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FASCIN-1 IS A NOVEL PROGNOSTIC BIOMARKER ASSOCIATED WITH TUMOR INVASIVENESS IN ADRENOCORTICAL CARCINOMA

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Running Title: Fascin-1 is a marker of ACC invasiveness

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48 **PRECIS**

49 Tumor invasion markers are urgently needed to better stratify adrenocortical cancer patients and
50 improve the current limited therapies. This study demonstrates the robust independent prognostic
51 power of Fascin-1 in a local adrenocortical cancer series further validated in two independent cohorts.
52
53

54 **ABSTRACT**

55 CONTEXT: Novel tumor markers are urgently needed to better stratify adrenocortical cancer (ACC)
56 patients and improve therapies for this aggressive neoplasm.

57 OBJECTIVE: To assess the diagnostic and prognostic value of the actin-bundling protein fascin-1
58 (FSCN1) in adrenocortical tumours.

59 DESIGN, SETTING AND PARTICIPANTS: A local series of 37 malignant/37 benign adrenocortical
60 tumours at Careggi University Hospital and two independent validation ACC cohorts (Cochin and
61 TCGA) from the European Network for the Study of Adrenal Tumors were studied.

62 MAIN OUTCOME MEASURES: FSCN1 expression was quantified by immunohistochemistry,
63 Western Blot and quantitative RT-PCR analyses in ACC specimens; overall and disease-free survival
64 associated with FSCN1 expression were assessed by Kaplan-Meier analysis and compared with that of
65 Ki67 labelling index and tumor stage.

66 RESULTS: In spite of the low diagnostic power, in the Florence ACC series, FSCN1
67 immunohistochemical detection appeared as an independent prognostic factor, also refining results
68 obtained with staging and Ki67 labelling index. The robust prognostic power of FSCN1 levels was
69 further confirmed in two independent ACC cohorts. A positive correlation was found between FSCN1
70 and Steroidogenic Factor-1 (SF-1), with a significant higher expression of both factors in ACCs at
71 advanced stages and with at least one of the three Weiss score parameters associated with invasiveness.
72 Moreover, we demonstrated FSCN1 role in promoting cell invasion in a human ACC cell line only in
73 the case of increased SF-1 dosage.

74 CONCLUSIONS: These findings show that FSCN1 is a novel independent prognostic marker in ACC
75 and may serve as a potential therapeutic target to block tumor spread.

76

77

78 **INTRODUCTION**

79 Adrenocortical cancer (ACC) is a rare endocrine tumor with poor prognosis, particularly when
80 metastatic at diagnosis. It lacks selective and efficacious therapies, which currently consist of surgical
81 resection (R0) and administration of the adrenolytic drug mitotane [1] in association with cytotoxic
82 agents in advanced stages [2]. Recently, significant advances in the molecular diagnosis and prognosis
83 of the tumor have been achieved by integrated genetic profile analysis of large patient cohorts [3-4].
84 Nevertheless, the mechanisms inducing an aggressive and metastatic phenotype in ACC remain elusive
85 [5]. So far, few studies have searched for protein markers capable of not only discriminating between
86 benign forms of adrenocortical tumours (ACA) and ACC, but also predicting tumor progression [5]. A
87 recent proteomic study [6] identified Fascin-1 (FSCN1) as a potential malignancy marker by a two-
88 dimensional-differential-in-gel-electrophoretic (2D-DIGE) approach performed in ACC versus normal
89 adrenals. FSCN1 is an actin-bundling protein involved in formation of filopodia and invadopodia [7-8].
90 It is almost absent in most normal epithelial tissues and highly expressed in many human carcinomas
91 [9]. Its upregulation has been associated with a poor prognostic value and metastatic spread in several
92 carcinomas, as revealed by recent meta-analyses and systemic reviews [10-11].

93 The aim of the present study was to assess the diagnostic and prognostic value of FSCN1 compared
94 with the current histopathological parameters in a local monocentric series of 74 adrenocortical
95 tumours (37 ACCs and 37 ACAs). Results were further confirmed in two independent ACC validation
96 cohorts. Finally, we investigated the role of this protein in promoting tumor cell invasion in a human
97 ACC cell line that overexpresses the transcription factor Steroidogenic Factor-1 (SF-1) resulting in a
98 more aggressive and invasive phenotype [12].

