

1 Targeting Oncogenic Src Homology 2 Domain-Containing Phosphatase 2

2 (SHP2) by Inhibiting its Protein-Protein Interactions

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4 Sara Bobone,¹ Luca Pannone,^{2,3} Barbara Biondi,⁴ Maja Solman,⁵ Elisabetta Flex,³ Viviana
5 Canale,¹ Paolo Calligari,¹ Chiara De Faveri,⁶ Tommaso Gandini,⁶ Andrea Quercioli,¹ Giuseppe
6 Torini,¹ Martina Venditti,² Antonella Lauri,² Giulia Fasano,² Jelmer Hoeksma,⁵ Valerio Santucci,¹
7 Giada Cattani,¹ Alessio Bocedi,¹ Giovanna Carpentieri,^{2,3} Valentina Tirelli,⁷ Massimo Sanchez,⁷
8 Cristina Peggion,⁶ Fernando Formaggio,^{4,6} Jeroen den Hertog,^{4,8} Simone Martinelli,^{3,†} Gianfranco
9 Bocchinfuso,^{1,†} Marco Tartaglia,^{2,†} Lorenzo Stella^{1,*}

10
11 1. Department of Chemical Sciences and Technologies, University of Rome Tor Vergata, Rome,
12 00133, Italy.

13 2. Genetics and Rare Diseases Research Division, Ospedale Pediatrico Bambino Gesù, IRCCS,
14 Rome, 00146, Italy.

15 3. Dipartimento di Oncologia e Medicina Molecolare, Istituto Superiore di Sanità, Rome, 00161,
16 Italy.

17 4. Institute of Biomolecular Chemistry, Padova Unit, CNR, Padova, 35131, Italy.

18 5. Hubrecht institute - KNAW and University Medical Center Utrecht, Utrecht, 3584 CT, The
19 Netherlands.

20 6. Department of Chemical Sciences, University of Padova, Padova, 35131, Italy.

21 7. Centre of Core Facilities, Istituto Superiore di Sanità, Rome, 00161, Italy.

22 8. Institute of Biology Leiden, Leiden University, Leiden, 2333 BE, the Netherlands.

23
24 † These authors contributed equally.

25 * To whom correspondence should be addressed:
26
27

28 **Abstract**

29 We developed a new class of inhibitors of protein-protein interactions of the SHP2 phosphatase,
30 which is pivotal in multiple signaling pathways and a central target in the therapy of cancer and
31 rare diseases. Currently available SHP2 inhibitors target the catalytic site or an allosteric pocket but
32 lack specificity or are ineffective on disease-associated SHP2 mutants. Based on the consideration
33 that pathogenic lesions cause signaling hyperactivation due to increased SHP2 association with
34 cognate proteins, we developed peptide-based molecules with low nM affinity for the N-terminal
35 Src homology domain of SHP2, good selectivity, stability to degradation and an affinity for
36 pathogenic variants of SHP2 up to 20 times higher than for the wild-type protein. The best peptide
37 reverted the effects of a pathogenic variant (D61G) in zebrafish embryos. Our results provide a
38 novel route for SHP2-targeted therapies and a tool to investigate the role of protein-protein
39 interactions in the function of SHP2.

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43 **Keywords:** *SHP2, PTPN11, Src homology 2 domains, inhibitors, protein-protein interactions*

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45 **Introduction**

46 *SHP2 in physiology and pathology*

47 Tyrosine phosphorylation, regulated by protein-tyrosine kinases (PTKs) and protein-tyrosine
48 phosphatases (PTPs), is a fundamental mechanism of cell signaling. Aberrant tyrosine
49 phosphorylation, caused by hyperactive PTKs, occurs in many malignancies and most current
50 targeted anticancer drugs are PTK inhibitors. PTPs counteract the effects of kinases, and therefore
51 they are generally considered negative regulators of cell signaling and tumor suppressors [Elson
52 2018]. However, the Src homology 2 (SH2) domain-containing phosphatase 2 (SHP2), encoded by
53 the *PTPN11* gene, is a non-receptor PTP that does not conform to this simplistic picture [Tajan
54 2015].

55 SHP2 is ubiquitously expressed and mediates signal transduction downstream of various receptor
56 tyrosine kinases (RTKs): it is required for full and sustained activation of the RAS/MAP kinase
57 pathway [Saxton 1997] and modulates signaling also through the PI3K-AKT and JAK-STAT
58 pathways, among others. Therefore, it is involved in the regulation of multiple cell processes,
59 including proliferation, survival, differentiation, and migration [Tajan 2015]. Therefore, it is not
60 surprising that dysregulated SHP2 function contributes to oncogenesis and underlies developmental
61 disorders [Tajan 2015].

62 *PTPN11* was the first proto-oncogene encoding a tyrosine phosphatase to be identified [Tartaglia
63 2003]. Somatic, gain of function mutations in *PTPN11* are the major cause of juvenile
64 myelomonocytic leukemia (JMML), accounting for approximately 35% of cases [Tartaglia 2003].
65 JMML is a rare and aggressive myelodysplastic/myeloproliferative disorder of early childhood with
66 a very poor prognosis, for which no drugs are presently available. Somatic *PTPN11* mutations also
67 occur in childhood myelodysplastic syndromes, acute monocytic leukemia (AMoL, FAB M5) and
68 acute lymphoblastic leukemia (ALL, “common” subtype) [Tartaglia 2003; 2004]. More rarely,
69 activating mutations in this gene are found in adult myelodysplastic syndromes, chronic

70 myelomonocytic leukemia, as well as solid tumors, including neuroblastoma, glioma, embryonal
71 rhabdomyosarcoma, lung cancer, colon cancer and melanoma.

72 In addition to malignancies driven by *PTPN11* mutations, several forms of cancer are linked to the
73 activity of wild type (WT) SHP2, too. By screening hundreds of cancer cell lines with a shRNA
74 library, a landmark study showed that SHP2 is required for survival of RTK-driven cancer cells
75 [Chen 2016]. SHP2 is also a central node in intrinsic and acquired resistance to targeted cancer
76 drugs [Prahallad 2015], which is often caused by RTK activation through feedback loops.

77 SHP2 is a mediator of immune checkpoint pathways, such as PD-1 [Okazaki 2013]. These signaling
78 cascades inhibit the activation of immune cells, thus allowing self-tolerance and modulation of the
79 duration and amplitude of physiological immune responses. SHP2 binds to the activated receptors
80 and is responsible for starting the signaling cascade that prevents immune cell activation [Okazaki
81 2013]. Some cancer cells are able to hijack these signaling pathways, thus evading antitumor
82 immune defenses; therefore, SHP2 is currently being considered as a possible target for cancer
83 immunotherapy [Marasco 2020a]. Finally, it is worth mentioning that induction of gastric
84 carcinoma by *H. pylori* is mediated by the interaction of its virulence factor CagA with SHP2,
85 causing aberrant activation of the phosphatase [Higashi 2002, Hayashi 2017].

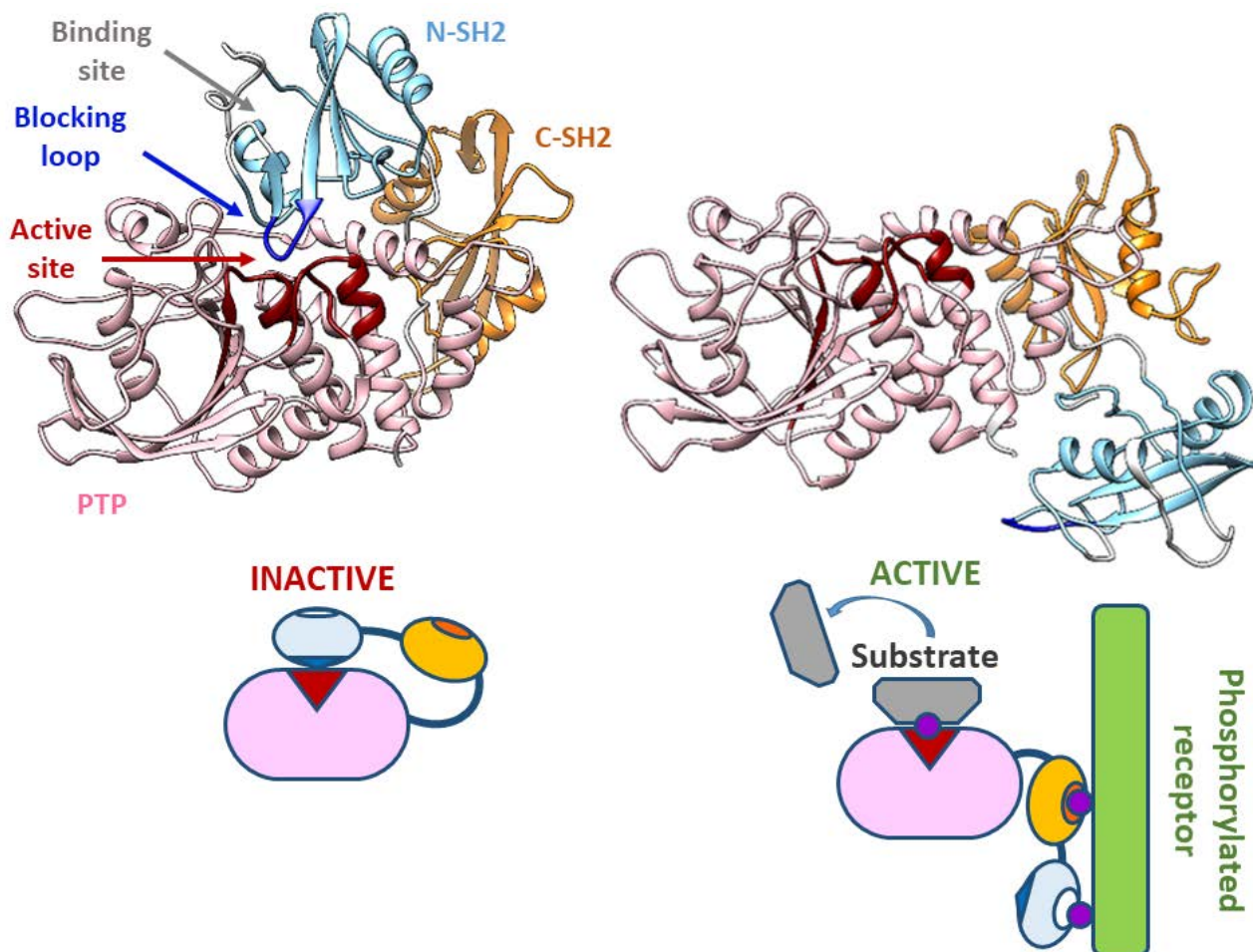
86 In addition to its role in cancer, SHP2 is involved in a family of rare diseases collectively known
87 as RASopathies. Germline missense mutations in *PTPN11* occur in ~50% of individuals affected
88 by Noonan syndrome (NS) [Tartaglia 2001], one of the most common non-chromosomal disorders
89 affecting development and growth [Roberts 2013], and in ~90% of patients affected by the
90 clinically related Noonan syndrome with multiple lentigines (NSML, formerly known as
91 LEOPARD syndrome) [Digilio 2002]. RASopathies are characterized by congenital cardiac
92 anomalies, hypertrophic cardiomyopathy, short stature, musculoskeletal anomalies, facial
93 dysmorphisms, variable intellectual disability and susceptibility to certain malignancies [Tartaglia

94 2010]. To date, the only treatment in use for NS and related disorders is growth hormone therapy,
95 to improve linear growth [Roberts 2013].

96 *Structure and allosteric regulation of SHP2*

97 The structure of SHP2 includes two Src homology 2 (SH2) domains, called N-SH2 and C-SH2,
98 followed by the catalytic PTP domain, and an unstructured C-terminal tail (Figure 1) [Tajan 2015].
99 SH2 domains are recognition elements that bind protein sequences containing a phosphorylated
100 tyrosine (pY) [Liu 2006, Anselmi 2020]. In SHP2, they mediate association to RTKs, cytokine
101 receptors, cell adhesion molecules and scaffolding adaptors. Therefore, SHP2 (together with the
102 closely related SHP1) is recruited (through its SH2 domains) by motifs containing two pYs and
103 dephosphorylates other (or even the same) pYs through its PTP domain. The crystallographic
104 structures of SHP2 [Hof 1998, LaRochelle 2018], complemented by biochemical analyses
105 [Keilhack 2005; Tartaglia 2006; Bocchinfuso 2007; Martinelli 2008], have elucidated the main
106 features of the allosteric regulation of SHP2 activity. Under basal conditions, the N-SH2 domain
107 blocks the active site of the PTP domain, inserting a loop (DE or “blocking” loop) in the catalytic
108 pocket. Consistently, the basal activity of SHP2 is very low. Association of SHP2 to its binding
109 partners through the SH2 domains favors the release of this autoinhibitory interaction, making the
110 catalytic site available to substrates, and causing activation (Figure 1). Specifically, structures of
111 the N-SH2 domain associated to phosphopeptide sequences show that association to binding
112 partners induces a conformational change in the blocking loop, which loses complementarity to the
113 active site [Lee 1994]. At the same time, the N-SH2/PTP interaction allosterically controls the
114 conformation of the N-SH2 domain binding site. Structures of the autoinhibited protein show that
115 the binding site of the N-SH2 domain is closed by two loops (EF and BG). By contrast, in structures
116 of the isolated N-SH2 domain [Lee 1994], or the recently reported structure of the active state of
117 SHP2 [LaRochelle 2018], the binding site is open (Figure 1). Consequently, we and others have
118 hypothesized that the transition between the closed, autoinhibited state and the open, active

119 conformation is coupled to an increased affinity for binding partners [Keilhack 2005; Bocchinfuso
120 2007, Martinelli 2008, LaRochelle 2018].
121 The spectrum of pathogenic *PTPN11* mutations is generally consistent with this picture of SHP2
122 regulation. Most mutations cluster at the N-SH2/PTP interface, destabilizing the interaction
123 between these two domains and causing constitutive activation of the phosphatase [Keilhack 2005;
124 Tartaglia 2006; Bocchinfuso 2007]. These mutations concomitantly induce an increased
125 responsiveness to activation by association of bisphosphorylated sequences to the SH2 domains
126 [Keilhack 2005; Bocchinfuso 2007; Martinelli 2008; LaRochelle 2018]. Other mutations localize
127 in the binding site of the SH2 domains, and simply cause an increased affinity for phosphorylated
128 binding partners [Tartaglia 2006]. In all cases, the final effect is an upregulation of the RAS/MAPK
129 signal transduction pathway.



