Pathogenic *PTPN11* variants involving the poly-glutamine Gln^{255}-Gln^{257} stretch highlight the relevance of helix B in SHP2's functional regulation

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Abstract

Germline *PTPN11* mutations cause Noonan syndrome (NS), the most common disorder among RASopathies. *PTPN11* encodes SHP2, a protein tyrosine-phosphatase controlling signaling through the RAS-MAPK and PI3K-AKT pathways. Generally, NS-causing *PTPN11* mutations are missense changes destabilizing the inactive conformation of the protein or enhancing its binding to signaling partners. Here, we report on two *PTPN11* variants resulting in the deletion or duplication of one of three adjacent glutamine residues (Gln255-to-Gln257). While p.Gln257dup caused a typical NS phenotype in carriers of a first family, p.Gln257del had incomplete penetrance in a second family. Missense mutations involving Gln^{256} had previously been reported in NS. This poly-glutamine stretch is located on helix B of the PTP domain, a region involved in stabilizing SHP2 in its autoinhibited state. Molecular dynamics simulations predicted that changes affecting this motif perturb the SHP2's catalytically inactive conformation and/or substrate recognition. Biochemical data showed that duplication and deletion of Gln^{257} variably enhance SHP2's catalytic activity, while missense changes involving Gln^{256} affect substrate specificity. Expression of mutants in HEK293T cells documented their activating role on MAPK signaling, uncoupling catalytic activity and modulation of intracellular signaling. These findings further document the relevance of helix B in the regulation of SHP2's function.

Key words: Noonan syndrome, ERK phosphorylation studies, *in vitro* phosphatase assay

Clinical features associated with the identified *PTPN11* variants

Family 1. The p.Gln257dup variant identified in the index case and his mother is present in the ClinVar database (variation ID:40519) and is classified as pathogenic by the submitter (GeneDx). This variant

has not been reported in the ExAC (http://exac.broadinstitute.org/) and gnomAD (https://gnomad.broadinstitute.org/) population databases, but it has been linked to NS in three members of a Spanish family with typical facial dysmorphism, pterygium colli, short stature, and lowset ears (*Collazo et al., 2012, Rev. Esp. Endocrinol. Pediatr. 3(1):37–46*).

Family 2. We found the heterozygous p.Gln257del variant in a three - generation family (index case, father and paternal grandmother). No other variant in known RASopathy genes was identified. Two alleles with this variant have been reported in gnomAD v.2.1 (frequency: 8.081e - 6; 247,494 alleles).

Facial appearance of affected subjects are visible in Figure 1, while the clinical features of individuals carrying the single residue duplication and deletion are reported in Table 1.

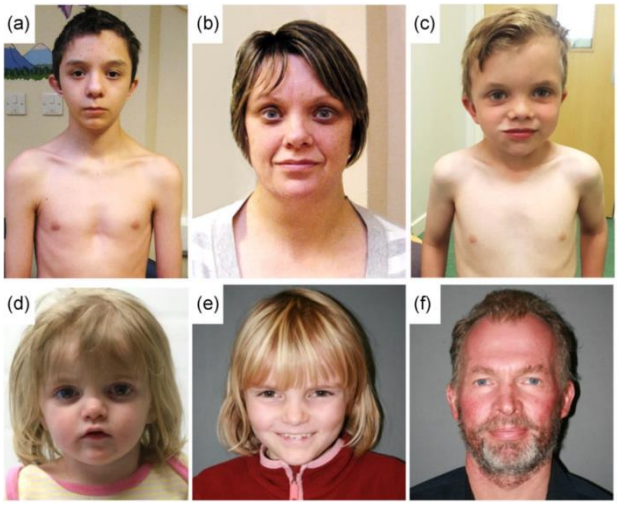

Figure 1. Facial appearance of subjects heterozygous for the p.Gln257dup and p.Gln257del pathogenic variants.

(a) Dysmorphic features of the index patient from family 1 at the age of 16 years; note mild pectus carinatum. (b) Facial features of the index case mother (family 1). (c) Facial features of the index case brother (family 1) at the age of 5 years and 9 months; note mild pectus carinatum and widely spaced nipples. (d, e) Facial aspect of the index subject from family 2 at the age of 2.4 and 9 years, respectively, mild NS features arevisible. (f) The index case father (family 2) has no obvious signs of NS.