99

100

101 **MATERIALS AND METHODS**

102 **Patients and ethical approval**

103 All patients, or their parents in the case of paediatric patients, gave their written informed consent to
104 the study. The study consists of a local cohort of n=37 patients affected by malignant (ACC) and n=37
105 by benign (ACA) adrenal tumours, whose clinical characteristics are detailed in Tables 1 and 2,
106 respectively. All patients underwent surgical removal of the tumor at Careggi University Hospital in
107 Florence (Florence series). Tumor samples were snap frozen and stored at -80°C until protein/mRNA
108 extraction, or were formalin-fixed/ paraffin embedded for immunohistochemistry. The study was
109 approved by the Local Ethical Committee (Prot.2017-277 BIO 59/11, 27/09/2017).

110

111 **Histology and immunohistochemistry**

112 Histological diagnosis of ACC and ACA was recorded by a reference pathologist (G.N.) on tumor
113 tissue removed at surgery. Tumor specimens were evaluated according to the Weiss score system in
114 which the presence of three or more criteria highly correlates with malignant behaviour [13].

115 The Ki67 labelling index (Ki67 LI) was evaluated as a proliferation marker to assess ACC prognosis
116 using the anti-human Ki67 antibody (1:40 dilution, MIB-1, Dako, Carpenteria, CA, USA). Ki67
117 positive nuclei were counted in 1000 tumor cells and Ki67 LI was expressed as the percentage of
118 labelled cells.

119 Tumor stage was evaluated according to the revised TNM classification of ACC proposed by the
120 European Network for the Study of Adrenal Tumours [14].

121 Immunohistochemical analysis was performed as previously described [6]. Briefly, 3 μm sections were
122 de-paraffinised, hydrated with graded ethanol concentrations until distilled water. Sections were stained
123 with anti FSCN1 antibody (1:100 dilution, sc-46675, Santa Cruz Biotechnology, Santa Cruz, CA,
124 USA) and anti synaptophysin antibody (prediluted, MRQ-40, Cell Marque, Rocklin, CA, USA) after

125 treatment with 3.0% hydrogen peroxide in PBS. Immunohistochemical analysis was carried out using
126 DAKO EnVision™ FLEX (Dako, Carpenteria, CA, US). Negative controls were incubated without the
127 primary antibody.
128 Immunostaining results were analysed using a light microscope at high magnification. FSCN1 staining
129 intensity was evaluated independently by two investigators blind to the clinical data (G.N. and R.S.).
130 The inter-observer agreement for the scoring system was evaluated by using the Cohen k coefficient
131 (0.98) and confirmed by using Pearson's correlation coefficient (0.95). In the case of discrepancy, a
132 score was agreed on by a joint re-evaluation of the slides. Cytoplasmic staining intensity was estimated
133 using a score of 0, 1, 2, or 3, which corresponded to negative, weak, moderate, and strong intensity.
134 The proportion of positive tumor cells was calculated for each specimen and scored 0 if 0%, 0.1 if 1 to
135 9%, 0.5 if 10 to 49%, and 1 if >50% of tumor cells were positive for FSCN1. A semiquantitative H-
136 score was then calculated by multiplying the staining intensity grade by the proportion score [15]. The
137 cut-off point for separating samples with high or low FSCN1 expression was between the H-scores < 2
138 or ≥ 2.
139

140 **SDS-PAGE and Western blot analysis**

141 Tissue samples were homogenised by mechanical disruption with Ultraturrax T10 basic IKA (Werke
142 Gmbh & Co, Staufen, Germany) in RIPA lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 0.5%
143 Triton-100, 1 mM Na_3VO_4, 1 mM PMSF). Cell samples were prepared as previously described [16].
144 After protein measurement using the Bradford method, equal amounts of proteins for each cell and
145 tissue sample (30 µg) were separated by 10% SDS–PAGE and transferred onto PVDF membranes
146 (Immobilon, Merck Millipore, Milan, Italy). Membranes were incubated overnight at 4°C with the
147 following primary antibodies: anti FSCN1 (sc-46675, Santa Cruz Biotechnology), anti VAV2