131 **Figure 1: SHP2 structure and scheme of the activation process.**

132 **Top:** crystallographic structures for the closed, auto-inhibited and the open, active states of SHP2
133 (left and right, respectively). The N-SH2, C-SH2 and PTP domain are colored in light blue,
134 orange and pink, respectively. The N-SH2 blocking loop (DE loop) is colored in blue, while the
135 PTP active site is highlighted in dark red. The EF and BG N-SH2 loops, controlling access to the
136 binding site for phosphorylated sequences of binding partners, are represented in white. PDB
137 codes of the two structures are 2SHP and 6CRF. Segments missing in the experimental structures
138 were modeled as previously described [Bocchinfuso 2007].

139 **Bottom:** schematic model of the allosteric regulation mechanism.

140

141

142 *SHP2 as a pharmacological target*

143 All the findings reported above clearly indicate SHP2 as an important molecular target for cancer
144 and RASopathies [Tang 2020]. Since SHP2 is a convergent node for multiple signaling pathways,
145 SHP2 inhibitors may represent a way to suppress the effect of disease-causing mutations involving
146 different proteins along the signaling cascade with a single molecule [Mullard 2018].

147 Research efforts in SHP2-targeted drug discovery have long been focused mainly on active-site
148 inhibitors [Yuan 2020, Mostinski 2020]. Several molecules inhibiting the catalytic activity of SHP2
149 have been reported, but many of them are affected by the same limitations that led PTPs in general
150 to be considered “undruggable”, *i.e.* lack of target specificity and poor bioavailability [Mullard
151 2018, Yuan 2020]. Some compounds with good affinity and apparent selectivity have been
152 described, but more recent studies demonstrated that these molecules have several off-target effects
153 [Tsutsumi 2018].

154 An alternative pharmacological strategy has been pursued by researchers at Novartis [Chen 2016,
155 Garcia Fortanet 2016, Bagdanoff 2019, Sarver 2019], followed by others [Xie 2017, Mullard
156 2018;Wu 2018; Tang 2020], who reported allosteric inhibitors stabilizing the autoinhibited
157 structure of SHP2 by binding to a pocket located at the interdomain interface in the closed
158 conformation of the phosphatase. SHP099, the inhibitor developed by Novartis, is finding
159 promising applications in the treatment of RTK-driven cancers [LaMarche 2020] and in combined
160 therapy against drug resistant cells [Prahallad 2015]. These results have spurred a renewed interest
161 in the inhibition of phosphatases [Mullard 2018]. Currently, several allosteric inhibitors stabilizing
162 the closed conformation of SHP2 are undergoing clinical trials [Tang 2020]: TNO155
163 [LaMarche2020] by Novartis (derived from SHP099), RMC-4630, by Revolution Medicines and
164 Sanofi, JAB-3068 and JAB-3312, developed by Jacobio Pharmaceuticals and RLY-1971, by Relay
165 Therapeutics. Allosteric inhibitors have also been used to target SHP2 to proteolytic degradation
166 [Wang 2020]. However, these compounds are generally poorly effective in the case of activating

167 *PTPN11* mutants, since the allosteric binding site is lost in the open conformation of the enzyme
168 [LaRoche 2018, Tang 2020].

169 *Inhibition of protein-protein interactions as an alternative pharmacological strategy*

170 Due to the allosteric mechanism described above, SHP2 activation and its association to binding
171 partners are coupled events. Therefore, the effect of NS- and leukemia-causing mutations
172 destabilizing the autoinhibited conformation is twofold: they cause an increase in the phosphatase
173 activity of the protein, but at the same time favor the N-SH2 conformation suitable for binding
174 phosphorylated proteins, thus increasing the overall responsiveness of SHP2 to its interaction
175 partners. Several lines of evidence indicate that the second event, rather than the enhanced basal
176 activity, is essential for the abnormal activation of the RAS/MAPK pathway.

177 Some pathogenic mutations, such as the NS-associated p.T42A, simply increase the binding affinity
178 of the N-SH2 domain, without causing basal activation [Martinelli 2008, Keilhack 2005]; on the
179 other hand, the ability of SHP2 to associate to binding partners is preserved in all the disease-
180 associated *PTPN11* mutations [Tartaglia 2006, Martinelli 2012, 2020].

181 Truncated constructs with deletion or partial deletion of the N-SH2 domain cause a dramatic
182 increase in the enzymatic activity of SHP2 and, at the same time, a complete loss of its ability to
183 bind signaling partners. These constructs do not affect development in heterozygous mice [Saxton
184 1997] and do not cause any aberrant phenotype in cells [Saxton 1997, Higashi 2002]. Indeed,
185 RAS/MAPK signaling in homozygous cells with the mutated construct was reduced with respect
186 to the WT cells [Shi 1998]. However, cellular morphological changes (hummingbird phenotype)
187 were observed when the truncated construct was targeted to cellular membranes by adding a
188 membrane-localization signal [Higashi 2002], demonstrating the importance of proper cellular
189 localization, normally mediated by SH2 domains. The relevance of SHP2 association to its binding
190 partners for its role in aberrant signaling has been demonstrated also by a study on monoclonal
191 antibodies targeting the N-SH2 domain and disrupting its association with adaptor proteins. Expression of

192 these monobodies in cancer cells carrying the activating *p.V45L* mutation abolished ERK1/2
193 phosphorylation almost completely [Sha 2013]. Similarly, Kertész and coworkers [Kertész 2006]
194 reported that the natural SHP2-binding motif of Gab1, when delivered into immune cells,
195 modulated phosphorylation patterns.

196 An example of the opposite situation, where binding is preserved and the catalytic activity is
197 impaired, is provided by *PTPN11* mutations causing NSML, such as T468M. This class of amino
198 acid substitutions are located in proximity of the PTP active site, at the PTP/N-SH2 interface, and
199 have a twofold effect: they destabilize the closed state of the protein, and consequently promote
200 SHP2 association to signaling partners; at the same time, they perturb the active site and therefore
201 strongly impair the catalytic activity of the phosphatase. Interestingly, the phenotype of NSML is
202 very similar to that of NS, and these mutations still allow the activation of multiple effector
203 pathways [Martinelli 2008; Yu 2014].

204 Overall, these findings strongly suggest that a mere enhancement in SHP2 catalytic activity is not
205 sufficient to cause disease and indicate that increased association to binding partners plays a major
206 role in the pathogenic mechanism associated with *PTPN11* mutations. Therefore, inhibition of
207 SHP2 binding to other proteins through its SH2 domains represents a promising alternative
208 pharmaceutical strategy. No molecules targeting the SH2 domains of SHP2 for therapeutic purposes
209 have been developed so far, even though SH2 domains in general have received much attention as
210 potential pharmaceutical targets [Machida 2005, Cerulli 2020]. These recognition units generally
211 have only moderate affinity and selectivity for cognate phosphorylated sequences, with dissociation
212 constants in the 0.1 – 10 μ M range [Kuriyan 1997, Machida 2005, Wagner 2013, Marasco 2020b].
213 However, we recently characterized the structural determinants of phosphopeptide binding by the
214 N-SH2 domain of SHP2 [Anselmi 2020], and our data indicate this particular domain as a favorable
215 exception, since its peculiar features make significantly higher affinities possible.

216 Based on these considerations, we explored the possibility to target SHP2 protein-protein
217 interactions (PPIs), rather than its catalytic activity. We developed a peptide-based molecule with
218 low nM affinity to the N-SH2 domain of SHP2, high specificity and resistance to degradation. This
219 inhibitor rescued the mortality and developmental defects induced by a pathogenic mutation *in vivo*.
220 Our results provide a novel route for SHP2-targeted therapies and offer a new tool to further
221 investigate the role of SHP2 PPIs in the signaling cascades controlled by this phosphatase.

222

223 **Results**

224 *1) Characterization of IRS-1 pY1172/N-SH2 binding*

225 1.1) The IRS-1 pY1172 peptide binds the N-SH2 domain with a low nanomolar affinity

226 The peptide corresponding to pY 1172 (rat sequence, SLN-pY-IDL DLV KD) or pY 1179 (human
227 sequence, GLN-pY-IDL DLV KD) of insulin receptor substrate 1 (IRS-1) has one of the highest
228 known binding affinities for the N-SH2 domain of SHP2 [Anselmi 2020, Marasco 2021]. Based on
229 our study of the structural determinants of high binding affinity to this domain, the IRS-1 pY 1772
230 sequence is near to optimal under several respects, since it has apolar residues at positions +1, +3
231 and +5, which point towards the hydrophobic groove in the N-SH2 structure, and anionic amino
232 acids at positions +2 and +4, which can interact with a peculiar KxK motif in the BG loop [Anselmi
233 2020].

234 The binding affinity of the IRS-1 pY1172 peptide has been characterized in several literature
235 studies. Unfortunately, these results are extremely contradictory, as reported in Table S1, with
236 dissociation constants ranging from ~10 nM to ~10 μ M. Several possible factors can be invoked to
237 explain these discrepancies, including the effect of radioactive labels [Case 1994], dimerization of
238 GST-N-SH2 constructs [Sugimoto 1994, Ladbury 1995] even at low nM concentrations [Fabrini
239 2009], or the sensitivity of the technique [Kelihack 2005]

240 Considering these difficulties, in the present study, we developed a fluorescence anisotropy binding
241 assay. In a direct binding experiment, the fluorescently labeled peptide IRS-1 pY1172 analog CF-
242 P9 (Table 1) was titrated with increasing concentrations of the N-SH2 domain. The fraction of
243 protein-bound peptide was determined from the increase in fluorescence anisotropy (Figure 2), and
244 a K_d of 53 ± 2 nM was obtained (Table 2).

245

246 **Table 1. Peptide sequences investigated in this study**

Abbreviation	Sequence
P9	GLN-pY-IDLDL
P9Y0	GLN- Y-IDLDL
P8	LN-pY-IDLDL
P7	N-pY-IDLDL
P8W5	LN-pY-IDLDW
P8F5	LN-pY-IDLDF
P8E4W5	LN-pY-IDLEW
P9ND0W5 (or OP)	GLN-ND-IDLDW
CF-P9	CF-GLN-pY-IDLDL
CF-P9Y0	CF-GLN- Y-IDLDL
CF-P9W5	CF-GLN-pY-IDLDW
Cy3-P9W5	Cy3-GLN-pY-IDLDW
CF-P9E4W5	CF-GLN-pY-IDLEW
CF-P9ND0W5 (or CF-OP)	CF-GLN-ND-IDLDW
P8W5-TAT	LN-pY-IDLDW-GRKKRRQRRR
CF-P9W5-TAT	CF-GLN-pY-IDLDW-GRKKRRQRRR

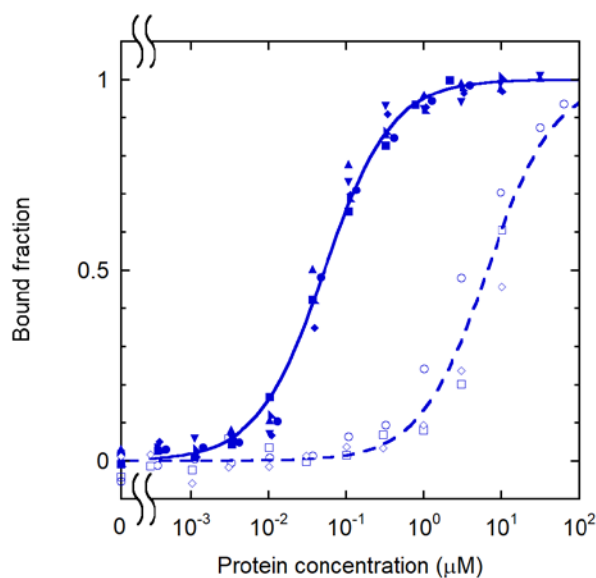
247 All peptides were amidated at the C-terminus. Unlabeled peptides were acetylated at the N-
 248 terminus. CF is 5,6 carboxyfluorescein, Cy3 is Cyanine 3 carboxylic acid and ND is the non-
 249 dephosphorylatable pY mimic phosphonodifluoromethyl phenylalanine (F₂Pmp). The optimized
 250 peptides are highlighted in grey. RP-HPLC retention times (R_t), purities, theoretical molecular
 251 weights and those determined experimentally by ESI-MS spectrometry are reported in Table S2.

252

253

1.2) Phosphorylation contributes only 30% of the standard binding free energy

Association of SH2 domains with the partner proteins is regulated by phosphorylation, and therefore the phosphate group is necessarily responsible for a large fraction of the binding affinity. On the other hand, to have a good selectivity, the rest of the sequence must also contribute significantly to the peptide/protein interaction. To quantify this aspect, we performed a binding experiment (Figure 2) with an unphosphorylated analog of the labeled IRS-1 pY1172 peptide, CF-P9Y0 (Table 1). The affinity was approximately 100 times lower, with a K_d of $6.6 \pm 0.6 \mu\text{M}$, compared with $53 \pm 2 \text{ nM}$ for the phosphorylated peptide. The corresponding values for the standard free energy of binding (assuming a 1 M standard state) are $-29.6 \pm 0.2 \text{ kJ/mol}$ and $-41.6 \pm 0.1 \text{ kJ/mol}$, respectively. Assuming additivity of contributions, the phosphate group results to be responsible for the difference of $-12.0 \pm 0.2 \text{ kJ/mol}$, *i.e.* for less than 30% of the total standard binding free energy of the phosphorylated peptide. This result indicates that the contribution of the rest of the peptide predominates in the binding interactions and bodes well for our design efforts.



268 **Figure 2: Binding curves for the phosphorylated and unphosphorylated IRS-1 pY1172**
269 **peptides.**

270 [CF-P9]=1.0 nM (full symbols and solid line), [CF-P9Y0]=10 nM (empty symbols and dashed
271 line); Replicate experiments are reported with different symbols and were fit collectively.

272

273

274 2) Sequence optimization

275 2.1) The sequence can be reduced to 8 amino acids without loss in affinity

276 Literature data are partially contradictory regarding the effect of shortening the IRS-1 pY1172
277 sequence on the binding affinity. Kay [1998] reported that the sequence could be shortened at the
278 C-terminus down to the +5 residue and at the N-terminus down to the -2 position, without any loss
279 in affinity. By contrast, Case [1994] observed a significant reduction in affinity by shortening the
280 sequence from SLN-pY-IDLDLVKD to LN-pY-IDLDLV. Our previous study clearly indicated
281 that residues -2 to +5 are the most important for the interaction [Anselmi 2020]. To clarify the role
282 of N-terminal residues in determining the N-SH2 domain binding affinity, we performed
283 displacement studies (Figure S1) with the unlabeled peptide P9, and with the shortened analogues
284 P8 and P7 (Table 1), where residues -3 or -2 and -3 were removed, respectively. No significant loss
285 in affinity was observed by reducing the sequence to 8 residues, while removal of the amino acid
286 at position -2 caused a drastic perturbation of complex stability (Figure S1). The -2 to +5 IRS-1
287 sequence is the minimal peptide with a low nM dissociation constant.

