytoplasmic K5 aggregates, red foci, that are
 974 more abundantly present in iASPP-deficient keratinocytes compared to
 975 wild-type keratinocytes as quantified in the right panel. (Right panel)
 976 Quantification of percentage of cells with keratin aggregation in four
 977 independent experiments with at least 60 cells scored per experimental
 978 replicate. Statistical analysis between control and iASPP-deficient
 979 keratinocyte groups was performed using unpaired *t*-test, bars denote \pm
 980 2SEM **(C)** Representative images of iASPP^{+/+} and iASPP^{Δ8/Δ8}
 981 keratinocyte monolayers before and after subjection to a dispase
 982 mechanical dissociation (mechanical stress), as indicated. The bar chart
 983 shows the number of fragments released as a result of the mechanical
 984 stress with increasing numbers of fragments indicating weaker cell-cell
 985 adhesion. The red horizontal bar denotes the group mean. Values are
 986 means \pm 1SEM (black vertical bars) from three different experiments,
 987 where each dot represents individual data point. Statistical analysis
 988 between groups was done using unpaired *t*-test; * denotes $p < 0.05$.
 989 Immunoblot for iASPP and tubulin (as a loading control) expression in
 990 lysates from keratinocytes isolated from wild-type and
 991 iASPP^{Δ8/Δ8} epidermis. **(D)** Immunostaining for desmoplakin (DSPI/II,

992 white) in wild-type (upper panels) and iASPP-deficient (lower panels)
 993 primary keratinocytes cultured at confluence in high calcium medium
 994 (HCM) for either 2 or 6 days, with subsequent subjection to calcium
 995 depletion for 90 minutes by switching to low calcium media (LCM)
 996 containing 3mM EGTA. **(E)** Representative images of *in vitro* wound
 997 scratch closure by MCF10A cells treated with control or iASPP siRNA;
 998 images at indicated time points of timelapse microscopy. The efficiency
 999 of the iASPP knockdown is shown by immunoblots (right). **(F)**
 1000 Representative Image J tracks of selected iASPP^{+/+} and iASPP^{Δ8/Δ8}
 1001 keratinocytes whose migratory behavior within a cell cluster was
 1002 examined to determine their tendency to remain and migrate together.
 1003 Each colored line represents one cell.

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1005 **Figure 5. Wound healing and cell adhesion is impaired in iASPP-**
 1006 **deficient mice**

1007 **(A)**(Left) Representative images of wound healing after skin biopsy in
 1008 germline iASPP^{Δ8/Δ8} adult mice, over the first 6 days. (Right) Plot
 1009 showing the average percentage (mean ± 2SEM) of wound area closed in
 1010 iASPP^{+/+} and iASPP^{Δ8/Δ8} mice. ***p*<0.001, **p*<0.05. The number of
 1011 iASPP^{+/+} and iASPP^{Δ8/Δ8} mice are, respectively: 19 and 17 at day 2; 14
 1012 and 13 at day 4; 12 and 11 at day 6; 6 and 4 at day 8. Statistical analysis
 1013 between groups was performed using the Mann-Whitney U test. **(B)**
 1014 (Left) Images of wound healing as in panel A, for K14-iASPP^{+/+} and
 1015 K14-iASPP^{Δ8/Δ8} mice. (Right) Plot showing average percentage (mean ±
 1016 2SEM) of wound area closed in K14-iASPP^{+/+} and K14-iASPP^{Δ8/Δ8} mice.

1017 $**p<0.001$, $*p<0.05$. The number of K14-iASPP^{+/+} and K14-iASPP^{Δ8/Δ8}
1018 mice are, respectively: 10 and 9 at day 2; 11 and 9 at day 4; 6 and 7 at
1019 day 6; 4 and 3 at day 8. Statistical analysis between groups was
1020 performed using the Mann-Whitney U test. (C) H&E staining of
1021 sections through the advancing epithelial tongues of wounds from
1022 iASPP^{+/+} and iASPP^{Δ8/Δ8} mice at days 2 and 6, as indicated. Black
1023 arrowheads point to widened intercellular spaces in iASPP^{Δ8/Δ8} wounded
1024 epidermis. Black dotted lines indicate the dermal-epidermal boundary.

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1026 **Figure 6. Working model of iASPP functions in cardiomyocytes and**
1027 **keratinocytes**

1028 A schematic model of how iASPP might act at different stages of
1029 development in cardiomyocytes and keratinocytes. iASPP (small blue
1030 rectangles) shuttles between the nucleus and cytoplasm in dividing
1031 cardiomyocytes and keratinocytes during development (e.g. E16.5), and
1032 cell junctions (desmosomes) in mature cardiomyocytes (e.g. 12 weeks)
1033 and differentiating keratinocytes. At the desmosomes, iASPP is
1034 important to maintain the integrity of desmosomal adhesion by
1035 interacting with desmoplakin (DSP) and intermediate filaments (Keratin
1036 5 in keratinocytes or Desmin in cardiomyocytes). In the nucleus (e.g. at
1037 E16.5) iASPP can interact with transcription factors such as p53 or p63
1038 and trigger cell signalling events. For example, this may lead to
1039 inhibition of p53-dependent apoptosis in cardiomyocytes or inhibition of
1040 p63-dependent differentiation in keratinocytes.