Peptide-based Inhibitors of Protein-Protein Interactions

of Src Homology 2 Domain-Containing Phosphatase 2

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ABSTRACT

The Src homology 2 domain-containing phosphatase 2 (SHP2) is emerging as a central target in the therapy of cancer and some rare diseases, due to its pivotal role in multiple signaling pathways. Mutations of *PTPN11*, the gene coding for SHP2, cause childhood leukemias and developmental disorders. Previously reported SHP2 inhibitors target the catalytic site or an allosteric pocket, but lack specificity, or are ineffective on pathogenic SHP2 variants. Several lines of evidence indicate that increased association with cognate proteins, through its SH2 domains, rather than enhanced catalytic activity, is the main effect of pathogenic *PTPN11* mutations causing hyperactivation of SHP2-mediated signaling. Based on these considerations, we explored the possibility to target SHP2 protein-protein interactions. We developed peptide-based molecules with low nM affinity to the N-SH2 domain of SHP2 and negligible association to other SH2 domains, thus demonstrating that, contrary to the common consensus, phosphopeptide/SH2 domain association with high affinity and specificity is possible. The best non-dephosphorylatable peptide is proteolytically stable and binds to basally activated pathogenic variants of SHP2 with an affinity up to 20 times higher than to the wild-type protein, in contrast to allosteric inhibitors, which are poorly effective on disease-associated variants. Consistently, the peptide was able to revert in a dose-dependent manner the effects of a pathogenic SHP2 mutation (D61G) on development and mortality in zebrafish embryos, while the wild-type animals were unaffected. Our results provide a novel route for SHP2-targeted therapies and a tool to investigate the role of protein-protein interactions in the function of SHP2.

SIGNIFICANCE STATEMENT

Protein phosphorylation is a post-translational modification involved in intracellular signaling cascades regulating fundamental processes such as cell proliferation and differentiation. Consequently, kinases (enzymes catalyzing phosphorylation) are a main target of anticancer drugs. By contrast, phosphatases (catalyzing dephosphorylation) have long be considered undruggable. Allosteric inhibitors, blocking a phosphatase called SHP2 in a closed, autoinhibited state, have recently been discovered. However, these molecules are poorly effective on SHP2 mutants that cause leukemias or genetic disorders, for which no cure currently exists. Here we demonstrate the efficacy of an alternative approach, using peptide-based molecules to inhibit SHP2 interactions with other proteins, rather than its catalytic activity. These inhibitors are particularly effective on SHP2 mutants and revert the effects of the mutations *in vivo*.

INTRODUCTION

SHP2 in physiology and pathology

Tyrosine phosphorylation, regulated by protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs), is a fundamental mechanism of cell signaling. Aberrant tyrosine phosphorylation, caused by hyperactive PTKs, occurs in many malignancies and most current targeted anticancer drugs are PTK inhibitors [Wu 2015]. PTPs counteract the effects of kinases, and therefore they are generally considered as negative regulators of cell signaling and as tumor suppressors [Elson 2018]. However, the Src homology 2 (SH2) domain-containing phosphatase 2 (SHP2), encoded by the *PTPN11* gene, is a non-receptor PTP that does not conform to this simplistic picture [Tartaglia 2004 a; Tajan 2015].

SHP2 is ubiquitously expressed and mediates signal transduction downstream of various receptor tyrosine kinases (RTKs): it is required for full and sustained activation of the RAS/MAP kinase pathway [Saxton 1997] and modulates signaling also through the PI3K-AKT and JAK-STAT pathways, among others. Therefore, it is involved in the regulation of multiple cell processes, including proliferation, survival, differentiation and migration [Tajan 2015]. Considering these functions, it is not surprising that dysregulated SHP2 function contributes to oncogenesis and underlies developmental disorders [Grossmann 2010].

PTPN11 was the first proto-oncogene encoding a tyrosine phosphatase to be identified [Tartaglia 2003]. Somatically acquired, gain of function mutations in *PTPN11* are the major cause of juvenile myelomonocytic leukemia (JMML), accounting for approximately 35% of cases [Tartaglia 2003]. JMML is a rare and aggressive myelodysplastic/myeloproliferative disorder of early childhood with a very poor prognosis, for which no drugs are presently available [Niemeyer 2019]. Somatic *PTPN11* mutations also occur in childhood myelodysplastic syndromes, acute monocytic leukemia (AMoL, FAB M5) and acute lymphoblastic leukemia (ALL, “common” subtype) [Tartaglia 2003; 2004 b; Grossmann 2010]. More rarely, activating mutations in this gene are found in adult myelodysplastic syndromes, chronic myelomonocytic leukemia, as well as solid tumors, including neuroblastoma, glioma, embryonal rhabdomyosarcoma, lung cancer, colon cancer and melanoma [Bentires-Alj 2004, Loh 2005, Martinelli 2006 and 2009].

In addition to malignancies driven by *PTPN11* mutations, several forms of cancer are linked to the activity of wild type (WT) SHP2, too. By screening hundreds of cancer cell lines with a shRNA library, a landmark study showed that SHP2 is required for survival of receptor tyrosine kinases (RTK)-driven cancer cells [Chen 2016]. SHP2 is also a central node in intrinsic and acquired resistance to targeted cancer drugs [Prahallad 2015; Fedele 2018; Ruess 2018; Ahmed 2019], which is often caused by RTK activation through feedback loops.

SHP2 is a mediator of immune checkpoint pathways, such as PD-1 [Okazaki 2013]. These signaling cascades inhibit the activation of immune cells, thus allowing self-tolerance and modulation of the duration and amplitude of physiological immune responses. SHP2 binds to the activated receptors and is responsible for starting the signaling cascade that prevents immune cell activation [Okazaki 2013]. Some cancer cells are able to hijack these signaling pathways, thus evading antitumor immune defenses [Pardoll 2012]; therefore, SHP2 is currently being considered as a possible target for cancer immunotherapy [Zhao 2019, Liu 2019, Quintana 2020, Marasco 2020]. Finally, it is worth mentioning that induction of gastric carcinoma by *H. pylori* is mediated by the interaction of its virulence factor CagA with SHP2, causing aberrant activation of the phosphatase [Higashi 2002, Hayashi 2017].

In addition to its role in cancer, SHP2 is involved in a family of rare diseases collectively known as RASopathies. Germline missense mutations in *PTPN11* occur in ∼50% of individuals affected by Noonan syndrome (NS) [Tartaglia 2001], one of the most common non-chromosomal disorders affecting development and growth [Tartaglia 2010; Roberts 2013], and in ∼90% of patients affected by the clinically related Noonan syndrome with multiple lentigines (NSML, formerly known as LEOPARD syndrome) [Digilio 2002, Legius 2002, Tajan 2018]. RASopathies are characterized by congenital cardiac anomalies, hypertrophic cardiomyopathy, short stature, musculoskeletal anomalies, facial dysmorphisms, variable intellectual disability and susceptibility to certain malignancies [Tartaglia 2010]. To date, the only treatment in use for NS and related disorders is growth hormone therapy, to improve linear growth [Roberts 2013, Gelb 2015].