288

289 2.2) Single amino acids substitutions improve the K_d to the low nM range.

290 Hydrophobic residues are required at position +1, +3 and +5 of the phosphopeptide sequence
291 [Anselmi 2020], but aromatic residues are present in some natural high affinity binding sequences,
292 at position +5 only [Case 1994, Huyer 1995; Hayashi 2017, Bonetti 2018, Marasco 2020a]. The
293 crystallographic structures of some of these complexes [Lee 1994, Hayashi 2017, Marasco 2020]
294 show that an aromatic side chain can be accommodated by a relatively large hydrophobic pocket
295 and that the +5 peptide residue interacts with the BG and EF loops of the domain, which are
296 important for binding specificity [Lee 1994, Anselmi 2020]. Finally, a preference for aromatic
297 residues at position +5 has been indicated by several peptide library studies [De Souza 2002; Imhof
298 2006; Martinelli 2012; Tinti 2013].

299 Based on these considerations, we analyzed in silico the effect of different aromatic amino acids at
300 position +5. Free energy calculations indicated that substitution of L with the bulkier W (but not
301 with F) could be favorable (Figure 3). The additional substitution of D in +4 with the longer E was
302 evaluated as well, in view of a possible strengthening of the electrostatic interactions with the KxK
303 motif in the BG loop. However, in this case no further increase in binding affinity was predicted by
304 the free energy calculations (Figure 3).

305 Analogs with F or W at position +5 (P8F5 and P8W5), as well as a labeled analog with the L to W
306 substitution (CF-P9W5, Table 1) were synthesized and studied experimentally (Figure 4). As
307 predicted, introduction of W in +5 was highly favorable, leading to reduction in the dissociation
308 constant by an order of magnitude, both for the labeled and unlabeled analog (Tables 2 and 3).
309 Consequently, the dissociation constant for the P8W5 analog was 1.5 ± 0.3 nM. By contrast, the
310 additional D to E substitution resulted in a slight loss in binding affinity (Figure 4 and Table 2 and
311 3).

312 Based on these results, further studies concentrated on the peptide with W at position +5.

313

314 **Table 2. Dissociation constants obtained from the fluorescence anisotropy**
 315 **binding experiments.**

Peptide	Domain/Protein	K_d (nM)
CF-P9	N-SH2	53 ± 2
CF-P9Y0	N-SH2	6600 ± 600
CF-P9W5	N-SH2	3.3 ± 0.2
CF-P9W5	C-SH2	4200 ± 300
Cy3-P9W5	N-SH2	23 ± 2
CF-P9E4W5	N-SH2	8.2 ± 0.7
CF-P9ND0W5 (CF-OP)	N-SH2	68 ± 5
CF-P9ND0W5 (CF-OP)	PTP	10000 ± 800
CF-P9ND0W5 (CF-OP)	SHP2 (WT)	930 ± 70
CF-P9ND0W5 (CF-OP)	SHP2 (A72S)	400 ± 40
CF-P9ND0W5 (CF-OP)	SHP2 (E76V)	330 ± 10
CF-P9ND0W5 (CF-OP)	SHP2 (D61H)	170 ± 10
CF-P9ND0W5 (CF-OP)	SHP2 (F71L)	140 ± 10
CF-P9ND0W5 (CF-OP)	SHP2 (E76K)	48 ± 2

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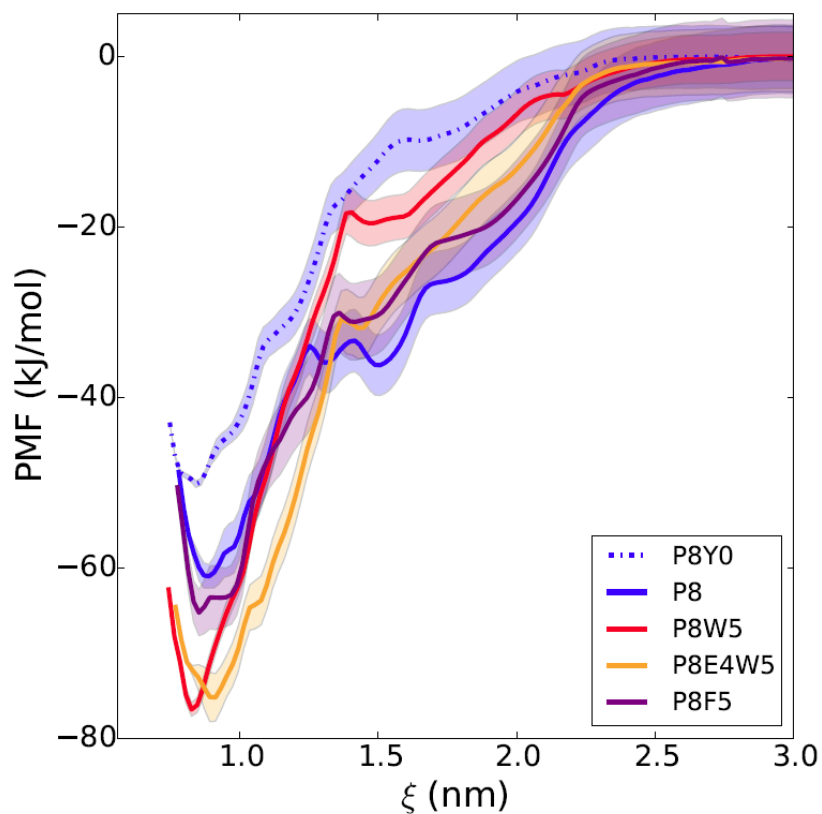
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319 **Table 3. Dissociation constants obtained from the displacement experiments**

Peptide	IC_{50} (nM)	K_i (nM)
P8	47 ± 4	22 ± 4
P8F5	16 ± 1	7 ± 2
P8W5	5.4 ± 0.3	1.5 ± 0.3
P8E4W5	11 ± 1	4 ± 2
P9ND0W5 (OP)	32 ± 5	14 ± 5

320 All measurements were performed on the N-SH2 domain of SHP2. Experiments were performed
321 at $[N-SH2] = 3.4$ nM and $[CF-P9W5] = 0.5$ nM (for P8 and P9ND0W5) or 0.1 nM (for the other
322 peptides).

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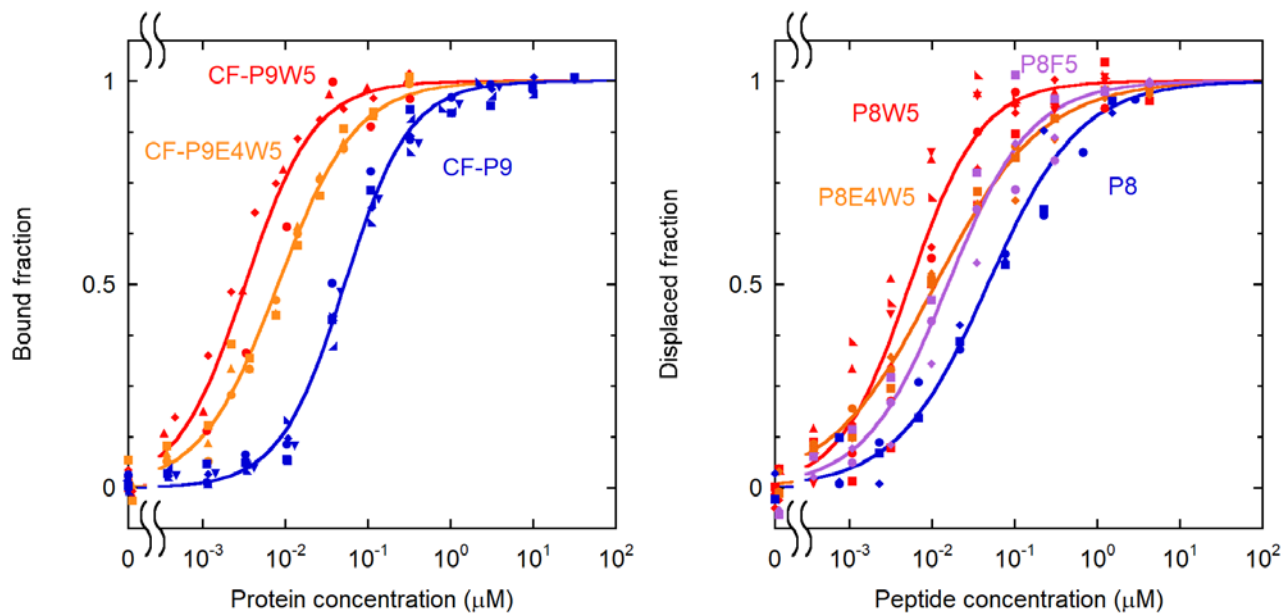
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325 **Figure 3: in silico free energy calculations for different modified sequences**

326 The free energy profile is reported as a function of the distance between the centers of mass of the
327 N-SH2 domain and of the phosphopeptide. The simulations predict a loss in affinity of P8 (blue
328 line) with dephosphorylation of the pY (dashed blue line), and a gain with substitution of the L in
329 position +5 with W (red line), but not with F (violet line). The additional substitution of D in +4
330 with E (orange) does not provide any further increase in affinity. Shaded areas correspond to
331 standard deviations in the PMF profile.

332

333



334

335 **Figure 4: effect of substitutions at position +5 on the binding affinity**

336 **Left:** direct binding experiments; [CF-P9W5]=0.10 nM, [CF-P9E4W5]=0.10 nM, [CF-P9]=1.0
337 nM. Data for CF-P9 are repeated here for comparison.

338 **Right:** displacement assay, [CF-P9W5]=0.10 nM, [N-SH2]=3.35 nM.

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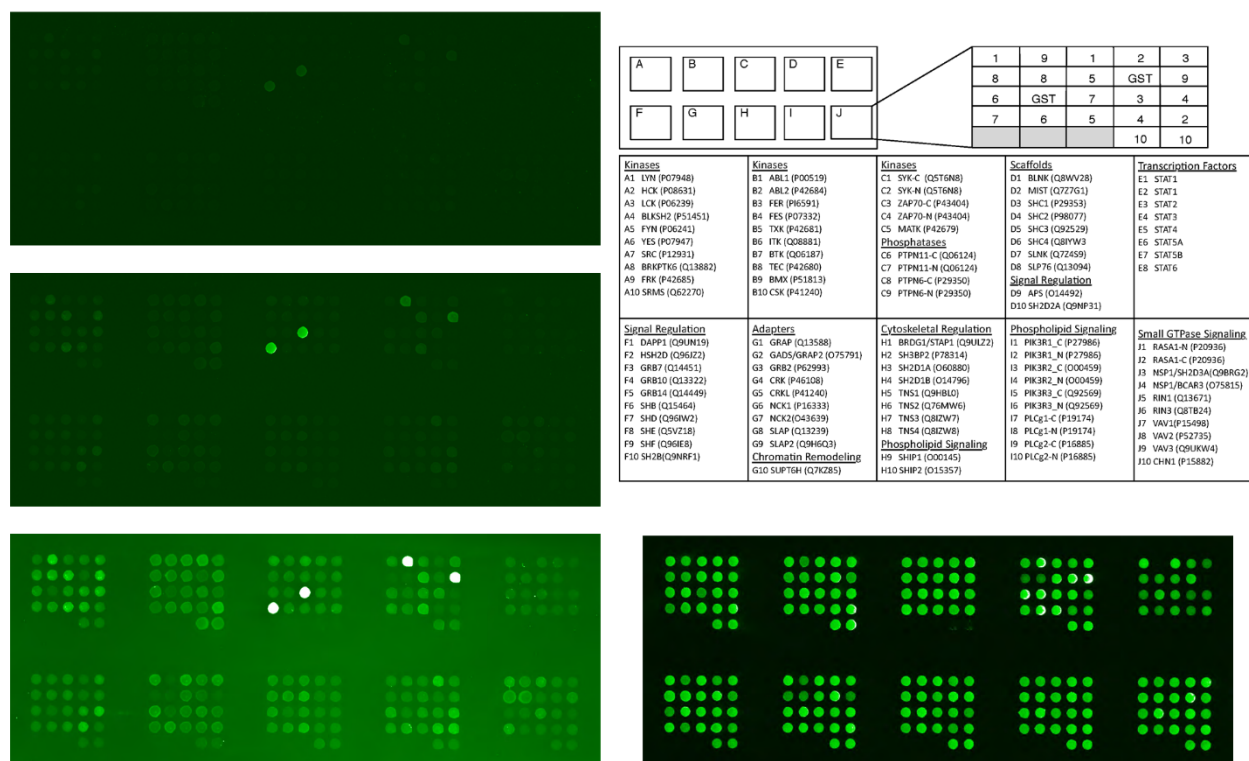
341 *3) Binding selectivity*

342 3.1) The modified sequence is highly selective for the N-SH2 domain of SHP2.

343 The selectivity of binding of CF-P9W5 was first assessed with respect to the C-SH2 domain of
344 SHP2, again with the fluorescence anisotropy assay (Figure S2). As reported in Table 2, the affinity
345 for the C-SH2 domain was almost 1,000 times less than that for the N-SH2 domain.

346 A more complete analysis of the binding selectivity was performed on a protein array of 97 human
347 SH2 domains (Figure 5). An analogue of CF-P9W5 was employed in this assay, where CF was
348 substituted with the Cy3 dye, suitable for detection in the array reader. Control binding experiments
349 showed that the change in fluorophore did affect peptide binding affinity only marginally (Table

350 2). Strikingly, significant binding was observed only with the N-SH2 domain of SHP2, and, to a
 351 lesser extent, to the SH2 domain of the adapter protein APS (also called SHP2B2). It is worth noting
 352 that binding to the N-SH2 domain of SHP1, which has the highest identity with that of SHP2 [Liu
 353 2006], was negligible.



354
 355 **Figure 5: binding selectivity of Cy3-P9W5 for an array of SH2 domains.**
 356 **Left:** fluorescence of the bound peptide, at a concentration of 0.5 nM (top), 5.0 nM (center) and
 357 50 nM (bottom). Each SH2 domain was spotted in duplicate, and negative control spots (with
 358 GST only) are also present. The bright spots correspond to the N-SH2 domain of SHP2 and to the
 359 SH2 domain of the SH2 and PH domain-containing adapter protein APS (also called SHP2B2).
 360 The intensity of all other spots is comparable to that of the negative controls.
 361 **Right:** position of each SH2 domain in the array (top), and control of the protein loading in each
 362 spot, performed with an anti-GST antibody (bottom).