148 (ab52640, Abcam, Cambridge, UK), anti SF-1 (07-618, Millipore), anti beta-tubulin (T4026, Sigma-
149 Aldrich), anti beta-actin (sc-1615, Santa Cruz Biotechnology), and anti GFP (ab6556, Abcam) followed
150 by species-specific peroxidase-conjugated secondary IgG (1:2000) incubation at room temperature for
151 1 hour. Image acquisition was performed with a ChemiDoc XRS instrument (BIO-RAD Labs, CA,
152 USA). Semiquantitative densitometric analysis of FSCN1 band intensity was performed by Quantity
153 One software of analysis (BIO-RAD Labs), with a grading score of low or high intensity,
154 corresponding to absence/band intensity below or above the intensity of the FSCN1 band of the internal
155 standards for ACC (IS_{ACC}) or ACA (IS_{ACA}). IS_{ACC} and IS_{ACA} consist of the same total protein amount
156 derived from equal amounts of all ACC or ACA samples, respectively, run in each gel. All Western
157 blots were repeated in at least three independent experiments. Beta-actin or beta-tubulin was used as
158 internal loading standards to normalise protein expression.
159
160 **RNA isolation and quantitative real-time RT-PCR**
161 mRNA isolated from tissues of the local ACC cohort was subjected to quantitative real-time RT-PCR
162 (qRT-PCR) for the following gene transcripts: *SF-1/NR5A1*, *FSCN1* and *GAPDH* (Taqman gene
163 expression assay, Applied Biosystems: Hs000610436_m1, Hs0060251_m1, 4352934). The amount of
164 target, normalised to the endogenous reference gene (*GAPDH*) and relative to a calibrator (Stratagene),
165 was calculated by $2^{-\Delta\Delta Ct}$.
166
167 **Cell culture**
168 The H295R/TR SF-1 cell line has been developed and fully characterized in our laboratory [12, 16].
169 Cells were cultured in Dulbecco's modified Eagle's medium-F12 supplemented with penicillin-
170 streptomycin, 2% NuSerum (BD Biosciences), 1% ITS+ (BD Biosciences) and blasticidin (5 µg/ml) -

171 zeocin (100 µg/ml) (both from Cayla InvivoGen). Doxycycline (Sigma-Aldrich) was used at the
172 concentration of 1 µg/ml in experiments involving increased SF-1 expression.
173
174 **Immunofluorescence and filopodia detection and quantification**
175 This was performed as described [16] using Alexa Fluor-594 phalloidin (Invitrogen) to visualise F-
176 actin. For each condition, between 120 and 360 cells were scored.
177
178 **Transwell invasion assay through Matrigel**
179 This was performed as described [16] using the CytoSelect 24-well cell invasion assay kit according to
180 the manufacturer's instructions (CBA-100, Cell Biolabs). In invasion experiments, the FSCN1 inhibitor
181 G2 (Xcessbio Biosciences) was used at the concentration of 10 µM.
182
183 **Knock-down and rescue experiments**
184 The following siRNAs were used in knockdown experiments:
185 - siFascin #1: GAGCAUGGCUUCAUCGGCU [17]
186 - siFascin #2: CACGGGCACCCUGGACGCCAA [17]
187 - VAV2: AGUCCGGUCCAUAGUCAAC [16]
188 - control siC (medium GC; Invitrogen)
189 Cells were transfected by Amaxa nucleofection, as described [16]. For rescue experiments, plasmids
190 encoding GFP (pEGFP-C2; Clontech), GFP-*X. tropicalis* fascin [18] and RNAi insensitive GFP-VAV2
191 [16] were co-transfected together with the siRNAs.
192
193 **Statistical analysis**

194 Data was expressed as mean±SD or in cell experiments as mean±SEM. Statistical analysis was
195 performed by SPSS 24.0 (Statistical Package for the Social Sciences, Chicago, US) for Windows.
196 Correlation analyses were carried out using a χ^2 test for categorical and Pearson's/Spearman's test for
197 parametric/ nonparametric continuous variables, respectively. The inter-observer agreement for the
198 scoring system was evaluated by using the Cohen k coefficient and confirmed by using Pearson's
199 correlation coefficient. The cut-off for strong agreement chosen for the k coefficient was 0.81, and 0.75
200 for the Pearson coefficient [19]. Differences in continuous variables were analysed by means of the
201 Student's t test for independent data to compare two classes of data, or 1-way ANOVA with
202 Bonferroni's correction for multiple testing.
203 For FSCN1 mRNA analysis three independent ACC cohorts were studied (local, Cochin and TCGA).
204 The local cohort consisted of n=21 ACC samples where the amount of FSCN1 normalised on GAPDH
205 expression was measured by qRT-PCR Taqman analysis (Taqman gene expression assay, Applied
206 Biosystems: Hs0060251_m1, 4352934). The Cochin cohort included 48 ACCs (Gene Expression
207 Omnibus data set GSE49280 and ArrayExpress data set E-TABM-311) [3], and the TCGA cohort
208 included 78 ACCs (https://gdc-portal.nci.nih.gov/ projects/TCGA-ACC) [4]. Patients' data is listed in
209 Suppl. Table S1. For the Cochin cohort, all samples were normalised using the Robust Multiarray
210 Average algorithm (Bioconductor affy package), and probe set intensities were then averaged per gene
211 symbol. For the TCGA cohort, mRNA sequencing data was extracted from Broad Institute GDAC
212 Firehose (TCGA data version 2015_08_21), and all calculations were performed on log2 values of
213 RSEM-normalised read counts. Differential abundance was measured with moderated t test (limma R
214 package). To avoid introducing bias by identification of the best cut-off, the median value was used in
215 each cohort.
216

217 **RESULTS**

218 **Diagnostic power of FSCN1 expression measurement**

219 FSCN1 expression was evaluated by immunohistochemistry and Western blotting in samples from a
220 series of n=37 ACCs and n=37 ACAs diagnosed and operated at Careggi University Hospital. Patients'
221 clinico-pathological characteristics are described in Table 1 (ACCs) and Table 2 (ACAs).