Structure and allosteric regulation of SHP2

The structure of SHP2 includes two Src homology 2 (SH2) domains, called N-SH2 and C-SH2, followed by the catalytic PTP domain, and an unstructured C-terminal tail with a still uncharacterized function (Figure 1) [Tajan 2015]. SH2 domains are recognition elements that bind protein sequences containing a phosphorylated tyrosine (pY) [Yaffe 2002, Anselmi 2020]. In SHP2, they mediate association to RTKs, cytokine receptors, cell adhesion molecules and scaffolding adaptors. Therefore, SHP2 (together with the closely related SHP1) is recruited (through its SH2 domains) by motifs containing two pYs and dephosphorylates other (or even the same) pYs through its PTP domain.

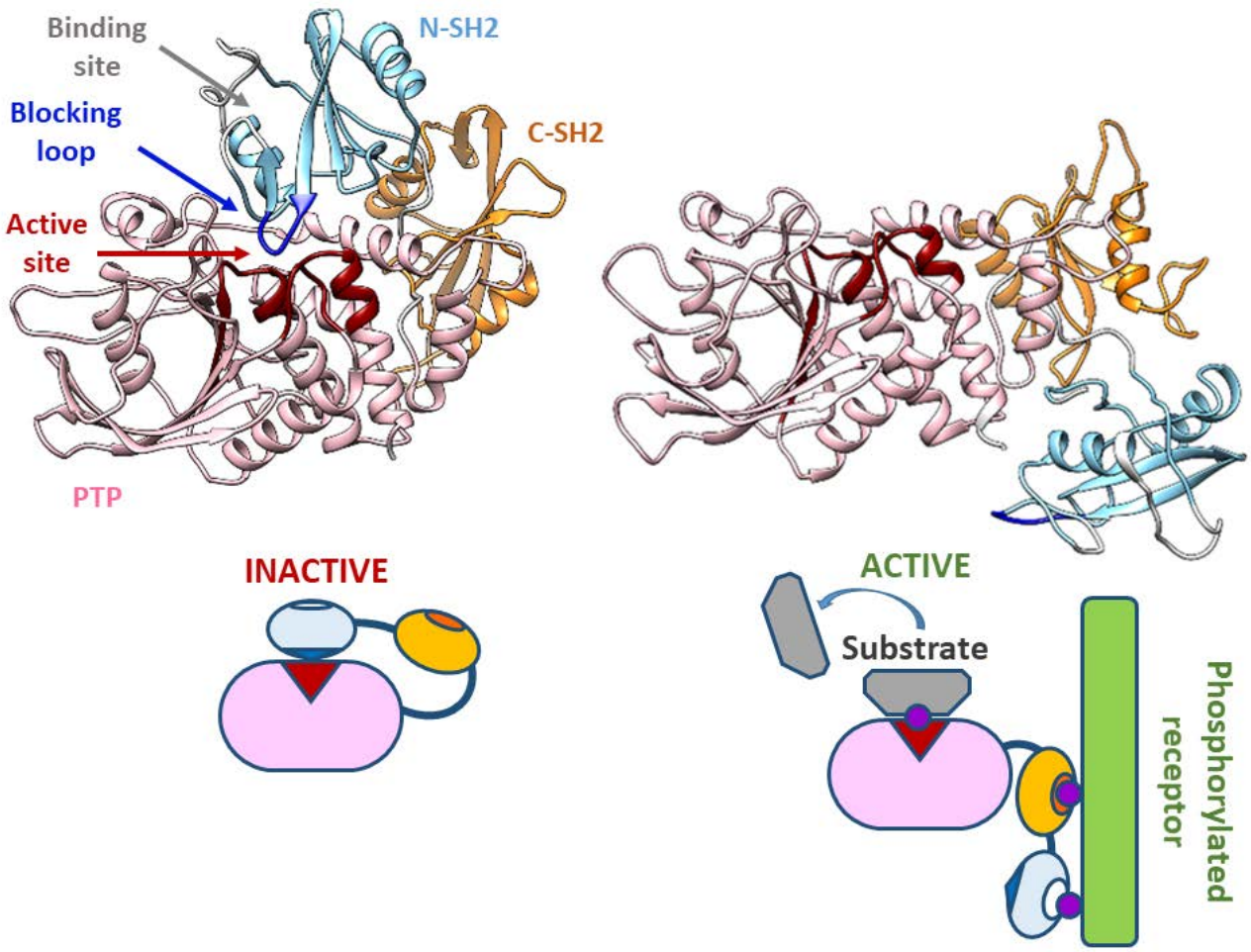

Figure 1: SHP2 structure and scheme of the activation process.

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

Bottom: schematic model of the allosteric regulation mechanism.

The crystallographic structures of SHP2 [Hof 1998, LaRochelle 2018], complemented by biochemical analyses [Keilhack 2005; Tartaglia 2006; Bocchinfuso 2007; Martinelli 2008], have elucidated the main features of the allosteric regulation of SHP2 activity. Under basal conditions, the N-SH2 domain blocks the active site of the PTP domain, inserting a loop (DE or "blocking" loop) in the catalytic pocket. Consistently, the basal activity of SHP2 is very low. Association of SHP2 to its binding partners through the SH2 domains favors the release of this autoinhibitory interaction, making the catalytic site available to substrates, and causing activation (Figure 1). Specifically, structures of the N-SH2 domain associated to phosphopeptide

sequences show that association to binding partners induces a conformational change in the blocking loop, which loses complementarity to the active site [Lee 1994]. At the same time, the N-SH2/PTP interaction allosterically controls the conformation of the N-SH2 domain binding site. Structures of the autoinhibited protein show that the binding site of the N-SH2 domain is closed by two loops (EF and BG). By contrast, in structures of the isolated N-SH2 domain [Lee 1994], or the recently reported structure of the active state of SHP2 [LaRochelle 2018], the binding site is open (Figure 1). Consequently, we and others have hypothesized that the transition between the closed, autoinhibited state and the open, active conformation is coupled to an increased affinity for binding partners [Keilhack 2005; Bocchinfuso 2007, Martinelli 2008, LaRochelle 2018].

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

SHP2 as a pharmacological target

All the findings reported above clearly indicate SHP2 as an important molecular target for cancer and RASopathies [Ran 2016; Frankson 2017]. Since SHP2 is a convergent node for multiple signaling pathways, SHP2 inhibitors may represent a way to suppress the effect of disease-causing mutations involving different proteins along the signaling cascade with a single molecule [Mullard 2018].