364 4) *Engineering resistance to degradation*

365 4.1) Introduction of a non-hydrolysable pY mimic is compatible with low nM binding affinity

366 In view of intracellular or *in vivo* applications of the phosphopeptide, it is essential to make it
367 resistant to degradation. The most labile moiety is the phosphate group of the pY residue, which
368 can be hydrolyzed by protein tyrosine phosphatase, possibly also including SHP2, of which IRS-1
369 pY 1172 has been shown to be a substrate [Noguchi 1994]. We substituted the pY with the non-
370 hydrolysable mimetic phosphonodifluoromethyl phenylalanine (F₂Pmp), which is isosteric with pY
371 and has a total negative charge comparable to that of pY under physiologic pH conditions [Burke
372 2006, Cerulli 2020].

373 Binding experiments demonstrated that the substituted analogue (CF-P9ND0W5, where ND0
374 indicates the introduction of the non-dephosphorylatable pY mimic at position 0, Table 1) has a
375 dissociation constant for the N-SH2 domain that is just an order of magnitude worse with respect
376 to that of CF-P9W5 (68 ± 5 nM with respect to 4.6 ± 0.4 nM) (Figure S3 and Table 2). Similarly,
377 the dissociation constant for the unlabeled peptide P9ND0W5 was 15 ± 0.4 nM (with respect to 1.6
378 ± 0.4 nM for P8W5) and thus remained in the nM range (Table 3).

379 For the sake of brevity, in the following text, CF-P9ND0W5 and its unlabeled analogue P9ND0W5
380 will be also referred to as the optimized peptides, or CF-OP and OP, respectively.

381

382 4.2) The optimized peptide OP is resistant to proteolytic degradation

383 To test the resistance to proteases, the optimized peptide OP was incubated in human serum for up
384 to 90 minutes, or in DMEM for three days, and then analyzed by HPLC. No significant degradation
385 was observed in these time frames (Figure S4). By contrast, the octadecapeptide HPA3NT3 [Park
386 2008], which we used as a positive control, was completely degraded already after 5 minutes (data
387 not shown). This result bodes well for potential *in vivo* applications of the peptide.

388

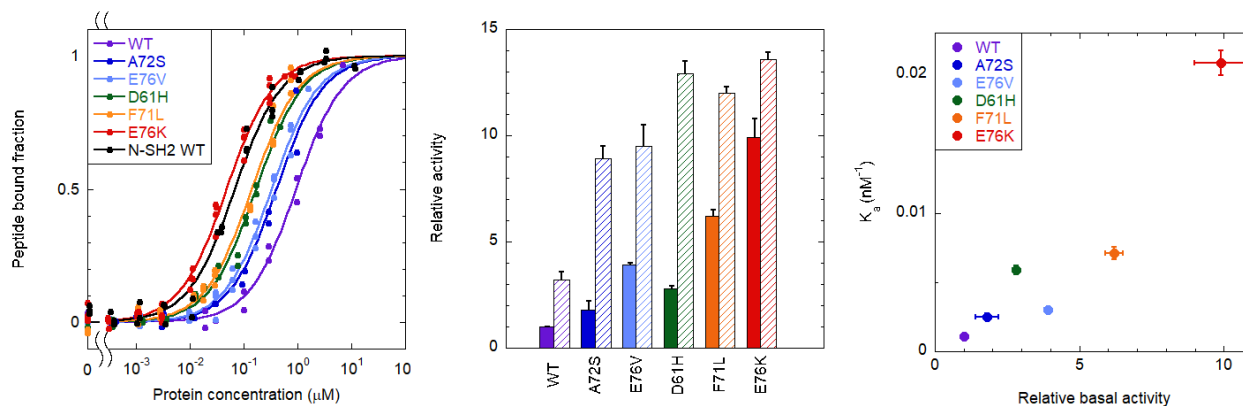
389 5) *Binding to and activation of the SHP2 protein*

390 5.1) OP binds to pathogenic mutants with much higher affinity than to the WT protein

391 As discussed in the introduction, we and others have hypothesized that, in its autoinhibited state,
392 the conformation of the N-SH2 domain prevents efficient association to binding partners, while
393 SHP2 binding affinity to phosphorylated sequences is maximized in the open, active state [Keilhack
394 2005; Bocchinfuso 2007, Martinelli 2008, LaRochelle 2018]. This model has many relevant
395 consequences, because it implies that pathogenic mutants have a twofold effect: they increase the
396 activity of the phosphatase, but also its affinity towards binding partners. In principle, both effects
397 could be the origin of the hyperactivation of the signal transduction pathways involved in the
398 pathologies caused by *PTPN11* mutations.

399 Notwithstanding the relevance of this aspect, to the best of our knowledge, no direct
400 phosphopeptide binding experiments to the whole SHP2 protein have ever been performed,
401 possibly due to the fact that pY can be dephosphorylated by the PTP domain. Now, OP and its
402 fluorescent analogue CF-OP allow us to directly assess the hypothesis described above. Figure 6
403 and Table 2 report the results of binding experiments performed with CF-OP and WT SHP2 or the
404 pathogenic mutants A72S, E76K, D61H, F71L and E76V. E76K is among the most common
405 somatic lesions associated with leukemia and has never been observed as germline event in
406 individuals with NS [Tartaglia 2003, 2006], as it results in early embryonic lethality [Xu 2011].
407 This mutation is strongly activating, with a basal activity of the corresponding mutant being at least
408 10 times higher than that of the WT protein. Conversely, A72S is a germline mutation specifically
409 recurring among subjects with NS. In this case, basal activation is only twofold [Bocchinfuso 2007].
410 The D61H, F71L and E76V amino acid substitutions have been identified as somatic events in
411 JMML and other leukemias [Tartaglia 2003] and, when transmitted in the germline, they are
412 associated with a high prenatal lethality (M. Zenker, personal communication, 9/2019).
413 Interestingly, we observed that the affinity for CF-OP nicely parallels the basal activity of these

414 mutants (Figure 6). This finding provides a first direct confirmation that the closed, autoinhibited
415 state has a lower affinity for the binding partners, compared to the open, active conformation.



416

417 **Figure 6: Binding of the CF-OP peptide to the whole SHP2 protein (WT and pathogenic**
418 **mutants)**

419 **Left:** fraction of CF-OP peptide bound to the WT protein and selected mutants, obtained from
420 fluorescence anisotropy experiments. The peptide bound fractions were obtained by following the
421 variation of the peptide fluorescence anisotropy during the titration with increasing amounts of
422 protein. [CF-OP]=1.0 nM.

423 **Center:** catalytic activity of the WT protein and selected mutants, under basal conditions (solid
424 bars) and after stimulation with 10 μM BTAM peptide (dashed bars). N=3

425 **Right:** correlation between basal activity and affinity (association constant).

426 Error bars represent standard deviations.

427

428

429 5.2) OP is also an inhibitor of the PTP domain.

430 Based on previous reports of the dephosphorylation of IRS-1 pY 1172 by SHP2 [Noguchi 1994],
431 we verified if P8 and P8W5 are also a substrate of this protein. These experiments were performed
432 with a truncated SHP2 construct lacking the N-SH2 domain (SHP2 Δ 104), as it is fully activated and
433 was shown to be more stable and less prone to aggregation compared to the isolated PTP domain
434 [Martinelli 2020]. As reported in Figure S5, dephosphorylation was indeed observed, although to a
435 lesser extent than for other phosphopeptides.

436

437 Using the non-dephosphorylatable peptide CF-OP, we measured directly binding to the PTP
438 domain of SHP2 (Figure S6 and Table 2). Significant association was observed, although with a
439 much lower affinity than with the N-SH2 domain ($K_d = 10.0 \pm 0.8 \mu\text{M}$). This finding indicates that
440 in principle, the non-dephosphorylatable OP could act as a double hit SHP2 inhibitor, acting on
441 both PPIs and catalytic activity.

442

443 *5.3) OP activates SHP2 only weakly*

444 Binding of mono- or bi-phosphorylated peptides causes activation of SHP2. We tested the effect of
445 OP on the WT protein, or on the A72S mutant (this experiment is not possible with E76K, as in
446 that case the protein is essentially fully activated also in the absence of phosphopeptides). As shown
447 in Figure S7, activation was very weak, compared with that induced by the bisphosphorylated
448 BTAM peptide, and a significant effect was observed only with the mutant protein. Interestingly,
449 under the experimental conditions used (10 μM peptide), the N-SH2 domain of both the WT and
450 the A72S proteins is nearly saturated by the OP, according to the binding experiments reported in
451 Figure 6. This finding indicates that the peptide could inhibit SHP2 PPIs, causing only a limited
452 increase in catalytic activity. In any case, as demonstrated by studies on truncated constructs lacking

453 the N-SH2 domain [Saxton 1997, Shi 1998, Higashi 2002], activation of SHP2 without proper PPIs
454 has no pathogenic effects.

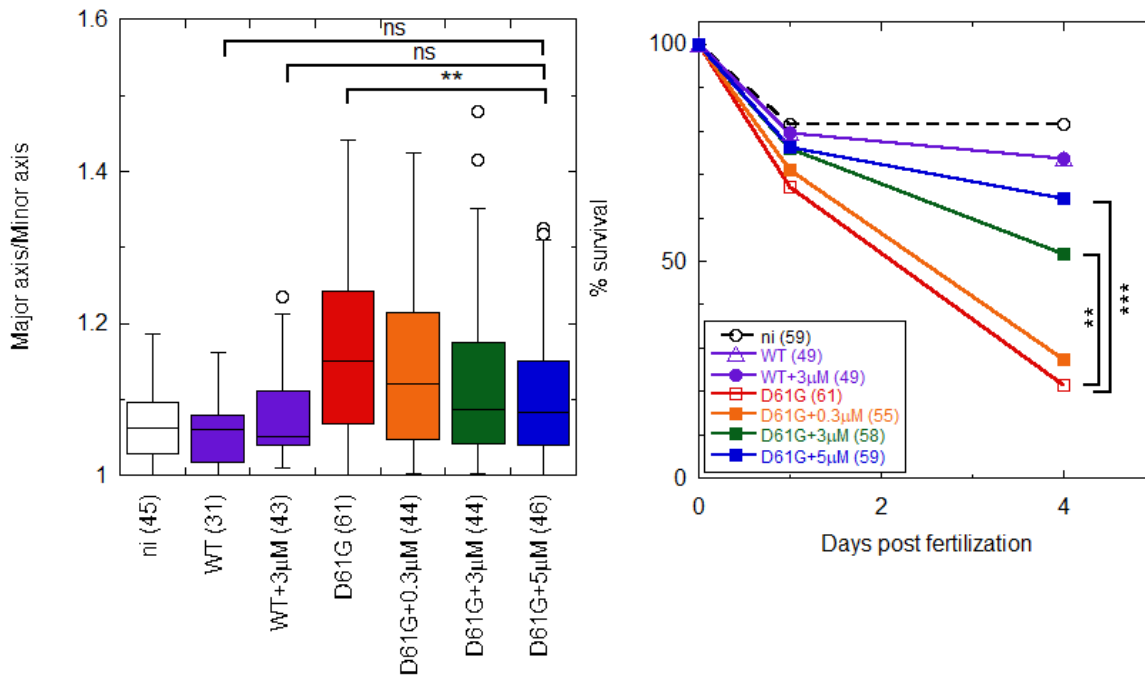
455

456 *6) OP effectively reverses the effects of D61G mutation in vivo.*

457 We used the zebrafish model system to explore the in vivo effect of the peptide. Zebrafish Shp2a
458 is highly homologous to human SHP2 (91.2% protein sequence identity); in particular, the sequence
459 of the N-SH2 domain and of the N-SH2/PTP interface are identical in the human and fish proteins.
460 RASopathies-associated mutants, including activating mutants of Shp2a, greatly impact zebrafish
461 development. In humans, the D61G substitution has been found in both NS and leukemia [Kratz
462 2005] and in animal models it induces both NS-like features and myeloproliferative disease [Araki
463 2004]. Microinjection of synthetic mRNA encoding NS-associated mutants of Shp2 at the one-cell
464 stage induces NS-like traits [Jopling 2007]. During gastrulation, convergence and extension
465 movement are affected, resulting in oval-shaped embryos, with increased major/minor axis length
466 ratio at 11 hpf [Jopling 2007]. Here we co-injected Shp2a-D61G mRNA with OP in zebrafish
467 embryos, to investigate whether OP rescues the defective cell movements during gastrulation.

468 As shown in Figure 7 and Figure S8, we observed a dose-dependent decrease in Shp2a-D61G-
469 induced major/minor axis ratios, with a rescue of the phenotype that was significant at 5 μ M peptide
470 concentration. On the other hand, embryos injected with Shp2a-WT were almost perfect spheres at
471 11 hpf, and co-injection with 3 μ M peptide had no impact on their shape. As expected, a large
472 portion of Shp2a-D61G injected embryos were severely affected and they died during embryonic
473 development, whereas injection of WT Shp2a did not induce significant lethality. We followed the
474 survival of Shp2a-D61G injected embryos and observed a significant and dose dependent
475 improvement in the survival of embryos upon co-injection with 0.3 μ M, 3 μ M and 5 μ M OP (Figure
476 8). By contrast, lethality of WT Shp2a embryos was not affected by co-injection of 3 μ M OP.

477 Altogether, these results indicate that co-injection of the OP rescued the developmental defects
478 induced by a pathogenic, basally activated Shp2a variant, while it had no effect on WT embryos.
479



480

481 **Figure 7: the OP partially rescues Shp2a-D61G-induced gastrulation defects and mortality**
482 **in a dose dependent manner in zebrafish embryos.**

483 Embryos were injected at the one-cell stage with mRNA encoding GFP-2A-Shp2-D61G or GFP-
484 Shp2-wt with or without peptide at 0.3 µM, 3 µM and 5 µM concentration. Non-injected embryos
485 (ni) were evaluated as a control.

486 **Left:** ovality of embryos at 11 hpf, as indicated by the ratio of the long and the short axis. Tukey's
487 honest significant difference test was done to assess significance. In the box plot, the horizontal
488 line indicates the median, box limits indicate the 25th and 75th percentiles (interquartile range),
489 whiskers (error bars) extend to the maximum and minimum values, or to 1.5 times the

490 interquartile range from the 25th and 75th percentiles, if some data points fall outside this range. In
491 the latter case, outliers are indicated as single data points.

492 **Right:** embryo lethality. Surviving embryos were counted at 1 dpf and 4 dpf. Survival was plotted
493 and Log-rank test was done to access differences between groups.

494 Non significant (ns) $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. The number of embryos that
495 were analyzed are indicated in parentheses.

496

497 **Discussion**

498 Our findings provide several insights in the interaction of phosphopeptides with SH2 domains, and
499 in particular, with the N-SH2 domain of SHP2, and on the suitability of this recognition units as
500 therapeutic targets.