222 FSCN1 immunohistochemical expression was high (H score $\geq$ 2) in 79% (26/33) of ACCs, whereas an
223 intense FSCN1 band was detected in 71% (15/21) of ACC by Western blot analysis. Conversely, only
224 55% of ACAs (20/36) showed a strong immunoreactivity for FSCN1 (H score $\geq$ 2) and the
225 corresponding band was observed with high intensity in 17% (6/36) of ACAs by Western blotting, with
226 χ^2=4.2, p=0.04 (in IH), and χ^2=17.1, p<0.001 (in Western blotting), between ACCs and ACAs for the
227 two techniques, respectively. Correlation between the two techniques was more stringent and
228 significant in ACCs than ACAs (χ^2=11, p=0.001, and χ^2=6, p=0.014, respectively).

229 Representative images of FSCN1 staining scores are illustrated in Fig.1A. FSCN1 expression was
230 assessed by semiquantitative Western blot analysis of representative ACC and ACA samples,
231 demonstrating the relatively lower expression of FSCN1 in benign *vs*. malignant neoplasms (Fig.1B).

232 Notably, FSCN1 distribution was not homogenous in all ACC samples, tending to concentrate at the
233 periphery of the tumor mass in advanced ACCs (Fig.2B). Non-tumoral tissue areas were negative for
234 FSCN1 (Fig. 2B, asterisks). Any artefact due to non-homogenous fixation (rim effect) could be
235 excluded, since immunostaining of tumor serial sections showed strong and diffuse reactivity for
236 neuroendocrine markers, i.e.synaptophysin (Fig. 2C).

237

238 **Prognostic power of FSCN1 expression**

239 In ACC cases, no significant correlation was found between clinico-pathological parameters (age, sex,
240 tumor diameter, functional activity, stage, Weiss score and Ki67 LI) and FSCN1 detected by
241 immunohistochemistry, Western blot or qRT-PCR.
242 To assess the value of FSCN1 in predicting disease-free (DFS) and overall (OS) survival, we
243 constructed Kaplan-Meier curves stratifying ACC patients according to high and low FSCN1
244 immunohistochemical expression: FSCN1 proved to be significantly associated with DFS (Fig.3A) and
245 OS (Fig.3B).
246 When estimating the prognostic value of tumor stage (Fig.3 C,D) and Ki67 LI (Fig. 3 E,F) in the
247 Florence ACC series, the addition of FSCN1 immunohistochemical reactivity as a second parameter
248 for stratification in Kaplan-Meier analysis significantly improved prediction of DFS (Fig. 3 G,I) and
249 OS (Fig. 3 H,J).
250 Expression levels of the adrenocortical lineage marker SF-1 have previously been proposed as an
251 independent immunohistochemical prognostic marker in ACC associated with tumor aggressiveness
252 [15,20]. We therefore measured both *SF-1* and *FSCN1* gene expression by qRT-PCR in our ACC cases,
253 and found a significant correlation between *SF-1* and *FSCN1* transcript levels ($r=0.773$, $p<0.001$),
254 $n=21$, Fig 4A).
255 When stratifying patients in two groups based on their clinical parameters (Stage: 3-4 *vs.* 1-2; Ki67 LI:
256 ≥ 10 *vs.* <10), *FSCN1* expression detected by qRT-PCR was significantly higher in tumor high-risk
257 groups (Fig. 4B). Interestingly, FSCN1 levels were significantly higher in tumors displaying at least
258 one of the three Weiss score parameters associated with invasion (i.e. sinusoidal, venous and capsular
259 invasion), "invasive" Weiss score (Fig. 4B). A significant positive correlation was found between
260 FSCN1 expression classes (low and high, with cut-off value defined as the median of FSCN1 gene
261 expression distribution) and Weiss score, stratified according to at least one of the three parameters
262 associated with invasion ($\chi^2=4.11$, $p=0.043$). Stratification of the Weiss score by invasion