Research efforts in SHP2-targeted drug discovery have been focused mainly on active-site inhibitors. Several molecules inhibiting the catalytic activity of SHP2 have been reported [Butterworth 2014], but many of them are affected by the same limitations that led PTPs in general to be considered “undruggable” [Ran 2016, Mullard 2018], *i.e.* lack of target specificity and poor bioavailability. Some compounds with good affinity and apparent selectivity have been described, and data supporting the potential anticancer efficacy of some SHP2 PTP inhibitors have been reported [Bunda 2015; Grosskopf 2015, Zhang 2017]. However, more recent studies demonstrated that these molecules have several off-target effects [Tsutsumi 2018].

An alternative pharmacological strategy has been pursued by researchers at Novartis [Chen 2016], followed by others [Xie 2017; Mullard 2018; Nichols 2018; Wu 2019], who reported allosteric inhibitors stabilizing the autoinhibited structure of SHP2 by binding to a pocket located at the interdomain interface in the closed conformation of the phosphatase. SHP099, the inhibitor developed by Novartis, is finding promising applications in the treatment of RTK-driven cancers [LaMarche 2020] and in combined therapy against drug

resistant cells [Fedele 2018; Ahmed 2019]. These results have spurred a renewed interest in the inhibition of phosphatases [Mullard 2018, Köhn 2020]. Currently, several allosteric inhibitors stabilizing the closed conformation of SHP2 are undergoing clinical trials [Tang, 2020]: TNO155 by Novartis (derived from SHP099), RMC-4630, by Revolution Medicines and Sanofi, JAB-3068 and JAB-3312, developed by Jacobio Pharmaceuticals and RLY-1971, by Relay Therapeutics. Allosteric inhibitors have also been used to target SHP2 to proteolytic degradation [Wang 2020]. However, these compounds are generally poorly effective in the case of activating *PTPN11* mutants, since the allosteric binding site is lost in the open conformation of the enzyme [Sun 2018; LaRochelle 2018, Tang 2020].

Inhibition of protein-protein interactions as an alternative pharmacological strategy

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

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

Truncated constructs with deletion or partial deletion of the N-SH2 domain cause a dramatic increase in the enzymatic activity of SHP2 and, at the same time, a complete loss of its ability to bind signaling partners. These constructs do not affect development in heterozygous mice [Saxton 1997] and do not cause any aberrant phenotype in cells [Saxton 1997, Higashi 2002]. Indeed, RAS/MAPK signaling in homozygous cells with the mutated construct was reduced with respect to the WT cells [Shi 1998]. However, cellular morphological changes (hummingbird phenotype) were observed when the truncated construct was targeted to cellular membranes by adding a membrane-localization signal [Higashi 2002], demonstrating the importance of proper cellular localization, normally mediated by SH2 domain binding. The relevance of SHP2 association to its binding partners for its role in aberrant signaling has been demonstrated also by a study on monobodies targeting the N-SH2 domain and disrupting its association with adaptor proteins. Expression of these monobodies in cancer cells carrying the activating p.V45L mutation abolished ERK1/2 phosphorylation almost completely [Sha 2013]. Similarly, Kertész and coworkers [Kertész 2006a and b] reported that the natural SHP2-binding motif of Gab1, when delivered into immune cells, modulated

phosphorylation patterns.

An example of the opposite situation, where binding is preserved and the catalytic activity is impaired, is provided by *PTPN11* mutations causing NSML, such as T468M. This class of amino acid substitutions are located in proximity of the PTP active site, at the PTP/N-SH2 interface, and have a twofold effect: they destabilize the closed state of the protein, and consequently promote SHP2 association to signaling partners; at the same time, they perturb the active site and therefore strongly impair the catalytic activity of the phosphatase. Interestingly, the phenotype of NSML is very similar to that of NS, and these mutations still allow the activation of multiple effector pathways [Martinelli 2008; Edouard 2010; Marin 2011, Yu 2013, 2014].

Overall, these findings strongly suggest that a mere enhancement in SHP2 catalytic activity is not sufficient to cause disease and indicate that increased association to binding partners plays a major role in the pathogenic mechanism associated with *PTPN11* mutations. Therefore, inhibition of SHP2 binding to other proteins through its SH2 domains represents a promising alternative pharmaceutical strategy. Although SH2 domains in general have received much attention as potential targets of pharmaceuticals [Machida 2005, Watson 2015, 2017, Cerulli 2019], no molecules targeting the SH2 domains of SHP2 for therapeutic purposes have been developed so far.

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

RESULTS

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

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

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

The binding affinity of the IRS-1 pY1172 peptide has been characterized in several literature studies. Unfortunately, these results are extremely contradictory, as reported in Table S1, with dissociation constants ranging from ~10 nM to ~10 µM. Several possible factors can be invoked to explain these discrepancies, including the effect of radioactive labels [Case 1994, Bolton 1973], dimerization of GST-N-SH2 constructs [Sugimoto 1994, Ladbury 1995] even at low nM concentrations [Fabrini 2009], or the sensitivity of the technique [Kelihack 2005, Wiseman 1989]

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

Table 1. Peptide sequences investigated in this study

Abbreviation	Sequence	R_t (min)	$[M+H]^+$	Purity
P9	GLN-pY-IDLDL	21.2 (10-40%B in30')	1156.5	95%
P9Y0	GLN- **Y**-IDLDL	10.9 (20-50%B in30')	1076.6	99%
P8	LN-pY-IDLDL	24.3 (10-40%B in30')	1099.4	92%
P7	N-pY-IDLDL	16.9 (10-40%B in30')	986.3	92%
P8W5	LN-pY-IDLD**W**	12.5 (20-60%B in20')	1172.5	95%
P8F5	LN-pY-IDLD**F**	16.5 (10-95%B in30')	1133.5	93%
P8E4W5	LN-pY-IDL**EW**	12.4 (20-60%B in 20')	1186.5	98%
P9ND0W5 (or OP)	GLN-**ND**-IDLD**W**	15.7 (10-50%B in20')	1263.4	94%
CF-P9	**CF**-GLN-pY-IDLDL	14.9 (20-50%B in30')	1473.5	93%
CF-P9Y0	**CF**-GLN- **Y**-IDLDL	18.7 (20-50%B in30')	1392.6	99%
CF-P9W5	**CF**-GLN-pY-IDLD**W**	14.0 (20-60%B in20')	1545.5	96%
Cy3-P9W5	**Cy3**-GLN-pY-IDLD**W**	19.6 (20-60%B in20')	1626.7	95%
CF-P9E4W5	**CF**-GLN-pY-IDL**EW**	13.9 (20-60%B in20')	1559.6	97%
CF-P9ND0W5 (or CF-OP)	**CF**-GLN-**ND**-IDLD**W**	16.1 (5-65%B in20')	1580.4	93%
P8W5-TAT	LN-pY–IDLD**W**-**GRKKRRQRRR**	16.1 (5-65%B in30')	2550.4	95%
CF-P9W5-TAT	**CF**-GLN-pY-IDLD**W**-**GRKKRRQRRR**	18.2 (5-65%B in30')	2924.6	90%

All peptides were amidated at the C-terminus. Unlabeled peptides were acetylated at the N-terminus. CF is 5,6 carboxyfluorescein, Cy3 is Cyanine 3 carboxylic acid and ND is the non-dephosphorylatable pY mimic phosphonodifluoromethyl phenylalanine (F_2Pmp). RP-HPLC retention times (R_t), molecular weights experimentally determined by ESI-MS spectrometry, and purities are reported. The RP-HPLC elution conditions used for each peptide are shown in parentheses. The optimized peptides are highlighted in grey.