501 Soon after their discovery, the affinities of SH2 domains for their binding partners (*i.e.* the
502 dissociation constants) were considered to fall in the 10-100 nM range [Pawson 1995]. However,
503 it turned out that most of the binding studies performed in that period were affected by experimental
504 artifacts, leading to an overestimation of the binding affinities [Ladbury 1995, Kuriyan 1997]. A
505 reassessment of the affinity values led to a commonly accepted range in the order of 0.1 to 10 μ M
506 [Kuriyan 1997, Machida 2005, Wagner 2013, Marasco 2020b]. Such moderate affinities are
507 considered to be crucial for allowing transient association and dissociation events in cell signaling.

508 Consistently, SH2 domains artificially engineered to reach low nanomolar affinities for
509 phosphorylated sequences (known as superbinders) [Kaneko 2012], by increasing the domain
510 affinity for the pY residue, have detrimental consequences for signal transduction. However,
511 micromolar binding affinities make SH2 domains in general less than optimal therapeutic targets.

512 In the case of the N-SH2 domain of SHP2, literature results on the affinity for the IRS-1 pY 1172
513 peptide were contradictory, with dissociation constants varying by three orders of magnitude [Case
514 1994, Sugimoto 1994, Keilhack 2005]. Here, we showed that, at least for this peptide, the
515 dissociation constant is in the low nM range. For the N-SH2 domain, similar affinities have been
516 reported also for the GRB2-associated-binding protein 1 (Gab1), pY 627 [Koncz 2001], and Gab2,
517 pY 614 [Bonetti 2018]; several other peptides have a dissociation constant within an order of
518 magnitude of that of IRS-1 pY 1172 [Anselmi 2020, Marasco 2020a]. In addition, in the present
519 study, we were able to further improve the affinity with respect to the parent peptide. Therefore,
520 the N-SH2 domain of SHP2 might constitute an exception in the panorama of SH2 domains,
521 regarding the binding affinity. In most cases, interaction of phosphopeptides with SH2 domains is

522 dominated by the hydrophobic effect (except for the pY pocket). The N-SH2 domain of SHP2 has
523 a peculiar KxK motif in the region of the BG loop pointing towards the binding groove, which can
524 interact electrostatically with basic residues present in the peptide sequence at positions +2 and +4
525 [Anselmi 2020]. Therefore, by contrast to the superbinders, the high binding affinity of the N-SH2
526 domain is a result of additional interactions in the selectivity-determining region, and not in the pY
527 pocket. Indeed, our data showed that the pY phosphate contributed less than 30% of the standard
528 binding free energy. This finding is comparable to what has been reported for other SH2 domains
529 [Waksman 2004].

530 Our data also showed that residue -2 contributes significantly to the binding affinity. Indeed, while
531 the specificity of most SH2 domains is determined by residues C-terminal to the pY, peptide library
532 and array studies have shown that, contrary to most other SH2 domains, the N-SH2 domain of
533 SHP2 has specific preferences for position -2 [Anselmi 2020]. This peculiarity is due to the fact
534 that, in place of the commonly conserved arginine at position 2 in the first α -helix (α A2), the N-
535 SH2 domain of SHP2 has G13. Consequently, a hydrophobic peptide residue at position -2 can
536 insert in the space left free by the missing protein side chain and interact with V14 α A3 and with
537 the phenol ring of pY, stabilizing its orientation and the overall complex [Anselmi 2020].

538 A preference of the N-SH2 domain of SHP2 for hydrophobic residues at positions +1, +3 and +5 is
539 well established. These side chains insert in the groove on the surface of the domain and interact
540 with exposed hydrophobic patches [Anselmi 2020]. Now our data demonstrate that the bulky,
541 aromatic side chain of tryptophan at position +5 is 10 times better (in terms of dissociation constant)
542 than the leucine residue, which is present in high-affinity natural sequences such as those of IRS-
543 1, Gab1 and Gab2. Overall, these data confirm that the phosphopeptide sequence in the -2 to +5
544 stretch contributes significantly to the binding affinity. In principle, highly specific binding should
545 be possible.

546 In general, SH2 domains are only moderately discriminating for binding target sequences, and a
547 range of residues is tolerated at each site [Kuriyan 1997, Waksman 2004]. Consequently,
548 nonspecific tyrosine phosphorylated sequences are usually bound only 10- to 100-fold more weakly
549 than specific targets [Bradshaw 1998, Waksman 2004, Machida 2005, Wagner 2013]. Indeed,
550 additional specificity is often provided by tandem SH2 domains [Waksman 2004]: two closely-
551 spaced tyrosine phosphorylated motifs bind to tandem SH2 domains with 20- to 50-fold greater
552 affinity and specificity compared with the binding of a single SH2 domain with a single tyrosine
553 phosphorylated motif [Wagner 2013]. SHP2 and its SH2 domains are no exception in this case, as
554 peptide library and array studies, together with the sequences of known natural binding partners,
555 showed a significant variability in the sequences of peptides bound by SHP2 [Anselmi 2020].
556 However, our results indicate that some peptides (like those developed here) can bind specifically
557 to a single SH2 domain. Among an array of 97 human SH2 domains, we found some interference
558 only with adapter protein APS (also called SHP2B2). The structure of the APS SH2 domain in
559 complex with a cognate protein shows that the phosphorylated sequence binds in a folded, kinked
560 conformation, rather than in the usual extended binding mode [Hu 2003]. This observation should
561 facilitate the further development of our peptides, to avoid the unwanted interaction with APS,
562 without affecting the affinity for the target N-SH2.

563 Finally, it is worth mentioning that approximately one order of magnitude in affinity was lost by
564 substituting the pY residue with the non-dephosphorylatable mimic F₂Pmp. This finding is
565 consistent with previous studies showing that F₂Pmp is tolerated differently by various SH2
566 domains, and its insertion in place of pY in a phosphopeptide sequence can lead either to a loss or
567 to an increase in affinity, by approximately one order of magnitude [Burke 2006]. Further
568 optimization of this aspect is warranted, but the dissociation constant of our non-
569 dephosphorylatable peptide remained in the nM range.

570 The non-dephosphorylatable peptide allowed novel experiments on several aspects of SHP2
571 function and regulation. As discussed in the introduction, in the autoinhibited state of SHP2, the N-
572 SH2 binding groove is closed, apparently making phosphopeptide association impossible. By
573 contrast, the N-SH2 binding site is open in the structure of the isolated N-SH2 domain or of active
574 SHP2. Consequently, it has been hypothesized that mutations destabilizing the closed state and
575 favoring SHP2 activation could lead to an increase binding affinity [Bocchinfuso 2007]. This idea
576 is indirectly supported by the fact that basally activated mutants require lower concentrations of
577 SH2 domain-binding phosphopeptides to reach full activation [Keilhack 2005; Bocchinfuso 2007,
578 Martinelli 2008, LaRochelle 2018]. However, no direct measurements of phosphopeptide binding
579 to different SHP2 variants had been reported until now. Our data directly demonstrate that the
580 affinity for phosphopeptides of activated variants of SHP2 can increase by a factor of 20, reaching
581 the same value as the isolated domain in the most active mutants (Figure 6). This consequence of
582 pathogenic mutations adds to the increase in basal activity and might be the main responsible for
583 hyperactivation of signaling pathways modulated by SHP2. Interestingly, in view of possible
584 therapeutic applications, in a cellular environment, our peptide would act as an effective inhibitor
585 of the PPIs of mutant, hyperactivated SHP2, while it would have a much lower effect on the WT
586 protein. This behavior is the exact opposite of what has been observed for allosteric inhibitors, such
587 as SHP099, which have a significantly impaired activity in pathogenic variants of SHP2
588 [LaRochelle 2018; Tang 2020].

589 A second link between SHP2 activity and binding functions is provided by literature data indicating
590 for SHP2 interactors a role both as ligands of the SH2 domains and as substrates of the catalytic
591 PTP domain, often with the same phosphorylated sequence. Examples include IRS-1 [Sugimoto
592 1994, Noguchi 1994], Gab1 [Cunnick 2001, Zhang 2002], Gab2 [Gu 1997], PDGFR [Rönstrand
593 1999, Sugimoto 1992], PD-1 [Marasco 2020a] and SHPS-1 [Fujioka 1996, Takada 1998]. We also
594 showed here that our modified sequence can be dephosphorylated. These data indicate the possible

595 presence of a still uncharacterized feedback mechanism to regulate SHP2 signaling. Using our non-
596 dephosphorylatable peptide, we could demonstrate that a N-SH2-binding sequence associates to the
597 catalytic PTP domain, too, although with a lower affinity. This finding suggests that it might be
598 possible to develop double-edged sword molecules, able to inhibit both the catalytic activity and
599 the PPIs of SHP2.

600 Phosphorylated sequences cause SHP2 activation by binding to the N-SH2 domain and inducing or
601 stabilizing a domain conformation that is incompatible with the N-SH2/PTP interaction. In
602 principle, it is possible that different phosphopeptide sequences do not have the same aptitude for
603 causing or favoring the conformational transition of the N-SH2 domain needed for SHP2 activation.
604 In this case, the binding affinity and activating potential of phosphopeptides would not be strictly
605 coupled. Some literature data indicate that this might be the case. For instance, the sequences
606 corresponding to pY546, pY895 and pY1222 of IRS-1 (rat ortholog numbering) [Sugimoto 1994,
607 Case 1994], or the artificial sequences AALNpYAQLMFP and AALNpYAQLWYA [Imhof 2006]
608 have similar dissociation constants for the N-SH2 domain (within a factor of 2), but the
609 concentrations of these phosphopeptides needed for full activation of SHP2 differ by orders of
610 magnitude. The interpretation of these studies is complicated by the fact that in principle these
611 sequences could be dephosphorylated by SHP2, to different extents, during the activation
612 experiments. Our data show that a concentration of nonhydrolyzable phosphopeptide that almost
613 completely saturates the N-SH2 domain causes only partial activation. While an inability of this
614 specific sequence to favor activation cannot be ruled out, it is possible that partial activation is
615 caused by inhibition of SHP2 activity due to peptide association to the PTP domain. In any case,
616 even if activation of SHP2 without proper PPIs is inconsequential [Saxton 1997, Shi 1998, Higashi
617 2002], it is important to note that the molecules developed here can inhibit the association of SHP2
618 to its partners, without causing complete activation, particularly for the WT protein.

619 Inhibition of PPIs, particularly using peptides, is currently a hot area of pharmaceutical research.
620 For the RAS/MAPK pathway alone, at least 30 inhibitors of PPI have been developed and several
621 of them are undergoing clinical trials [García-Gómez 2018]. However, no studies on SHP2 have
622 been reported, notwithstanding the central role of this phosphatase in the pathway. Peptides are
623 particularly appealing for the inhibition of PPIs, where large interfaces are involved, which are
624 difficultly targeted selectively by small molecules. An increasing number of drugs based on
625 peptides or peptidomimetics is progressing in the drug development pipeline [Henninot 2018].
626 Possible challenges in the therapeutic applications of peptide-based molecules are their rapid
627 degradation and a poor cell uptake, particularly for highly charged sequences [Henninot 2018].
628 Here we successfully overcame the first hurdle, thanks to the introduction of non-natural amino
629 acids. Several studies have demonstrated that efficient intracellular delivery of phosphopeptide
630 mimics is possible, for instance by conjugation to cell-penetrating sequences [Kertesz 2006, Ye
631 2007, Choi 2009, Nasrolahi Shirazi 2013, Cerulli 2020b]. Optimization of the cell uptake of our
632 molecules, through different strategies, is currently underway.

633 Our *in vivo* findings on zebrafish embryos are very promising in view of potential applications,
634 particularly considering that the peptide is more effective on activating mutants than on the WT
635 protein, contrary to allosteric inhibitors such as SHP099 [LaRoche 2018, Tang 2020]. Indeed,
636 besides their possible use as a research tool to study the role of PPIs in the function of SHP2, and
637 regulation of the pathways controlled by this protein, including RAS/MAPK and PI3K/AKT
638 signaling, the reported peptides constitute lead compounds for the development of new drugs
639 against malignancies driven by *PTPN11* mutations, such as JMML, AMoL, and ALL, also
640 considering that allosteric inhibitors have low activity against basally activated SHP2 variants
641 [Larochelle 2018, Tang 2020]. Finally, another possible field of therapeutic application is
642 represented by rare diseases such as NS and NSML, which are caused by activating mutations of
643 *PTPN11* (against which the available allosteric inhibitors are poorly active) and cause several

644 severe postnatal, evolutive clinical manifestations, particularly hypertrophic cardiomyopathy
645 [Tartaglia 2010].

646

647 **Materials and methods**

648 *Materials*

649 Fmoc (9-fluorenylmethyloxycarbonyl)-amino acids were obtained from Novabiochem (Merck
650 Biosciences, La Jolla, CA). Rink amide MBHA resin (0.65 mmol/g, 100-200 mesh) was purchased
651 from Novabiochem. All other protected amino acids, reagents and solvents for peptide synthesis
652 were supplied by Sigma–Aldrich (St. Louis, MO). The LB medium components, all the reagents
653 used to prepare the buffers and the Bradford reagent were purchased from Sigma Aldrich. Tris(2-
654 carboxyethyl)phosphine (TCEP) was obtained from Soltec Ventures, Beverly, MA, USA.
655 Spectroscopic grade organic solvents were purchased from Carlo Erba Reagenti (Milano, Italy).
656 Cell culture media growth factors and antibodies were purchased from VWR International PBI
657 (Milan, Italy), EuroClone (Milan, Italy), Promega (Madison, WI, USA), Invitrogen (Carlsbad, CA,
658 USA), Cell Signaling (Danvers, MA, USA), Sigma-Aldrich (Saint Louis, MO, USA), and Santa
659 Cruz Biotechnology (Dallas, TX, USA).