263 characteristics significantly correlated (χ^2=11.5, p=0.001) with stage stratification by tumor
264 aggressiveness (stage 1-2 vs. 3-4). These findings suggest that FSCN1 is associated with invasive ACC
265 capabilities. Similar results were obtained for quantitative *SF-1* expression (Fig.3C), when cases were
266 stratified according to tumor stage, further confirming that SF-1 is overexpressed in aggressive ACCs
267 [15-20].
268 Similar to what was found for FSCN1 protein expression, Kaplan-Meier analysis obtained by
269 stratifying patients in low and high expression levels of *FSCN1* transcript showed that it correlated with
270 DFS (Fig.5A) and OS (Fig.5B). Notably, prognostic analysis performed on two independent validation
271 cohorts of ACC patients, the Cochin [3] and TCGA [4] series, confirmed the significant role of *FSCN1*
272 expression in both tumor recurrence (Fig.5C,E) and OS (Fig.5D,F). The calculated hazard risk (HR)
273 values were comparable for DFS and OS, respectively, in all three cohorts (Fig.5).
274

275 **FSCN1 is involved in supporting the invasive phenotype of ACC ~~H295R~~ cells overexpressing SF-**
276 **1**
277 Since FSCN1 has been implicated in the migration, invasion and metastasis of several cancer cell types
278 [18,21-22], we investigated its role in regulating the invasive phenotype of ACC H295R cells. We used
279 the H295R cell model overexpressing SF-1 in a doxycycline (Dox)-inducible fashion (H295R/TR SF-
280 1), having previously demonstrated that increased SF-1 dosage in H295R cells affects cytoskeleton
281 remodelling and invasive properties through upregulation of the guanine nucleotide exchange factor
282 VAV2 [16]. Two different siRNAs were able to efficiently downregulate *FSCN1* expression in
283 H295R/TR SF-1 cells (Fig. 6A). Importantly, *FSCN1* knock-down could significantly inhibit filopodia
284 formation associated with the increased SF-1 dosage in H295R cells (Fig. 6B), although it had no effect
285 on the number of cells showing filopodia under conditions of basal SF-1 dosage. Similarly, *FSCN1*

286 knock-down or pharmacological inhibition with G2, a small molecule that hinders FSCN1 actin-
287 bundling activity, blocked cell invasion through Matrigel only when SF-1 dosage was increased, with
288 no effect on their invasive properties in the presence of SF-1 basal levels (Fig. 6C-E). This phenotype
289 is strikingly similar to the effects of VAV2 knock-down, which also selectively inhibits H295R cell
290 invasion only under conditions of increased SF-1 dosage [16]. For this reason, we tested whether
291 VAV2 and FSCN1 could compensate for the effects of each other's knock-down in invasion assays. As
292 shown in Fig. 6F, transfection of expression vectors encoding GFP-Fascin-1 and GFP-VAV2 could
293 both rescue the effect of FSCN1 knock-down in H295R/TR SF-1 cells treated with Dox. GFP-VAV2
294 but not GFP-Fascin-1 completely rescued the effect of VAV2 knock-down under the same conditions.
295 Knock-down specificity by siFSCN1- siVAV2 and expression of the GFP-fusion proteins is shown in
296 Fig.7.
297

298 **DISCUSSION**

299 Recent ACC guidelines suggest the importance of integrating molecular analysis with classical clinical
300 parameters to improve the management of this rare and aggressive tumor [23]. Over the last decade,
301 significant advances have been made in identifying alterations in candidate genes and their expression.
302 Nevertheless, very few routinely screenable protein markers have been recognised as being associated
303 with classical clinical parameters, thus allowing improvement in ACC prognostication [5]. Given that
304 these proteins mark the metastatic signature of ACC, they could also represent potential targets for the
305 development of novel personalised anti-cancer therapies.

306 In the present paper, we confirm our previous finding that FSCN1 is differentially expressed in ACCs
307 and normal adrenals [6], with no FSCN1 reactivity in normal tissue areas, but strong positivity in
308 neoplastic areas. We now demonstrate that FSCN1 cannot statistically be considered a valid marker for
309 differential diagnosis between malignant and benign neoplasms; however, FSCN1 protein levels in the
310 Florence series were generally lower in ACA than ACC samples.

311 Our previous paper [6] also demonstrated that FSCN1 expression is absent in normal adrenals,
312 confirming results obtained for other organs and their tumor counterparts [9]. This suggests that
313 FSCN1 displaces other actin-bundling proteins in the transformed cells, promoting and stabilizing
314 formation of filopodia and invadopodia, specialized and organized actin-rich cell protrusions that
315 favour cell migration [8].