Table 2. Dissociation constants obtained from the fluorescence anisotropy 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

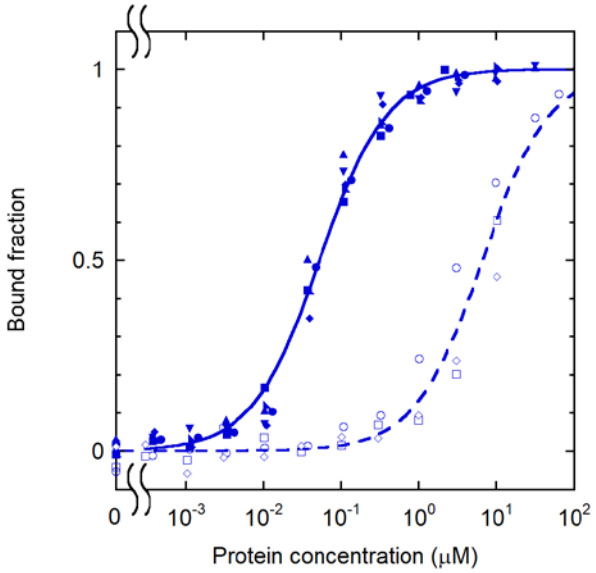

Figure 2: Binding curves for the phosphorylated and unphosphorylated IRS-1 pY1172 peptides.

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

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, in order 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±0.6 μM, compared with 53±2 nM for the phosphorylated peptide. The corresponding values for the standard free energy of binding (assuming a 1 M standard state) are -29.6±0.2 kJ/mol and -41.6±0.1 kJ/mol, respectively. Assuming additivity of contributions, the phosphate group results to be responsible for the difference of -12.0±0.2 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.

2) Sequence optimization

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

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

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

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

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

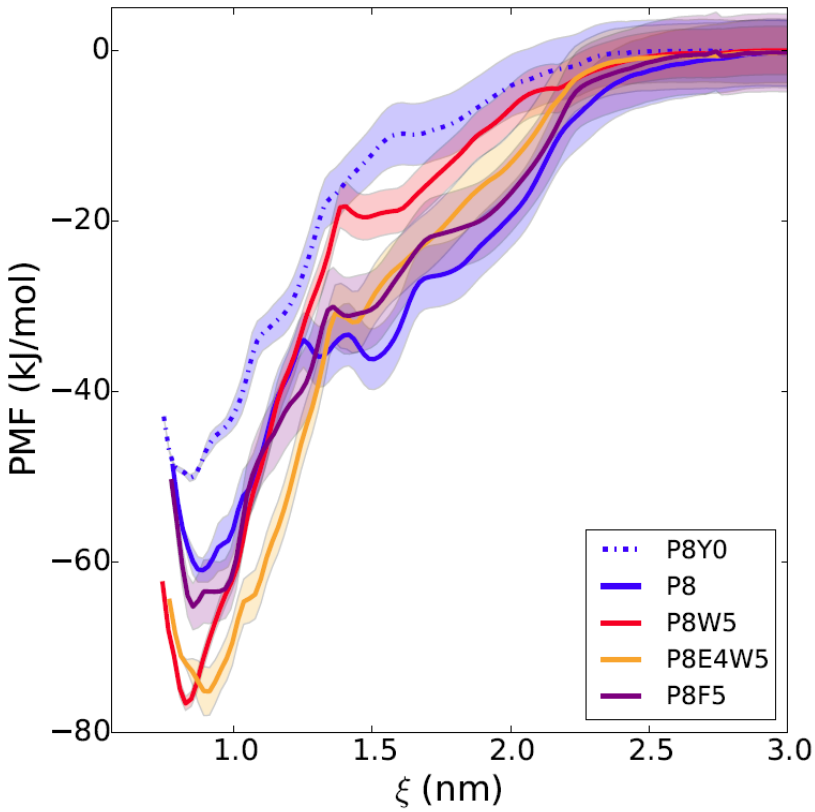

Figure 3: in silico free energy calculations for different modified sequences

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

Analogs with F or W at position +5 (P8F5 and P8W5), as well as a labeled analog with the L to W substitution (CF-P9W5, Table 1) were synthesized and studied experimentally (Figure 4). As predicted, introduction of W in +5 was highly favorable, leading to reduction in the dissociation constant by an order of magnitude, both for the labeled and unlabeled analog (Tables 2 and 3). Consequently, the K_i for the P8W5 analog was 1.6±0.4 nM. By contrast, the additional D to E substitution resulted in a slight loss in binding affinity (Figure 4 and Table 2 and 3).