660 *Peptide synthesis*

661 The solid-phase peptide synthesis of the analogs described in this manuscript was performed on the
662 Rink Amide MBHA resin using standard Fmoc chemistry protocols. using standard Fmoc
663 chemistry protocols. The deprotection of the Fmoc group was performed with a 20 % piperidine
664 solution in N,N-dimethylformamide. The single coupling steps were carried out in the presence of
665 HBTU/HOBt/DIPEA. The N-termini of the different peptides were manually acetylated using a
666 mixture of acetic anhydride (Ac₂O) and DIPEA in DMF. For the fluorescent analogs, the
667 introduction of the carboxyfluorescein probe was carried out activating 5(6)-carboxyfluorescein in

668 the presence of HBTU/HOBT/DIPEA, repeating the acylation step twice. The fluorescent analogs
669 were obtained as a mixture of isomers. At the end of the synthesis, each peptide was cleaved from
670 the resin using a mixture of TFA, TIS, and water in 95:2.5:2.5 ratio. The solution was concentrated
671 under a flow of nitrogen, and the crude peptide precipitated by addition on diethyl ether. The crude
672 peptides were purified by flash chromatography on Isolera Prime chromatographer (Biotage,
673 Uppsala, Sweden) using a SNAP Cartridge KP-C18-HS 12g or preparative RP-HPLC on a
674 Phenomenex C18 column (22.1x250 mm, 10 μ m, 300 Å) using an Akta Pure GE Healthcare (Little
675 Chalfont, UK) LC system equipped with an UV-detector (flow rate 15 mL/min) and a binary elution
676 system: A, H₂O; B, CH₃CN/H₂O (9: 1 v/v); gradient 25-55% B in 30 min. The purified fractions
677 were characterized by analytical HPLC-MS on a Phenomenex Kinetex XB-C18 column (4.6 x 100
678 mm, 3.5 μ m, 100 Å) with an Agilent Technologies (Santa Clara, CA) 1260 Infinity II HPLC system
679 and a 6130 quadrupole LC/MS. The purity and the characterization data are reported in Table S2.
680 Peptides were dissolved in DMSO to obtain stock solutions between 1 and 1.5 mM. The exact
681 concentration was obtained by UV measurements, exploiting the signal of carboxyfluorescein for
682 the labeled peptides and of pTyr, Tyr and Trp for the unlabeled peptides. To this end, CF-labeled
683 peptides were diluted from the stocks (1:100) in buffer (pH 9), and their concentration was
684 calculated from the CF signal at 490 nm using a molar extinction coefficient of 78000 M⁻¹ cm⁻¹
685 [Esbjörner 2007]. Unlabeled peptides were diluted 1:100 in a pH 7.4 buffer; molar extinction
686 coefficients of Tyr, Phe and Trp were taken from reference [Pace 1995], while molar extinction
687 coefficient of pY was taken from [Bradshaw 2003].

688

689 *Protein expression and purification*

690 The human esaHis-tagged *PTPN11* (residues 1-528) cDNA was cloned in a pET-26b vector
691 (Novagen, MA, USA). Nucleotide substitutions associated with NS or leukemia were introduced
692 by site-directed mutagenesis (QuikChange site-directed mutagenesis kit; Stratagene, CA, USA). A

693 construct containing the cDNA encoding the isolated PTP domain preceded by the C-SH2 domain
694 (residues 105-528) was generated by PCR amplification of the full-length wild-type cDNA and
695 subcloned into the pET-26b vector (SHP2 Δ 104). A similar procedure was followed for the constructs
696 of the N-SH2 (residues 2-111), C-SH2 (109-217) and PTP (212-528) domains, and of the N-SH2/C-
697 SH2 tandem (2-217). Primer sequences are available upon request.

698 Recombinant proteins were expressed as previously described [Martinelli 2012], using *E. coli*
699 (DE3) Rosetta2 competent cells (Novagen). Briefly, following isopropyl β -D-1-
700 thiogalactopyranoside (Roche) induction (2 hr at 30 °C, or overnight at 18 °C), bacteria were
701 centrifuged at 5,000 rpm, 4 °C for 15 minutes, resuspended in a lysozyme-containing lysis buffer
702 (TRIS-HCl 50 mM, pH=8.5, NaCl 0.5 M, imidazole 20 mM, tris(2-carboxyethyl)phosphine (TCEP)
703 1mM, lysozyme 100 mg/ml, 1 tablet of complete protease inhibition cocktail) and sonicated. The
704 lysate was centrifuged at 16,000 rpm, 4 °C for 30 minutes. The supernatant was collected, and the
705 protein of interest was purified by affinity chromatography on a Ni-NTA column (Qiagen, Hilden,
706 Germany), using a TRIS-HCl 50 mM, NaCl 0.5 M, TCEP 1 mM buffer containing 100 mM or 250
707 mM imidazole, for washing and elution, respectively. To remove imidazole, the samples were then
708 dialyzed in a 20 mM TRIS-HCl (pH 8.5) buffer, containing 1 mM TCEP and 1 mM EDTA and
709 50mM NaCl (or 150 mM NaCl if no further purification steps followed). Full length proteins and
710 the SHP2 Δ 104 construct were then further purified by sequential chromatography, using an Äkta
711 FPLC system (Äkta Purifier 900, Amersham Pharmacia Biotech, Little Chalfont, UK). The
712 samples were first eluted within an anion exchange Hi-Trap QP 1ml-column (GE Helathcare,
713 Pittsburgh, PA, USA); the elution was carried out using TRIS-HCl 20 mM (pH 8.5) in a NaCl
714 gradient from 50 to 500 mM. The most concentrated fractions were then eluted in a gel filtration
715 Superose column using TRIS-HCl 20 mM buffer containing NaCl (150 mM) as mobile phase.
716 Sample purity was checked by SDS PAGE with Coomassie Blue staining and resulted to be always
717 above 90%. Proteins were quantitated by both the Bradford assay and the UV absorbance of

718 aromatic residues, calculating extinction coefficients according to [Pace 1995]. In general, the two
719 methods were in agreement, but the values derived from UV absorbance were more precise and are
720 reported in the Figures and Tables. The protein samples were used immediately after purification
721 or stored at -20 °C and used within the following week. In this case, after thawing TCEP 2.5 mM
722 was added, the samples were centrifuged at 13,000 rpm for 20 minutes, and the new concentration
723 was re-evaluated. In the few cases where residual apparent absorbance due to light scattering was
724 present in the UV spectra, it was subtracted according to [Castanho 1997].

725 *Phosphatase activity assays*

726 Catalytic activity was evaluated *in vitro* using 20 pmol of purified recombinant proteins in a 200 µl
727 reaction buffer supplemented with 20 mM *p*-nitrophenyl phosphate (Sigma) as substrate, either
728 basally or following stimulation with the protein tyrosine phosphatase nonreceptor type substrate 1
729 (PTPNS1) bisphosphotyrosyl-containing motif (BTAM peptide)
730 (GGGGDIT(pY)ADLNLPKGKKPAPQAAEPNNHTE(pY)ASIQTS) (Primm, Milan, Italy), as
731 previously described [Martinelli 2008]. Proteins were incubated for 15 min (SHP2_{Δ104}) or 30 min
732 (SHP2) at 30 °C. Phosphate release was determined by measuring absorbance at 405 nm.

733 DiFMUP (6,8-difluoro-4-methylumbelliferyl phosphate) assay was carried out as previously
734 described [Chen 2016], with minor changes. Briefly, reactions were performed at room temperature
735 in 96-well flat bottom, low flange, non-binding surface, black polystyrene plates (Corning, cat. no.
736 3991), using a final volume of 100 µl and the following assay buffer: 60 mM HEPES, pH 7.2, 75
737 mM NaCl, 75 mM KCl, 1 mM EDTA, 0.05% Tween-20, 5 mM DTT. Catalytic activity was
738 checked using 1 nM SHP2 and different concentrations of activating peptides. After 45 min at 25
739 °C, 200 µM of surrogate substrate DiFMUP (Invitrogen, cat. no. D6567) was added to the mix, and
740 incubated at 25 °C for 30 min. The reaction was stopped by addition of 20 µl of 160 µM bpV(Phen)
741 (Potassium bisperoxo(1,10-phenanthroline) oxovanadate (V) hydrate) (Enzo Life Sciences cat. no.

742 SML0889-25MG). The fluorescence was monitored using a microplate reader (Envision, Perkin-
743 Elmer) using excitation and emission wavelengths of 340 nm and 455 nm, respectively.
744 The ability of SHP2 to dephosphorylate the phosphopeptides was evaluated through a malachite
745 green phosphatase assay (PTP assay kit 1 Millipore, MA, USA). The BTAM peptide and the
746 following monophosphorylated peptides derived from known SHP2 substrates were used for
747 comparison: DKQVEpYLDL_{DL} (GAB1_{Y657}), EEENIpYSVPHD (p190A/RhoGAP_{Y1105}), and
748 VDADEpYLIPQQ (EGFR_{Y1016}) (Primm). SHP2_{Δ104} (2.4 pmol) was incubated with 100 μM of each
749 phosphopeptide (total volume 25 μl) for different times. The reaction was stopped by adding 100
750 μl of malachite green solution. After 15 min, absorbance was read at 655 nm, using a microplate
751 reader, and compared with a phosphate standard curve to determine the release of phosphate. Data
752 obtained in the linear region of the curve were normalized on the reaction time (1 min).

753 *Fluorescence anisotropy binding assay*

754 Anisotropy measurements were carried out using a Horiba Fluoromax 4 spectrofluorimeter.
755 For the binding assays, the requested peptide amount (1nM or 0.1 nM) was diluted in buffer
756 (HEPES 10 mM, NaCl 150 mM, EDTA 1 mM, TCEP 1 mM, fluorescence buffer henceforth) and
757 its anisotropy signal was recorded. The peptide was then titrated with increasing protein amounts,
758 until the anisotropy signal reached a plateau at its maximum value, or up to a protein concentration
759 where protein aggregation and consequent light scattering affected the anisotropy values (usually
760 above 1 μM). The measurements of CF-labeled peptides were carried out using an excitation
761 wavelength of 470 nm and collecting the anisotropy values at an emission wavelength of 520 nm.
762 A 495 nm emission filter was used. For the Cy3-labeled peptides, excitation and emission
763 wavelengths of 520 and 560 nm were used. The lowest peptide concentration needed to have a
764 sufficient fluorescent signal (0.1 nM) was used in the binding experiments. Higher concentrations
765 (1 or 10 nM) were used for peptides with lower affinities, and therefore higher K_d values.

766 The displacement assays were carried out with the same experimental settings. In this case, the
767 labeled peptide-protein complex was titrated with increasing amounts of the unlabeled peptide,
768 following the decrease in anisotropy. Measurements were carried out at the same CF-peptide
769 concentration used for the corresponding binding experiments. Regarding the protein
770 concentration, a compromise between two requirements is needed [Huang 2003]. On one hand, it
771 is desirable to have a significant fraction of the CF-peptide bound to the protein, to maximize the
772 dynamic range in the anisotropy signal, which decreases during the displacement experiment. On
773 the other hand, the protein concentration should be comparable or lower than the dissociation
774 constant of the unlabeled peptide (K_i), to allow a quantitative and reliable determination of its
775 binding affinity. Since several unlabeled peptides had a higher affinity than their fluorescent
776 counterparts, in the displacement assays we used a protein concentration $[P]_T \sim K_d$, or in some cases
777 even $\sim K_d/2$.

778 The equations used for data fitting are described in the supplementary information appendix.

779 *SH2 domain microarray*

780 The microarray experiment was conducted by the Protein Array and Analysis Core at the MD
781 Anderson Cancer Center (University of Texas, USA), as previously described [Roth 2019]. Briefly,
782 a library of SH2 domains [Huang 2008] was expressed as GST fusion in *E. coli* and purified on
783 glutathione-sepharose beads. The domains were spotted onto nitrocellulose-coated glass slides
784 (Oncyte Avid slides, Grace Bio-Labs) using a pin arrayer. Each domain was spotted in duplicate.
785 After incubation with a Cy3-P9W5 solution (0.5, 5.0 nM, or 50 nM), fluorescence signals were
786 detected using a GeneTACTM LSIV scanner (Genomic Solutions).

787 *In silico studies*

788 System preparation

789 The initial structure of the N-SH2 complexed with phosphopeptide P8 (Table 1) was obtained by
790 amino acid substitutions (and deletions) in the crystallographic structure of the protein complexed
791 with the GAB1 peptide (sequence GDKQVE-pY-LDLDL) (PDB code 4QSY). The obtained
792 complex was then used as the starting structure for subsequent amino acid substitutions in the bound
793 peptide.

794 System equilibration

795 MD simulations were performed using the GROMACS 2018.2 simulation package [Abraham 2015]
796 and a variant of AMBER99SB force field with parameters for phosphorylated residues [Homeyer
797 2006]. Water molecules were described by the TIP3P model. All the simulated systems were
798 inserted in a pre-equilibrated triclinic periodic box (15x7x7 nm³), containing about 24000 water
799 molecules and counterions to neutralize system total charge. They were relaxed first by doing a
800 minimization with 5000 steepest descent cycles, by keeping protein positions fixed and allowing
801 water and ions to adjust freely, followed by a heating protocol in which temperature was
802 progressively increased from 100K to 300K. The system was then equilibrated for 100 ps in the
803 NVT ensemble at 300 K, using velocity rescaling with a stochastic term (relaxation time 1 ps)
804 [Bussi 2007] and then for 500 ps at constant pressure (1 atm) using the Parrinello-Rhman barostat
805 (relaxation time 5 ps). Long-range electrostatic interactions were calculated using the particle mesh
806 Ewald method and the cut-off distance for the non-bonded interaction was set equal to 12.0 Å. The
807 LINCS constraint to all the hydrogen atoms and a 2 fs time-step were used.

808 Preparation of the initial configurations for Umbrella Sampling

809 For each system, a set of initial configurations was prepared by performing a center-of-mass (COM)
810 pulling simulation. The distance between the peptide and N-SH2 domain COMs was constrained

811 with a harmonic force ($K=1000 \text{ kJ mol}^{-1} \text{ nm}^{-2}$). Pulling was performed by gradually increasing the
812 value of the equilibrium distance with a constant-rate of 0.0025 nm/ps . The length of each
813 simulation was about 2.5 ns . During the whole simulation, a positional restraint ($1000 \text{ kJ mol}^{-1} \text{ nm}^{-2}$)
814 was applied to all heavy atoms in the N-SH2 domain except for atoms in loops around the binding
815 region (residues 30-45, 52-75, 80-100). For the choice of the optimal unbinding pathway, three
816 different directions were tested, corresponding to: i) the vector from the phosphate to the alpha
817 carbon in pY, in the equilibrated complex; ii) the vector defined by the initial positions of the two
818 COMs; iii) the vector perpendicular to the surface of the cavity flanked by the EF and BG loops,
819 passing through the N-SH2 domain center of mass. Among the three different pathways, the third
820 direction encountered less steric occlusion by the EF and BG loops, and was thus selected for further
821 analyses.

822 Umbrella sampling simulations

823 A set of starting configurations was extracted from the pull-dynamics trajectory saving the peptide-
824 protein center-of-mass distances every 2 \AA in the range from 9 to about 40 \AA , thus obtaining about
825 20 windows along the COM distance. The system in each window was preliminarily equilibrated
826 for 1 ns with a strong positional restraint ($1000 \text{ kJ mol}^{-1} \text{ nm}^{-2}$) to all carbon alpha atoms except for
827 those in loops flanking the binding region (as in the pull simulation), followed by a production run
828 of 150 ns with the restraints. During this stage, a harmonic potential ($K=1000 \text{ kJ mol}^{-1} \text{ nm}^{-2}$) was
829 applied on the distance between the two COMs. Additional sampling windows were added every 1
830 \AA along the distance between the two COMs up to a distance of 15 \AA . The resulting asymmetric
831 distribution of sampling windows was used to calculate PMF on the production run trajectories.
832 The Weighted Histogram Analysis Method (WHAM) was used, with default settings (50 bins and
833 tolerance of $10^{-6} \text{ kJ mol}^{-1}$), using the `gmx wham` GROMACS tool. The analysis of the simulation
834 was carried out on the 150 ns production dynamics, during which configurations were stored every

835 0.1 ns. The statistical uncertainty of the obtained PMF was estimated by bootstrapping analysis
836 [Hub 2010].