316 Interestingly, FSCN1 localisation in ACCs is highly heterogeneous, from diffuse to focalized
317 expression. The tendency to localise at the tumor border may be the consequence of acquiring
318 migratory and invasive properties in certain subpopulations of the tumor cells, which become more
319 prone to invading the surrounding tissues. FSCN1 expression in breast cancer has also been
320 demonstrated as a key feature in supporting trans-endothelial migration [24], a pivotal step in the
321 metastatic process.

322 Our data shows that FSCN1 levels are significantly higher in the more aggressive tumours, when
323 stratified for stage and Weiss score parameters of invasion (at least for one positive parameter among
324 sinusoidal, venous and capsular invasion), but not for Ki67 LI, implying that FSCN1 correlates with
325 the metastatic potential rather than the proliferative properties of the tumour. We suggest that the Weiss
326 score parameters may differ in weight in relation to the proliferative or invasive properties of the
327 tumour, an aspect that could be considered for better stratification of ACCs. Notably, cases in the
328 Florence series revealed a significant correlation between advanced stages (3-4) and "invasive" Weiss
329 score. We observed similar behaviour for SF-1 immunostaining, with higher levels in aggressive than
330 indolent ACCs, and a significantly high correlation between the expression of these two markers
331 obtained by qRT-PCR.
332 As described for SF-1 [15, 20], Kaplan-Meier analysis of the Florence ACC cohort showed that FSCN1
333 immunohistochemical expression can also significantly predict disease recurrence and OS. Moreover,
334 when combined with stage or Ki67 LI, FSCN1 can refine their prognostication power, so providing a
335 useful protein marker for a more accurate stratification of recurrence risk in patients with ACC. Until
336 now, Ki67 LI has been considered the most powerful predictor of disease recurrence and survival in
337 ACC patients after complete tumor resection [25]. However, due to difficulties in its standardization
338 and reproducibility for many tumor types, including ACC [26], other histopathological parameters,
339 such as the mitotic index [27] or VAV2 [28] have been tested. Here, we have shown that FSCN1 and
340 Ki67 LI are independent parameters and are likely to be associated with two different tumor
341 characteristics, i.e. invasive/metastatic potential and proliferation. Notably, when considering the risk
342 of recurrence, FSCN1 greatly improved the predictive prognostic power of Ki67 LI and stage. In
343 addition, the similar results obtained for the three independent ACC cohorts show that quantitative
344 *FSCN1* gene expression is a robust independent prognosticator, as no significant correlation was found
345 between FSCN1 and clinico-pathological parameters. Interestingly, three different and independent

346 analytical techniques were employed for each of the three cohorts regarding gene expression
347 quantitative evaluation and correlation analysis, but very similar results in terms of HR were obtained
348 in all three cohorts. These findings not only strengthen our results, but also suggest that different
349 methods can be routinely applied to measure *FSCN1* abundance.
350 Together with VAV2, FSCN1 is one of the few histopathological markers associated with invasion, and
351 combined with genetic parameters, that could be used as an independent factor to further improve
352 patient stratification.
353 In addition to its prognostic value, FSCN1 may also represent a novel therapeutic target for ACC,
354 particularly in the advanced stages, where therapeutic options are rather disappointing and call for new
355 and more effective drugs. Our results demonstrate that interfering with FSCN1 by gene silencing or
356 chemical inhibition significantly reduced the increased tumor migration and invasiveness observed in a
357 H295R cell line conditionally overexpressing SF-1, and consequently more aggressive than its wild
358 type counterpart.
359 Repression of invadopodia and filopodia formation by inhibiting FSCN1 is not only important in
360 deterring the migratory ability of cancer cells, but it may also be pertinent to potential immunotherapy
361 strategies. It has been demonstrated that glioma cells silenced for *FSCN1* were more susceptible to
362 cytotoxic lymphocyte attack [29].
363 A recent paper reports that the specific FSCN1 competitive inhibitor G2, we used in our *in vitro*
364 experiments, could block breast cancer invasion and metastatic colonization in a mouse model, with no
365 toxicity or side effects after 2-month treatment [22]. This molecule is highly specific for FSCN1 as it
366 binds directly to one of the actin-binding sites on the protein, stabilizing FSCN1 in its inactive
367 conformation and blocking filopodia formation. Since FSCN1 is expressed at low levels in normal
368 tissues and overexpressed in ACC and other tumours, its inhibition seems a promising strategy, as it
369 acts specifically on tumor cells.