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

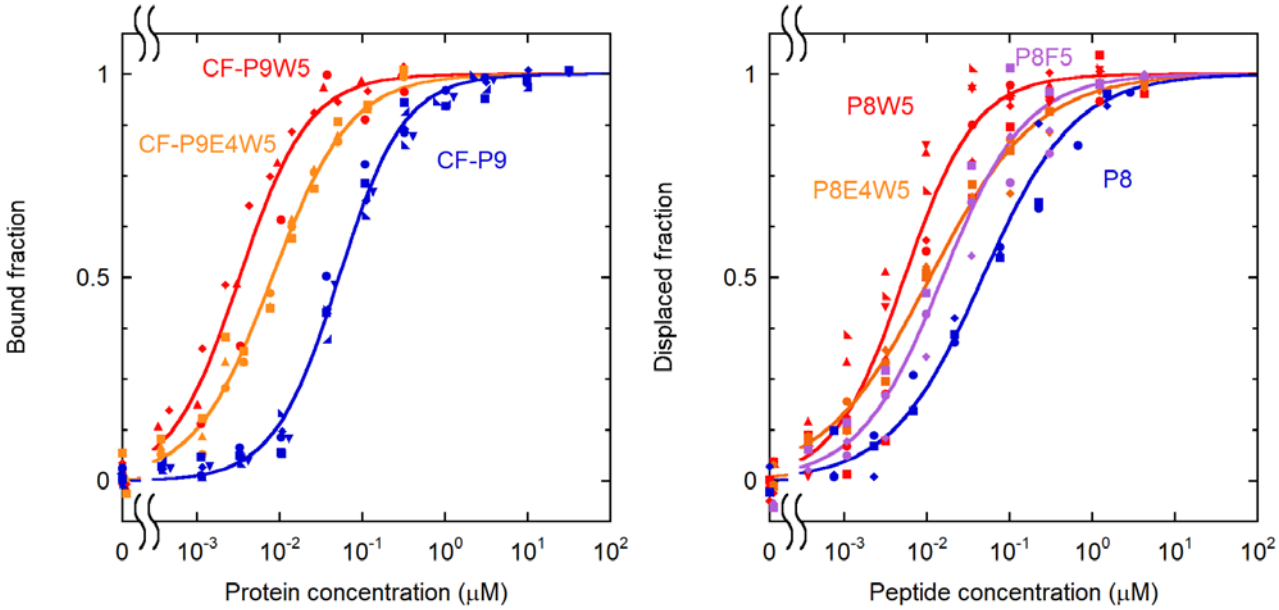

Figure 4: effect of substitutions at position +5 on the binding affinity

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

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

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

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

3) Binding selectivity

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

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

A more complete analysis of the binding selectivity was performed on a protein array of 97 human SH2 domains (Figure 5). An analogue of CF-P9W5 was employed in this assay, where CF was substituted with the Cy3 dye, suitable for detection in the array reader. Control binding experiments showed that the change in fluorophore did affect peptide binding affinity only marginally (Table 2). Strikingly, significant binding was observed only with the N-SH2 domain of SHP2, and, to a lesser extent, to the SH2 domain of the adapter protein APS (also called SHP2B2). It is worth noting that binding to the N-SH2 domain of SHP1, which has the highest identity with that of SHP2 [Liu 2006], was negligible.

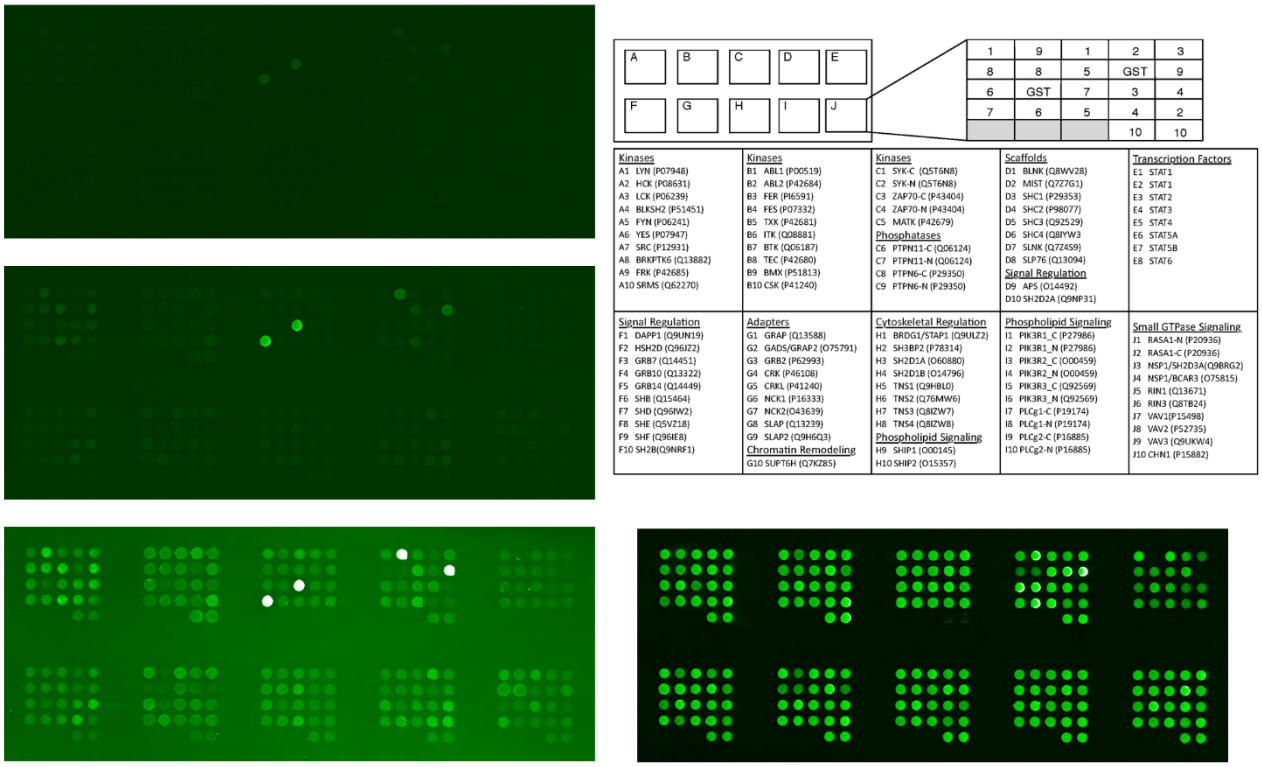

Figure 5: binding selectivity of Cy3-P9W5 for an array of SH2 domains.

The left panels report the fluorescence of the bound peptide, at a concentration of 0.5 nM (top), 5.0 nM (center) and 50 nM (bottom). Each SH2 domain was spotted in duplicate, and negative control spots (with GST only) are also present. The bright spots correspond to the N-SH2 domain of SHP2 and to the SH2 domain of the SH2 and PH domain-containing adapter protein APS (also called SHP2B2). The intensity of all other spots is comparable to that of the negative controls.

The top right panel shows the position of each SH2 domain in the array, while the top bottom panel is a control of the protein loading in each spot, performed with an anti-GST antibody.

4) Engineering resistance to degradation

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

In view of intracellular or *in vivo* applications of the phosphopeptide, it is essential to make it resistant to degradation. The most labile moiety is the phosphate group of the pY residue, which can be hydrolyzed by protein tyrosine phosphatase, possibly also including SHP2, of which IRS-1 pY 1172 has been shown to be a substrate [Noguchi 1994]. We substituted the pY with the non-hydrolysable mimetic phosphonodifluoromethyl phenylalanine (F_2Pmp), which is isosteric with pY and has a total negative charge comparable to that of pY under physiologic pH conditions [Burke 2006].