837 *Peptide stability in serum and in DMEM*

838 The peptides were dissolved in DMSO (5mg/mL). In eppendorf tubes, 1 mL of HEPES buffer (25
839 mM, pH = 7.6) was temperature equilibrated at 37 °C before adding 250 µL of human serum and
840 20 µL of peptide solution; the reaction was followed for 90 minutes. At fixed intervals, 100 µL of
841 the solution were withdrawn and added to 200 µL of absolute ethanol. These samples were kept on
842 ice for 15 minutes, then centrifuged at 13,000 rpm for 5 minutes; the supernatant solutions were
843 analyzed by HPLC and HPLC-MS with 20-60% B gradient in 20 minutes to follow the reaction. In
844 parallel, samples containing peptide, buffer and ethanol only were analyzed. A degradation
845 resistance test was also conducted in DMEM (Dulbecco's Modified Eagle Medium). The
846 experimental conditions are similar to those described above; the reaction was followed for 72
847 hours. The enzymatic degradation resistance tests were followed by HPLC using a 5-50% B
848 gradient in 20 minutes.

849 *In vivo zebrafish rescue experiments*

850 One cell stage zebrafish embryos were injected with a mixture of 120 ng/µl of mRNA encoding
851 either GFP-2A-Shp2-D61G or GFP-2A-Shp2-wt (as a control), with or without OP, at 0.3 µM, 3
852 µM and 5 µM concentration. Embryos were selected based on proper GFP expression and imaged
853 at 11 hours post fertilization (hpf) in their lateral position using the Leica M165 FC
854 stereomicroscope. Images were analyzed using ImageJ [Schneider 2012], by measuring the ratio of
855 the major and minor axis from a minimum of 31 embryos. Statistical analysis was performed in
856 GraphPad Prism, using the analysis of variance (ANOVA) complemented by Tukey's honest
857 significant difference test (Tukey's HSD). To measure the survival of injected embryos, a minimum
858 of 48 embryos per group were grown up to 4 days post fertilization (dpf) and counted at 1 dpf and

859 4 dpf. Survival curves were plotted using GraphPad Prism, and the differences between samples
860 were determined using the Log-rank (Mantel-Cox) test.

861

862

863 **Supporting Information:**

864 supplementary Materials and Methods, Figures S1 to S7, Table S1 and S2

865

866 **Corresponding Author Information:**

867 correspondence should be addressed to Prof. Lorenzo Stella,

868 Department of Chemical Science and Technologies,

869 University of Rome Tor Vergata,

870 Via della Ricerca Scientifica, 1, 00133, Rome, Italy

871 Tel. +390672594463

872 stella@stc.uniroma2.it.

873

874 **Author Contributions:**

875 † these authors contributed equally

876

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885

886 **Competing interests:**

887 L.S., B.B., G.B., S.M. and M.T. are the inventors of a patent application, filed by the University of Rome Tor Vergata

888 and the Ospedale Pediatrico Bambino Gesù, regarding the molecules described in the present article.

889 **Abbreviations Used:** ALL, acute lymphoblastic leukemia; AmoL, acute monocytic leukemia; CagA, cytotoxicity-
890 associated immunodominant antigen; JMML, juvenile myelomonocytic leukemia; NS, Noonan syndrome; NSML,
891 Noonan syndrome with multiple lentigines; PTK, protein-tyrosine kinase; PTP, protein tyrosine phosphatase; pY,
892 phosphotyrosine; RTK, receptor tyrosine kinase; SH2, Src homology 2; SHP2, SH2 domain-containing phosphatase 2;
893 shRNA, short hairpin ribonucleic acid; WT, wild type; .

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1199

Supplementary materials

for

Targeting Oncogenic Src Homology 2 Domain-Containing Phosphatase 2 (SHP2) by Inhibiting its Protein-Protein Interactions

S. Bobone, L. Pannone, B. Biondi, M. Solman, E. Flex, V. Canale, P. Calligari, C. De Faveri, T. Gandini, A. Quercioli, G. Torini, M. Venditti, A. Lauri, G. Fasano, J. Hoeksma, V. Santucci, G. Cattani, A. Bocedi, G. Carpentieri, V. Tirelli, M. Sanchez, C. Peggion, F. Formaggio, J. den Hertog, S. Martinelli, G. Bocchinfuso, M. Tartaglia, Lorenzo Stella.^{1,*}

Corresponding author: Lorenzo Stella

Email: stella@stc.uniroma2.it

Contents

Supplementary Materials and Methods

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Table S1 and S2

Supplementary materials and methods

Analysis of binding curves

K_d values were obtained fitting the data with the following equation [Van de Weert 2011], which avoids the need for the commonly used (but often unjustified) approximation of the concentration of unbound protein with the total concentration:

$$\frac{r - r_0}{r_{max} - r_0} = \frac{[P]_T + [L]_T + K_d - \sqrt{([P]_T + [L]_T + K_d)^2 - 4[P]_T[L]_T}}{2[L]_T} \quad (1)$$

Here, $[P]_T$ and $[L]_T$ are the total protein and ligand concentrations, while r , r_0 and r_{max} are the anisotropy values at a given protein concentration, in the absence of protein and when the peptide is completely bound, respectively. When allowed by the experimental conditions (i.e. when $[L]_T \ll K_d$), this equation was simplified by assuming $[P]_T \cong [P]$ and obtaining:

$$\frac{r - r_0}{r_{max} - r_0} = \frac{[P]_t/K_d}{1 + [P]_t/K_d} \quad (2)$$

The affinity of unlabeled peptides was determined by competition experiments, in which a sample with fixed total protein and fluorescently labeled peptide concentrations ($[P]_T$ and $[L]_T$) was titrated with the unlabeled peptide, causing displacement of the fluorescent peptide and a decrease in anisotropy. From these data, the IC_{50} (i.e. the total concentration of unlabeled peptide that displaces half of the bound fluorescent analog) was determined, interpolating the displacement curve using a phenomenological Hill equation [Barlow 1989]:

$$\frac{r - r_0}{r_{fin} - r_0} = \frac{\{[I]_T/IC_{50}\}^n}{1 + \{[I]_T/IC_{50}\}^n} \quad (3)$$

where $[I]_T$ is the total concentration of the peptide causing the displacement, and r_{fin} is the anisotropy corresponding to total displacement, while in this case r_0 is the starting anisotropy, in the absence of displacing peptide.

Successively, the dissociation constant of the unlabeled peptide (K_i) was calculated from the know values of IC_{50} , K_d , $[P]_T$ and $[L]_T$, as described here below. Our treatment follows that of Nikolovska-Coleska [2004], through a slightly simplified route, and correcting some inaccuracies present in the equations of that article.

In the system where protein (P), ligand (L) and a competitive inhibitor (I) are present, both L and I can form complexes with P (PL and PI, respectively). The following dissociation constants can be defined for the two binding equilibria:

$$K_d = \frac{[P][L]}{[PL]}; \quad K_i = \frac{[P][I]}{[PI]} \quad (4)$$

and the following mass conservation laws apply:

$$[P]_T = [P] + [PI] + [PL]; \quad [L]_T = [L] + [PL]; \quad [I]_T = [I] + [PI] \quad (5)$$

Let's define $[PL]_0$ as the complex concentration in the absence of inhibitor. Then, by definition, at the IC_{50}

1250
$$[PL]_{50} = \frac{[PL]_0}{2} \quad (6)$$

1251 At the IC_{50} ,

1252
$$\frac{[PL]_{50}}{[P]_T} = \frac{[PL]_{50}}{[P]_{50} + [PL]_{50} + [PI]_{50}} = \frac{1}{1 + \frac{[P]_{50}}{[PL]_{50}} + \frac{[PI]_{50}}{[PL]_{50}}} = \frac{1}{1 + \frac{[P]_{50}}{[PL]_{50}} \left(1 + \frac{[PI]_{50}}{[P]_{50}}\right)} \quad (7)$$

1253 and therefore

1254
$$[PL]_{50} = \frac{[PL]_0}{2} = \frac{[P]_T}{1 + \frac{K_d}{[L]_{50}} \left(1 + \frac{[I]_{50}}{K_i}\right)} \quad (8)$$

1255 This equation can be inverted to calculate K_i

1256
$$K_i = \frac{[I]_{50}}{\left(\frac{2[P]_T}{[PL]_0} - 1\right) \frac{[L]_{50}}{K_d} - 1} \quad (9)$$

1257 For $[L]_{50}$ we can write:

1258
$$[L]_{50} = [L]_T - [PL]_{50} = [L]_T - \frac{[PL]_0}{2} \quad (10)$$

1259 Finally, for $[I]_{50}$ we can write:

1260
$$[I]_{50} = IC_{50} - [PI]_{50} = IC_{50} - ([P]_T - [P]_{50} - [PL]_{50}) =$$

1261
$$= IC_{50} - [P]_T + K_d \frac{[PL]_{50}}{[L]_{50}} + [PL]_{50} = IC_{50} - [P]_T + [PL]_{50} \left(1 + \frac{K_d}{[L]_{50}}\right) =$$

1262
$$= IC_{50} - [P]_T + \frac{[PL]_0}{2} \left(1 + \frac{K_d}{[L]_T - \frac{[PL]_0}{2}}\right) \quad (11)$$

1263 Substituting the above equations in the expression for K_i , we get:

1264
$$K_i = \frac{IC_{50} - [P]_T + \frac{[PL]_0}{2} \left(1 + \frac{K_d}{[L]_T - \frac{[PL]_0}{2}}\right)}{\left(\frac{2[P]_T}{[PL]_0} - 1\right) \frac{[L]_T - \frac{[PL]_0}{2}}{K_d} - 1} \quad (12)$$

1265 Finally, $[PL]_0$ can be substituted with the following expression, analogous to Eq. (1):

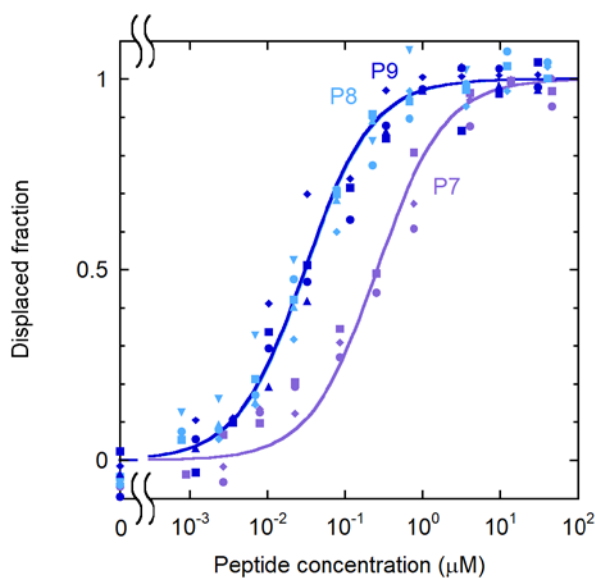
1266
$$[PL]_0 = \frac{[P]_T + [L]_T + K_d - \sqrt{([P]_T + [L]_T + K_d)^2 - 4[P]_T[L]_T}}{2} \quad (13)$$

1267 In this way, K_i is expressed as a function of the known quantities IC_{50} , K_d , $[P]_T$ and $[L]_T$, without any approximation.

1268

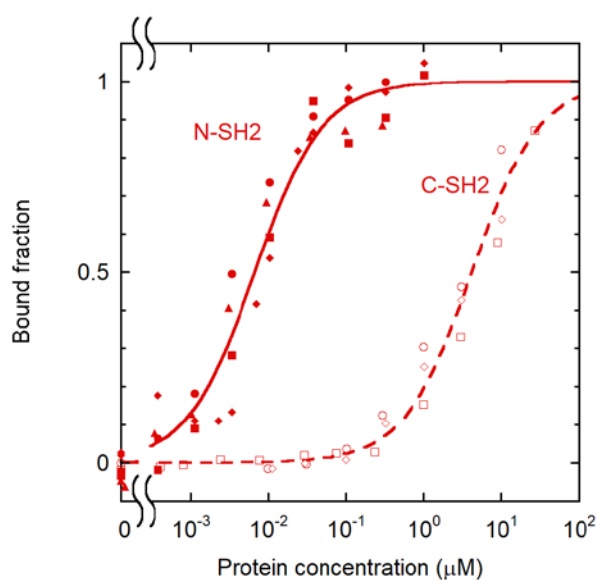
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1276 **Supplementary figures**



1277
1278 **Figure S1: effect of sequence length on the binding affinity.**

1279 Displacement experiments for analogs of different length. [CF-P9]=1.0 nM; [N-SH2]= 40 nM.



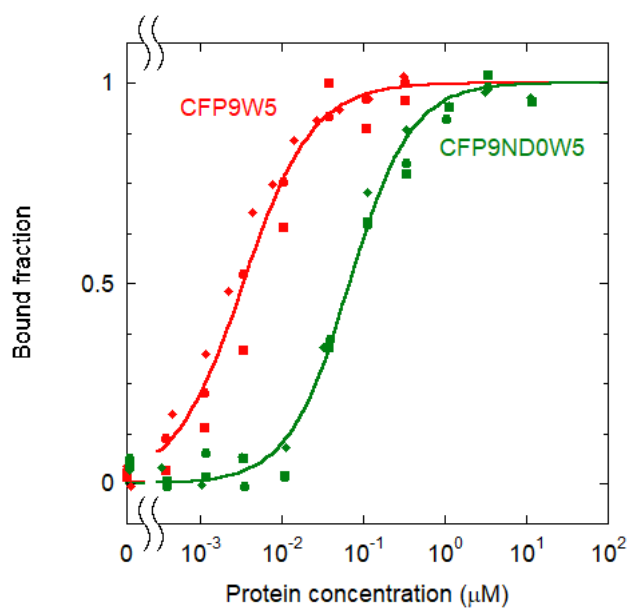
1280

1281 **Figure S2: binding selectivity of CF-P9W5 for the two SH2 domains of SHP2**