370 VAV2 is a direct SF-1 dosage-dependent target gene that, through its GEF activity, is essential to
371 induce increased migration in Matrigel when SF-1 expression is forced in the H295R ACC cell line
372 [16]. Remarkably, our results show that FSCN1 and VAV2 are both required in filopodia formation
373 and invasiveness only under conditions of increased SF-1 dosage in H295R cells. In particular,
374 silencing or inhibition of *FSCN1* suppressed the increased cell invasiveness when *SF-1/VAV2* is
375 overexpressed (Fig. 4C, D). Transfected *VAV2* could rescue the effect of *FSCN1* knock-down on cell
376 invasion, but *FSCN1* overexpression could not completely compensate for the effect of *VAV2* silencing.
377 These results are consistent with a model whereby VAV2 acts to enhance Cdc42 and Rac1 activation
378 and induce actin polymerization, and finally FSCN1 stabilizes the actin bundles. The final result of this
379 process is the induction of cytoskeletal remodelling and increased cell invasion (Fig. 8).
380 Recently, FSCN1-induced expression and filopodia formation have been reported to mediate the
381 migratory and invasive effects exerted by linoleic acid in an *in vitro* cell line model of breast cancer
382 (30). This unsaturated acid can also stimulate steroidogenesis in adrenocortical cells in vitro (31).
383 Functional ACCs, particularly cortisol-secreting forms, are associated with worse prognosis (32,33).
384 Interestingly, in the Florence series, association analysis between FSCN1 immunoreactivity and
385 cortisol secretion in ACCs displays a trend toward significance $\chi^2=3.5$, $p=0.06$, which suggests that
386 dietary unsaturated fatty acids, such as linoleic acid (LA), may trigger FSCN1 upregulation in ACC
387 cells, eliciting tumor progression and invasiveness. Further studies investigating any correlation
388 between LA levels and FSCN1 expression in ACC are mandatory to validate this hypothesis.
389 FSCN1 expression also occurs in benign tumors but not in normal tissue, thus excluding its use as a
390 diagnostic marker in adrenocortical neoplasms. A similar distribution of FSCN1 has been reported in
391 both benign and malignant colorectal tumors, despite the prognostic power maintained by this marker
392 in adenocarcinomas (34). However, progression from the benign to the malignant form is still debated
393 for adrenocortical tumors (35,36). All together, these findings indicate that FSCN1 may be an essential

394 but not exclusive factor to trigger malignancy, suggesting that additional factors are necessary for
395 acquiring the malignant phenotype. VAV2, which cooperates with FSCN1 to induce ACC cell
396 invasion, may be one of these factors. Further studies are required to elucidate the exact mechanisms by
397 which FSCN1, SF.-1 and VAV2 may concur to support ACC invasive properties.
398 We recognize as a limitation of this study that, for each analysis and correlation, a variable number of
399 ACC samples were considered, since some data were not available for all patients and some techniques
400 could not be applied to all samples (FSCN1 immunohistochemistry and Western blot analysis, FSCN1
401 and SF-1 RNA expression). In particular, FSCN1 immunostaining could not be performed in the
402 validation cohorts, due to the retrospective nature of data available and the lack of tissue samples.
403 In conclusion, survival analysis conducted in three ACC independent cohorts shows that FSCN1
404 expression is a robust independent prognostic marker. Since FSCN1 is a key protein in promoting
405 tumor cell functions involved in invasion, it could also serve as a potential therapeutic target to
406 specifically interfere with ACC spread and metastasis.
407

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411

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542

543 **FIGURE LEGENDS**

544 **Fig. 1. FSCN1 protein expression in adrenal tumours. (A)** Representative immunohistochemical
545 staining of FSCN1 **(right panel,** 20X magnification) for each intensity score (0-3) coupled with
546 haematoxylin-eosin section **(left panel)** in ACC specimens. **(B)** Representative Western blotting for
547 FSCN1 performed on ACCs and ACAs with different intensity grading (high and low, evaluated *vs.* the
548 respective internal standards, IS_{ACC} and IS_{ACA}). Lanes were normalised with respect to actin.

549

550 **Fig. 2. FSCN1 localization in ACC.** Haematoxylin-eosin staining of a representative advanced ACC
551 (5X magnification, **A**). Immunohistochemistry shows positivity concentrated at the tumor border for
552 FSCN1 (**B**) but not for synaptophysin (**C**). Asterisks indicate normal tissue areas, negative for FSCN1
553 immunohistochemistry.

554

555 **Fig. 3. FSCN1 as detected by IH predicts disease free survival and overall survival in ACC.**
556 Disease free (DFS) and overall (OS) survival Kaplan-Meier curves according to low/high classes of
557 FSCN1 immunohistochemical expression (**A** and **B**), low (1-2)/high (3-4) stages (**C** and **D**), Ki67 LI
558 $<10/\geq10$ classes (**E** and **F**), or when samples were stratified in 3 classes combining FSCN1 and stage
559 (**G** and **H**) or FSCN1 and Ki67 LI (**I** and **J**); p values determined using a Log-Rank test, and number of
560 cases in each group are indicated.