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

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

4.2) The optimized peptide OP is resistant to proteolytic degradation

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

5) Binding to and activation of the SHP2 protein

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

As discussed in the introduction, we and others have hypothesized that, in its autoinhibited state, the conformation of the N-SH2 domain prevents efficient association to binding partners, while SHP2 binding affinity to phosphorylated sequences is maximized in the open, active state [Keilhack 2005; Bocchinfuso 2007, Martinelli 2008, LaRochelle 2018]. This model has many relevant consequences, because it implies that pathogenic mutants have a twofold effect: they increase the activity of the phosphatase, but also its

affinity towards binding partners. In principle, both effects could be the origin of the hyperactivation of the signal transduction pathways involved in the pathologies caused by *PTPN11* mutations.

Notwithstanding the relevance of this aspect, to the best of our knowledge, no direct phosphopeptide binding experiments to the whole SHP2 protein have ever been performed, possibly due to the fact that pY can be dephosphorylated by the PTP domain. Now, OP and its fluorescent analogue CF-OP allow us to directly assess the hypothesis described above. Figure 6 and Table 2 report the results of binding experiments performed with CF-OP and WT SHP2 or the pathogenic mutants A72S, E76K, D61H, F71L and E76V. E76K is among the most common somatic lesions associated with leukemia, and has never been observed as germline event in individuals with NS [Tartaglia 2003, 2006], as it results in early embryonic lethality [Xu 2011]. This mutation is strongly activating, with a basal activity of the corresponding mutant being at least 10 times higher than that of the WT protein. Conversely, A72S is a germline mutation specifically recurring among subjects with NS. In this case, basal activation is only twofold [Bocchinfuso 2007]. The D61H, F71L and E76V amino acid substitutions have been identified as somatic events in JMML and other leukemias [Tartaglia 2003, Tartaglia 2005] and, when transmitted in the germline, they are associated with a high prenatal lethality (M. Zenker, personal communication). Interestingly, we observed that the affinity for CF-OP nicely parallels the basal activity of these mutants (Figure 6). This finding provides a first direct confirmation that the closed, autoinhibited state has a lower affinity for the binding partners, compared to the open, active conformation.

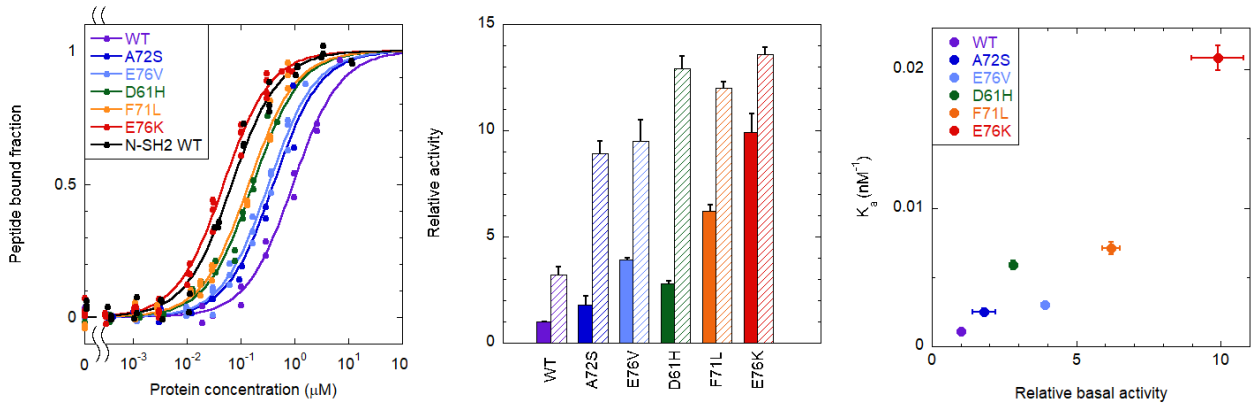

Figure 6: Binding of the CF-OP peptide to the whole SHP2 protein (WT and pathogenic mutants)

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

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

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

Error bars represent standard deviations.

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

Based on previous reports of the dephosphorylation of IRS-1 pY 1172 by SHP2 [Noguchi 1994], we verified if P8 and P8W5 are also a substrate of this protein. These experiments were performed with a truncated SHP2 construct lacking the N-SH2 domain ($SHP2_{\Delta 104}$), as it is fully activated and was shown to be more stable and less prone to aggregation compared to the isolated PTP domain [Martinelli 2020]. As reported in Figure S5, dephosphorylation was indeed observed, although to a lower extent than for other phosphopeptides.

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

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

We used the zebrafish model system to explore the *in vivo* effect of the peptide. Zebrafish Shp2a is highly homologous to human SHP2 (91.2% protein sequence identity); in particular, the sequence of the N-SH2 domain and of the N-SH2/PTP interface are identical in the human and fish proteins. RASopathies-associated mutants, including activating mutants of Shp2a, greatly impact zebrafish development. In humans, the D61G substitution has been found in both NS and leukemia [Kratz 2005] and in animal models it induces both NS-like features and myeloproliferative disease [Araki 2004]. Microinjection of synthetic mRNA encoding NS-associated mutants of Shp2 at the one-cell stage induces NS-like traits [Jopling 2007; Patterson 2020]. During gastrulation, convergence and extension movement are affected, resulting in oval-shaped embryos, with increased major/minor axis length ratio at 11 hpf [Bonetti 2014; Jindal 2016; Jopling 2007). Here we co-injected Shp2a-D61G mRNA with OP in zebrafish embryos, to investigate whether OP rescues the defective cell movements during gastrulation.

As shown in Figure 7 and Figure S7, we observed a dose-dependent decrease in Shp2a-D61G-induced major/minor axis ratios, with a rescue of the phenotype that was significant at 5 μM peptide concentration. On the other hand, embryos injected with Shp2a-WT were almost perfect spheres at 11 hpf, and co-injection with 3 μM peptide had no impact on their shape. As expected, a large portion of Shp2a-D61G injected embryos were severely affected and they died during embryonic development, whereas injection of WT Shp2a did not induce significant lethality. We followed the survival of Shp2a-D61G injected embryos

and observed a significant and dose dependent improvement in the survival of embryos upon co-injection with 0.3 µM, 3 µM and 5 µM OP (Figure 8). By contrast, lethality of WT Shp2a embryos was not affected by co-injection of 3 µM OP. Altogether, these results indicate that co-injection of the OP rescued the developmental defects induced by a pathogenic, basally activated Shp2a variant, while it had no effect on WT embryos.