1282 Comparison of the association curves of CF-P9W5 to the N-SH2 and C-SH2 domains of SHP2.

1283 Experimental conditions for the N-SH2 binding experiments: see Fig. 5; for the C-SH2 binding

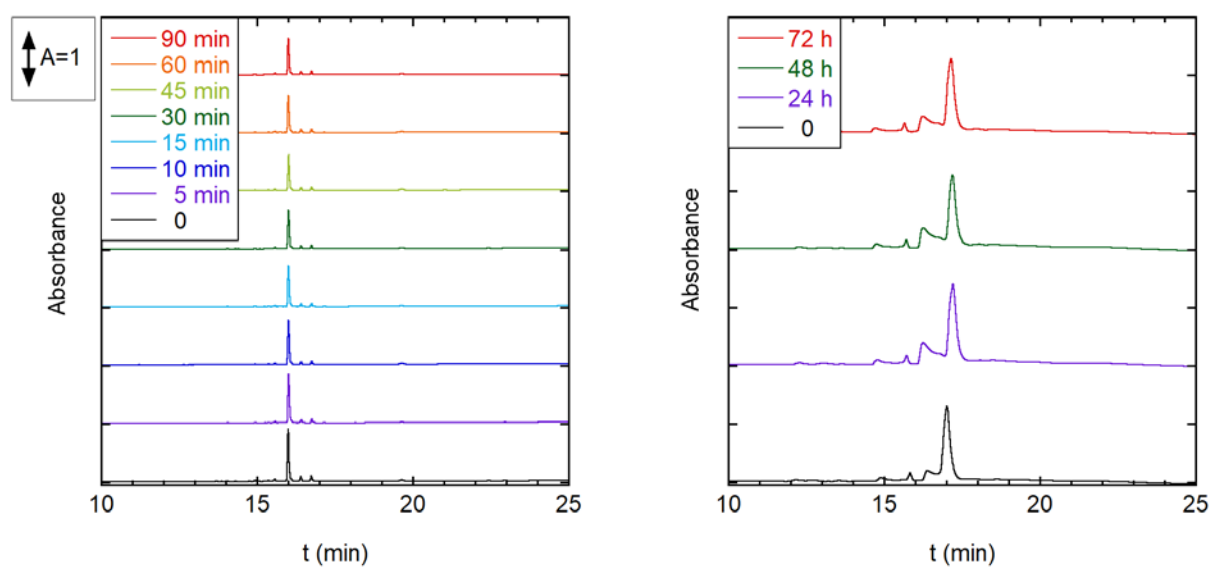
1284 experiments: [CF-P9W5]=1.0 nM.



1285

1286 **Figure S3: binding of the non-dephosphorylatable peptide CF-P9ND0W5 (or OP) to the N-**
1287 **SH2 domain.**

1288 For comparison, the curve for CF-P9W5 is also shown. [CF-P9ND0W5]=1.0 nM, [CF-
1289 P9W5]=0.10 nM.



1290

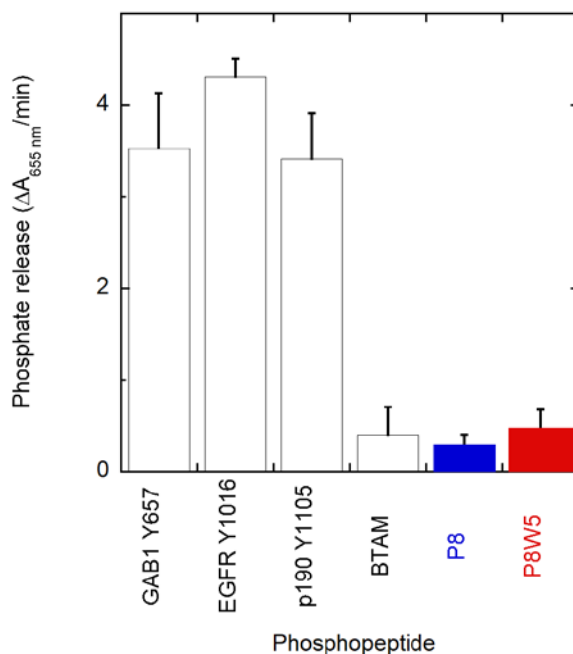
1291 **Figure S4. OP resistance to proteolytic degradation in human serum and in DMEM**

1292 HPLC profiles of OP (CF-P9ND0W5), after incubation with human serum (left) or DMEM

1293 (right), for different times.

1294

1295



1296

1297 **Figure S5: dephosphorylation of P8W5 and other phosphopeptides by SHP2 Δ 104.**

1298 The following phosphopeptides were used for comparison, in addition to P8 and P8W5.

1299 GAB1 Y657 (DKQVEpYLDDL)

1300 p190A/RhoGAP Y1105 (EENIpYSVPHD)

1301 EGFR Y1016 (VDADEpYLIPQQ)

1302 BTAM, or bisphosphorylated SHSP-1 TAM1

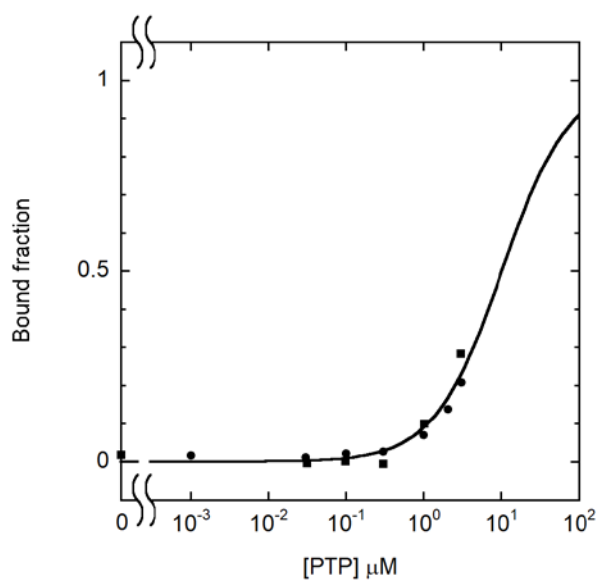
1303 (GGGGDIT(pY)ADLNLPKGKKPAPQAAEPNNHTE(pY)ASIQTS, with 4 N-terminal G
1304 residues)

1305 A SHP2 construct lacking the N-SH2 domain (i.e. the first 104 residues, SHP2 Δ 104) was used at a
1306 95 nM concentration. Phosphopeptides were added at a 100 μ M concentration and the phosphate
1307 released was measured at different times. From the linear region of the phosphate versus time

1308 curve, the variation in absorbance at 655 nm in 1 min, due to phosphate release, was calculated
1309 and plotted.

1310

1311

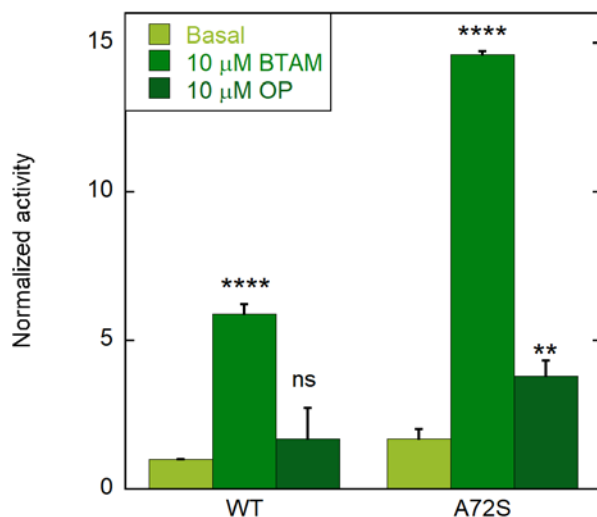


1312

1313 **Figure S6. CF-OP association to the PTP domain.**

1314 [CF-P9ND0W5]=1.0 nM

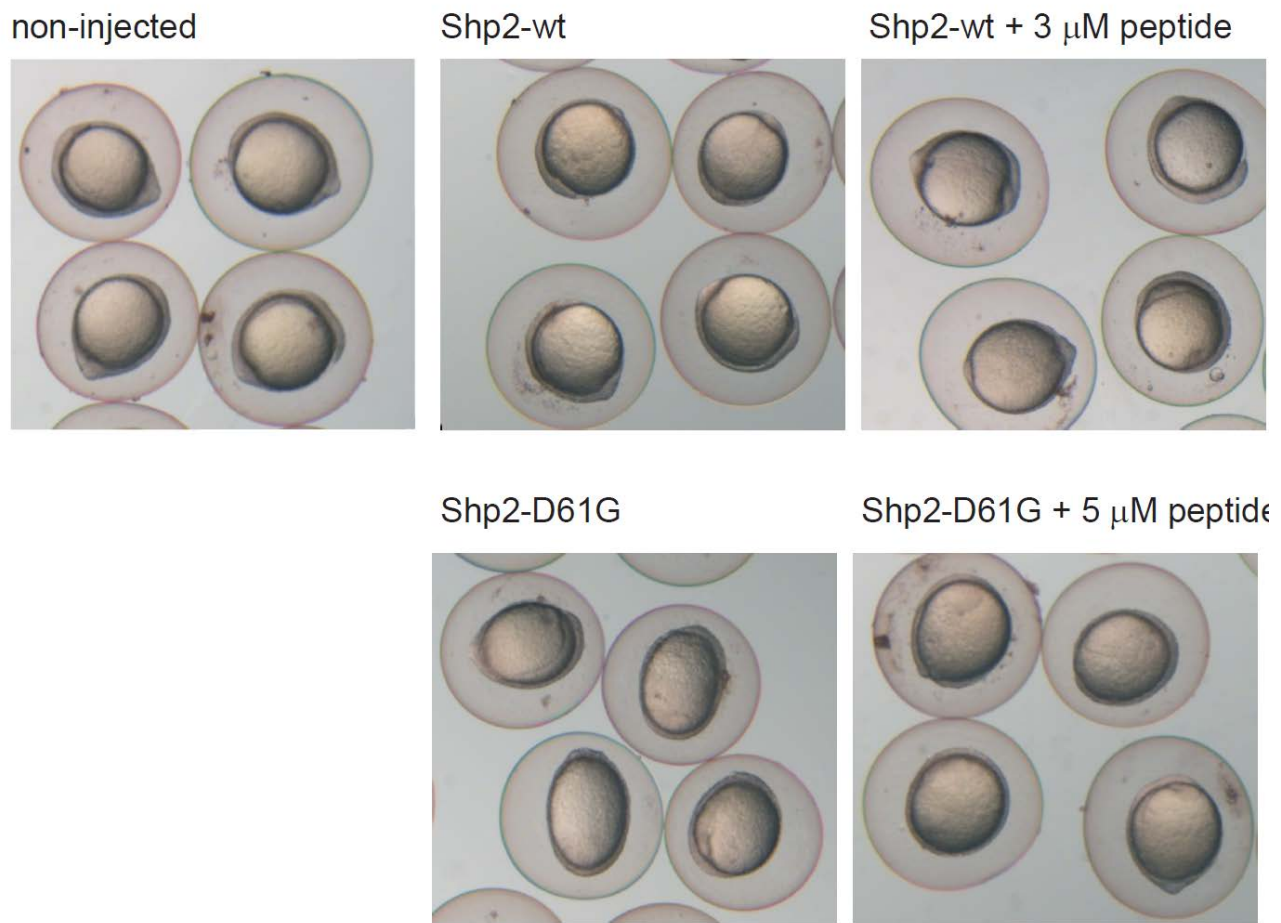
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1316

1317 **Figure S7: SHP2 activation by the OP**

1318 Basal activity is reported in light green, while activities in the presence of 10 μM BTAM or 10 μM
1319 OP are shown in green and dark green, respectively. Error bars represent standard deviations.
1320 Statistical significance of the difference between the basal activities and the activities in the
1321 presence of the peptides, was calculated by ANOVA, complemented by Tukey's test, and is
1322 indicated by asterisks (Non significant (ns) $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ****
1323 $p < 0.0001$)



1324

1325 **Figure S8. Representative images of zebrafish embryos at 11 hpf.**

1326 Embryos were injected at the one-cell stage with mRNA encoding GFP-2A-Shp2-D61G or GFP-
1327 Shp2-wt with or without peptide at 0.3 μ M, 3 μ M and 5 μ M concentration. Non-injected embryos
1328 (ni) were evaluated as a control.

1329

1330

1331 **Supplementary tables**

1332 **Table S1. Literature values for IRS-1 pY1172/N-SH2 domain dissociation**

1333 **constant.**

Reference	Method	K _D
Case 1994	Radioactively labeled peptide	~1-10 μM
Sugimoto 1994	Surface plasmon resonance	14±8 nM
Keilhack 2005	Isothermal titration calorimetry	51 nM

1334

1335

1336 **Table S2. Characterization of the synthesized peptides**

1337

Peptide	ESI-MS			
	HPLC* rt (min)	Purity**	calc [M+H] ⁺ [M+H] ⁺	exp
P9	21.2 ^a	95%	1156.5	1156.6
P9Y0	10.9 ^b	99%	1076.6	1076.6
P8	24.3 ^a	92%	1099.5	1099.4
P7	16.9 ^a	92%	986.4	986.3
P8W5	12.5 ^c	95%	1172.5	1172.5
P8F5	15.1 ^e	95%	1133.2	1133.5
P8E4W5	12.4 ^c	98%	1186.2	1186.5
P9ND0W5	14.3 ^e	97%	1263.4	1263.4
CF-P9	14.9 ^b	93%	1473.5	1473.6
CF-P9Y0	18.7 ^b	99%	1392.6	1392.6
CF-P9W5	14.0 ^c	96%	1545.4	1545.5
Cy3-P9W5	19.6 ^c	95%	1626.8	1626.7
CF-P9E4W5	13.9 ^c	97%	1559.1	1559.6
CF-P9ND0W5	16.3 ^e	97%	1579.6	1579.5
P8W5-TAT	16.1 ^f	95%	2550.4	2550.4
CF-P9W5-TAT	14.1 ^e	99%	2924.1	2924.6

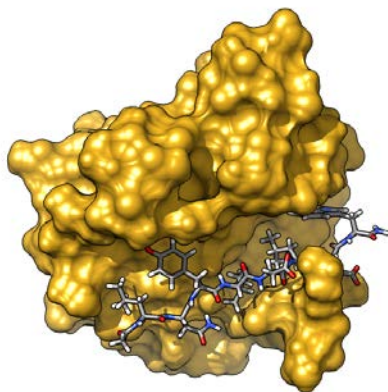
1338

1339 * Phenomenex Kinetex XB-C18 column (4.6 x 100 mm, 3.5 μm, 100 Å); mobile phase A (aqueous
1340 0.05% TFA) and B (acetonitrile, 0.05% TFA). The different elution conditions are reported below:

1341 ^a 10-40%B in 30 min; ^b 20-50%B in 30 min; ^c 20-60%B in 20 min; ^d 10-95%B in 30 min; ^e 5-95%B
1342 in 30 min;
1343 ^f 5-65%B in 30 min.
1344

1345

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