561

562 **Fig. 4. *FSCN1* and *SF-1* quantitative gene expression in ACC stratified for clinical**
563 **characteristics.** (A) Positive correlation between *FSCN1* and *SF-1* gene expression (with *GAPDH* as a
564 reference gene) evaluated by Taqman quantitative real time RT-PCR in ACC samples; $r=0.773$,
565 $p<0.001$, $n=21$.

566 Bar graphs represent mean±SEM *FSCN1* (B) and *SF-1* (C) gene expression (with *GAPDH* as a
567 reference gene) evaluated by Taqman quantitative RT-PCR in ACC samples stratified for stage, Ki67
568 LI, and Weiss score parameters of invasiveness ("invasive" Weiss score defined as at least one positive
569 parameter among sinusoidal invasion, venous invasion, capsular invasion); p values (*p<0.05,
570 **p<0.005, and ***p<0.001) were calculated by Student's t-test. Cut-off values to define each class and
571 number of samples (n) are indicated.
572
573 **Fig. 5. High FSCN1 transcript levels are a negative prognostic factor in ACC: a multicentre**
574 **analysis.** Disease free (DFS) and overall (OS) survival Kaplan-Meier curves according to FSCN1
575 transcript classification in low/high levels in three independent ACC cohorts, local **(A,B)**, Cochin
576 **(C,D)** and TCGA **(E,F)**. Log rank and Hazard Risk (HR) are indicated, with respective p values.
577
578 **Fig. 6. FSCN1 selectively regulates cytoskeleton remodelling and invasion in H295R cells**
579 **overexpressing SF-1.** (**A**) Immunoblots showing FSCN1, SF-1, VAV2 and beta-tubulin expression in
580 H295R/TR SF-1 cells transfected with a control siRNA (siC) or two different siRNAs directed against
581 *FSCN1* (siFSCN1 #1 and siFSCN1 #2), in basal culture conditions or after treatment with doxycycline
582 (Dox; 1 μg/ml) to induce SF-1 overexpression, as indicated. (**B**) Data is reported as mean±SEM
583 percentage of filopodia-forming cells in H295R/TR SF-1 cells transfected with siC or siFSCN1 #1 in
584 basal culture conditions or upon Dox treatment in n=3 independent experiments; ***p<0.001, 1-way
585 ANOVA with Bonferroni's correction for multiple testing. (**C,D**) Invasion through Matrigel by
586 H295R/TR SF-1 cells transfected with siC or siFSCN1 #1 **(C),** with siC or siFSCN1 #2 **(D),** as well as
587 treated or untreated with the FSCN1 inhibitor G2 (10 μM) **(E)**, under basal culture conditions or upon
588 Dox treatment. Data is reported as mean±SEM percentage of Matrigel-invading cells over ctrl in
589 H295R/TR SF-1 cells, under basal culture conditions or upon Dox treatment; *p<0.05 and ***p<0.001,

590 1-way ANOVA with Bonferroni's correction for multiple testing in n=3 **(C)** or n=4 **(E)** independent
591 experiments; **p<0.01 Mann-Whitney test in n=6 independent experiments **(D)**. (**F**) Data is reported as
592 mean±SEM percentage of Matrigel-invading cells over ctrl in H295R/TR SF-1 cells treated with Dox
593 transfected with siC, siFSCN1 #1 or siVAV2 and expression plasmids for GFP, GFP-FSCN1 or GFP-
594 VAV2, as evaluated in n=3 independent experiments; *p<0.05, **p<0.01, ***p<0.001, ns (not
595 significant). 1-way ANOVA with Bonferroni's correction for multiple testing.
596
597 **Fig. 7. Specificity of knock-down and rescue experiments in H295R cells. (A)** Specificity of *FSCN1*
598 and *VAV2* knock-down by the respective siRNAs. (**B**) Expression of GFP, GFP-FSCN1 and GFP-
599 VAV2 proteins in the experiments shown in Fig. 5F.
600
601 **Fig. 8. A schematic hypothesis of the role of VAV2 and FSCN1 in promoting cytoskeletal**
602 **remodelling and invasion of cancer cells.** By its GEF activity, VAV2, a gene transcriptionally
603 upregulated following SF-1 overexpression in ACC cells, favours activation of Cdc42 and Rac1, which
604 promote cytoskeleton remodelling and filopodia - lamellipodia/ruffles formation. These structures are
605 stabilized by FSCN1, leading to increased cell migration and invasion.
606