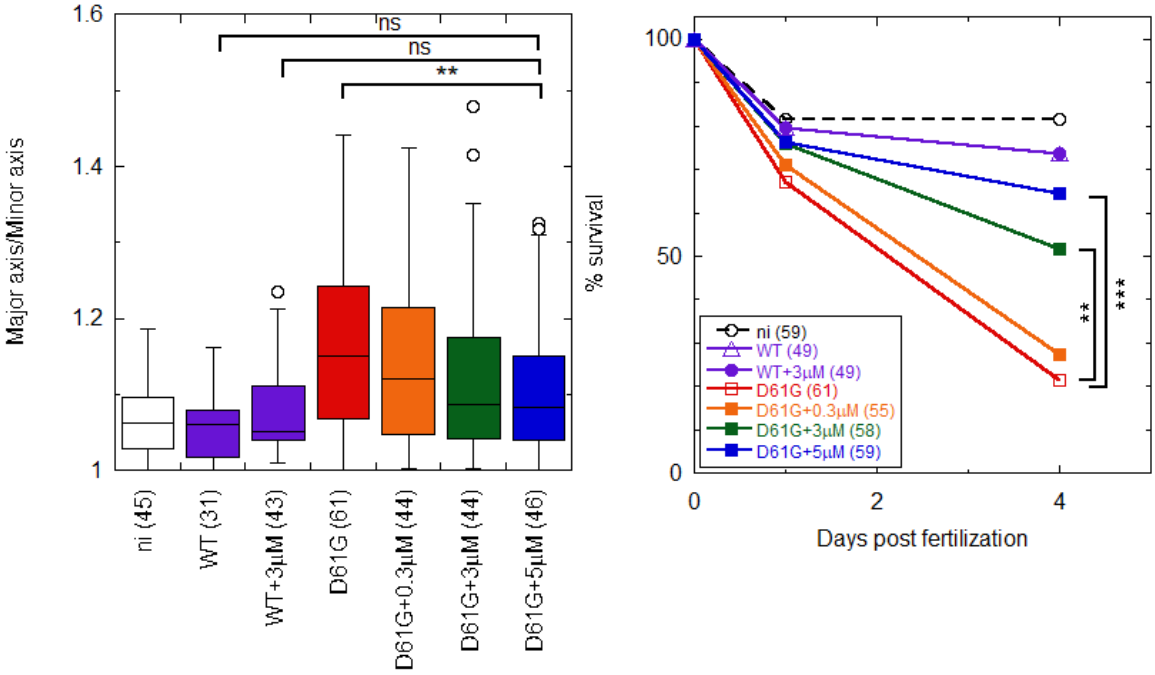

Figure 7: the OP partially rescues Shp2a-D61G-induced gastrulation defects and mortality in a dose dependent manner in zebrafish embryos.

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

Left panel: ovality of embryos at 11 hpf, as indicated by the ratio of the long and the short axis. Tukey's honest significant difference test was done to assess significance. In the box plot, the horizontal line indicates the median, box limits indicate the 25th and 75th percentiles (interquartile range), whiskers (error bars) extend to the maximum and minimum values, or to 1.5 times the interquartile range from the 25th and 75th percentiles, if some data points fall outside this range. In the latter case, outliers are indicated as single data points.

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

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

DISCUSSION

Our findings provide several insights in the interaction of phosphopeptides with SH2 domains, and in particular, with the N-SH2 domain of SHP2.

Soon after their discovery, the affinities of SH2 domains for their binding partners *(i.e.* the dissociation constants) were considered to fall in the 10-100 nM range [Cohen 1995, Pawson 1995]. However, it turned out that most of the binding studies performed in that period were affected by experimental artifacts, leading to an overestimation of the binding affinities [Ladbury 1995, Kuriyan 1997]. A reassessment of the affinity values led to a commonly accepted range in the order of 0.1 to 10 µM [Kuriyan 1997, Bradshaw 2002, Machida 2005, Kaneko 2012, Wagner 2013]. Such moderate affinities are considered to be crucial for allowing transient association and dissociation events in cell signaling. Consistently, SH2 domains artificially engineered to reach low nanomolar affinities for phosphorylated sequences (known as superbinders) [Kaneko 2012 b], by increasing the domain affinity for the pY residue, have detrimental consequences for signal transduction [Gopalasingam 2015].

In the case of the N-SH2 domain of SHP2, literature results on the affinity for the IRS-1 pY 1172 peptide were contradictory, with dissociation constants varying by three orders of magnitude [Case 1994, Sugimoto 1994, Keilhack 2005]. Here, we showed that, at least for this peptide, the dissociation constant is in the low nM range. For the N-SH2 domain, similar affinities have been reported also for the GRB2-associated-binding protein 1 (Gab1), pY 627 [Koncz 2001], and Gab2, pY 614 [Bonetti 2018]; several other peptides have a dissociation constant within an order of magnitude of that of IRS-1 pY 1172 [Anselmi 2020]. In addition, in the present study, we were able to further improve the affinity with respect to the parent peptide. Therefore, the N-SH2 domain of SHP2 might constitute an exception in the panorama of SH2 domains, regarding the binding affinity. In most cases, interaction of phosphopeptides with SH2 domains is dominated by the hydrophobic effect (except for the pY pocket). The N-SH2 domain of SHP2 has a peculiar KxK motif in the region of the BG loop pointing towards the binding groove, which can interact electrostatically with basic residues present in the peptide sequence at positions +2 and +4 [Anselmi 2020]. Therefore, by contrast to the superbinders, the high binding affinity of the N-SH2 domain is a result of additional interactions in the selectivity-determining region, and not in the pY pocket. Indeed, our data showed that the pY phosphate contributed less than 30% of the standard binding free energy. This finding is comparable to what has been reported for other SH2 domains [Waksman 2004].

Our data also showed that residue -2 contributes significantly to the binding affinity. Indeed, while the specificity of most SH2 domains is determined by residues C-terminal to the pY, peptide library and array studies have shown that, contrary to most other SH2 domains, the N-SH2 domain of SHP2 has specific preferences for position -2 [Anselmi 2020]. This peculiarity is due to the fact that, in place of the commonly conserved arginine at position 2 in the first α-helix (αA2), the N-SH2 domain of SHP2 has G13. As a

consequence, a hydrophobic peptide residue at position −2 can insert in the space left free by the missing protein side chain and interact with V14 αA3 and with the phenol ring of pY, stabilizing its orientation and the overall complex [Anselmi 2020].

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

In general, SH2 domains are only moderately discriminating for binding target sequences, and a range of residues is tolerated at each site [Kuriyan 1997, Bradshaw 2002, Waksman 2004]. Consequently, nonspecific tyrosine phosphorylated sequences are usually bound only 10- to 100-fold more weakly than specific targets [Bradshaw 1998, Bradshaw 2002, Waksman 2004, Machida 2005, Wagner 2013]. Indeed, additional specificity is often provided by tandem SH2 domains [Waksman 2004]: two closely-spaced tyrosine phosphorylated motifs bind to tandem SH2 domains with 20- to 50-fold greater affinity and specificity compared with the binding of a single SH2 domain with a single tyrosine phosphorylated motif [Wagner 2013]. SHP2 and its SH2 domains are no exception in this case, as peptide library and array studies, together with the sequences of known natural binding partners, showed a significant variability in the sequences of peptides bound by SHP2 [Anselmi 2020]. However, our results indicate that some peptides (like those developed here) can bind specifically to a single SH2 domain. Among an array of 97 human SH2 domains, we found some interference only with adapter protein APS (also called SHP2B2). The structure of the APS SH2 domain in complex with a cognate protein shows that the phosphorylated sequence binds in a folded, kinked conformation, rather than in the usual extended binding mode [Hu 2003]. This observation should facilitate the further development of our peptides, to avoid the unwanted interaction with APS, without affecting the affinity for the target N-SH2.

Finally, it is worth mentioning that approximately one order of magnitude in affinity was lost by substituting the pY residue with the non-dephosphorylatable mimic F_2Pmp. This finding is consistent with previous studies showing that F_2Pmp is tolerated differently by various SH2 domains, and its insertion in place of pY in a phosphopeptide sequence can lead either to a loss or to an increase in affinity, by approximately one order of magnitude [Burke 1994, Burke 2006]. Further optimization of this aspect is warranted, but the dissociation constant of our non-dephosphorylatable peptide remained in the nM range.

The non-dephosphorylatable peptide allowed novel experiments on several aspects of SHP2 function and regulation. As discussed in the introduction, in the autoinhibited state of SHP2, the N-SH2 binding groove is

closed, apparently making phosphopeptide association impossible. By contrast, the N-SH2 binding site is open in the structure of the isolated N-SH2 domain or of active SHP2. Consequently, it has been hypothesized that mutations destabilizing the closed state and favoring SHP2 activation could lead to an increase binding affinity [Bocchinfuso 2007]. This idea is indirectly supported by the fact that basally activated mutants require lower concentrations of SH2 domain-binding phosphopeptides to reach full activation [Keilhack 2005; Bocchinfuso 2007, Martinelli 2008, LaRochelle 2018]. However, no direct measurements of phosphopeptide binding to different SHP2 variants had been reported until now. Our data directly demonstrate that the affinity for phosphopeptides of activated variants of SHP2 can increase by a factor of 20, reaching the same value as the isolated domain in the most active mutants (Figure 6). This consequence of pathogenic mutations adds to the increase in basal activity and might be the main responsible for hyperactivation of signaling pathways modulated by SHP2. Interestingly, in view of possible therapeutic applications, in a cellular environment, our peptide would act as an effective inhibitor of the PPIs of mutant, hyperactivated SHP2, while it would have a much lower effect on the WT protein. This behavior is the exact opposite of what has been observed for allosteric inhibitors, such as SHP099, which have a significantly impaired activity in pathogenic variants of SHP2 [Sun 2018; LaRochelle 2018].

A second link between SHP2 activity and binding functions is provided by literature data indicating for SHP2 interactors a role both as ligands of the SH2 domains and as substrates of the catalytic PTP domain, often with the same phosphorylated sequence. Examples include IRS-1 [Sugimoto 1994, Noguchi 1994], Gab1 [Cunnick 2001, Zhang 2002], Gab2 [Gu 1997], PDGFR [Rönnstrand 1999, Sugimoto 1992], PD-1 [Marasco 2020] and SHPS-1 [Fujioka 1996, Takada 1998]. We also showed here that our modified sequence can be dephosphorylated. These data indicate the possible presence of a still uncharacterized feedback mechanism to regulate SHP2 signaling. Using our non-dephosphorylatable peptide, we could demonstrate that a N-SH2-binding sequence associates to the catalytic PTP domain, too, although with a lower affinity. This finding suggests that it might be possible to develop double-edged sword molecules, able to inhibit both the catalytic activity and the PPIs of SHP2.

Inhibition of PPIs, particularly using peptides, is currently a hot area of pharmaceutical research [Modell 2016, Giralt 2011]. For the RAS/MAPK pathway alone, at least 30 inhibitors of PPI have been developed and several of them are undergoing clinical trials [García-Gómez 2018]. However, no studies on SHP2 have been reported, notwithstanding the central role of this phosphatase in the pathway. Peptides are particularly appealing for the inhibition of PPIs, where large interfaces are involved [Shin 2017], which are difficultly targeted selectively by small molecules. An increasing number of drugs based on peptides or peptidomimetics is progressing in the drug development pipeline [Henninot 2017]. Possible challenges in the therapeutic applications of peptide-based molecules are their rapid degradation and a poor cell uptake, particularly for highly charged sequences [Henninot 2018, Checco 2015, Johnson 2014]. Here we successfully overcame the first hurdle, thanks to the introduction of non-natural amino acids. Several

studies have demonstrated that efficient intracellular delivery of phosphopeptide mimics is possible, for instance by conjugation to cell-penetrating sequences [Watson 2017, Nasrolahi Shirazi 2013, Hsiao 2011]. Optimization of the cell uptake of our molecules, through different strategies, is currently underway.

Our *in vivo* findings on zebrafish embryos are very promising in view of potential applications, particularly considering that the peptide is more effective on activating mutants than on the WT protein, contrary to allosteric inhibitors such as SHP099 [Sun 2018; LaRochelle 2018, Tang 2020]. Indeed, besides their possible use as a research tool to study the role of PPIs in the function of SHP2, and regulation of the pathways controlled by this protein, including RAS/MAPK and PI3K/AKT signaling [Tajan 2018], the reported peptides constitute lead compounds for the development of new drugs against malignancies driven by *PTPN11* mutations, such as JMML, AMoL, and ALL, also considering that allosteric inhibitors have low activity against basally activated SHP2 variants [Sun 2018, Larochelle 2018]. Finally, another possible field of therapeutic application is represented by rare diseases such as NS and NSML, which are caused by activating mutations of *PTPN11* (against which the available allosteric inhibitors are poorly active) and cause several severe postnatal, evolutive clinical manifestations, particularly hypertrophic cardiomyopathy [Tartaglia 2010].

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AUTHOR CONTRIBUTIONS

S. B., V. C., A. Q., G. T, G.C., A.B. and L.S. performed the binding studies and participated to the protein purification; L.P., E.F., M.V., G.C., V.T., M.S. and S.M. contributed the DNA constructs, protein production and purification, and the phosphatase assays; B.B., C.D.F., T.G., C.P. and F.F. performed peptide synthesis and purification and the experiments on peptide degradation; M. S., A. L., G. F., J. H. and J. d. H. contributed the zebrafish experiments. P.C., V. S., L.S. and G. B. contributed the *in silico* investigations; S.M., G.B., M.T. and L.S. conceived the study; L.S. coordinated the research and wrote the manuscript. All the authors revised the article and contributed to its writing.

COMPETING INTERESTS

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

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