	Family 1			Family 2	
1-1 (Index)	1-2 (Mother)	1-3 (Brother)	2-1 (Index)	2-2 (Father)	
Gender	Male	Female	Male	Female	Male
Age at last evaluation	16 years	52 year	5 year 9 months	8 year 11 month	51 year
PTPN11 variant	p.(Gln257dup)	p.(Gln257dup)	p.(Gln257dup)	p.(Gln257del)	p.(Gln257del)
Inheritance	maternal	unknown	maternal	paternal	maternal
Prenatal features	–	NA	NE		
Birth measurements: weight, length, OFC (weeks GA)	weight 2700 g (−2.0 SD) (40 weeks)	NA	weight 2860 g (−0.5 SD); OFC: 34 cm (−0.3 SD) (37 weeks)	weight 2340 g (−2.4 SD); length 41 cm (−4.6 SD); OFC 31.5 cm (−1.3 SD) (39 weeks)	NA
Feeding difficulties	PF	NA	–	PF, GERD	NA
Height at last examination	160.8 cm (−2.1 SD)	161 cm (−1.1 SD)	105.5 cm (−2.3 SD)	110.2 cm (−4.1 SD)	171 cm (−1.4 SD)
Weight	45.6 kg (−2.4 SD)	NA	18.7 kg (−0.9 SD)	18.3 kg (−3.5 SD)	NA
OFC	53.5 cm (−1.9 SD)	NA	OFC 52.5 cm (+ 0.4 SD)	49.0 cm (−2.8 SD)	NA
Genito-urinary anomalies	–	–	CRY	–	–
Congenital heart defect	PVS	PVS	PVS	PVS, VSD	–
Lymphatic anomalies	–	–	–	–	–
Facial anomalies	typical	suggestive	suggestive	suggestive	atypical
Development	mild MD, mild LD	mild LD	mild LD, mild MD	LD	mild LD
Neurology	- (no MRI)	- (no MRI)	- (no MRI)	- (no MRI)	- (no MRI)
Skeletal	SN, TH	SN	SN, TH	NA	–
Hematology & oncology	–	–	–	–	–
Skin and hair	–	–	–	Psoriasis	Facial keratosis, ichthyosis[a]
Ocular	RE	NA	RE	RE	–
Other malformations/ anomalies	–	–	frequent infections	fragile bones, temper tantrums	–

Abbreviations: CRY, cryptorchidism; GA, gestational age; GERD, gastro-esophageal reflux disease; LD, learning difficulties; MD, motor delay; NA, not applicable/not available; NE, nuchal edema; OFC, occipitofrontal head circumference; PF, poor feeding reported; PH, polyhydramnios; PVS, pulmonary valve stenosis; RE, refractive error; SN, short neck; TF, tube feeding (>4 weeks); TH, thorax anomalies; VSD, ventricular septal defects; –, none/normal.
[a]This phenotype may be explained by the Xp22.32 microdeletion.

Table 1. Clinical features of individuals carrying the *PTPN11* variants resulting in the single residue duplication/deletion affecting the polyglutamine Gln255-Gln256-Gln257 stretch of SHP2.

Structural analysis

Gln255, Gln256, and Gln257 are located on helix B (residues 247 - 261) of the PTP domain (Hof *et al., 1998, Cell, 92:441-450*), a region that is involved in an extended network of inter-domain interactions stabilizing the autoinhibited state of SHP2 (Figure 2a). In particular, Gln255 is in contact with the N-SH2 domain (Arg4 and Tyr63) and with residues located in the PTP domain (Arg498 and Gln506), including the Q - loop motif (residues 505 - 513), which contribute to the active site pocket (Figure 2b). Gln256 has multiple interactions with the N - terminal region of the N - SH2 domain (Arg4, Phe7, and Pro9) and N - SH2/C - SH2 linker (Ser109 and Glu110; Figure 2c). Finally, Gln257 interacts with Arg111 (N - SH2/C - SH2 linker) and Gln495 on the PTP domain (Figure 2d). Elongation/shortening of this poly - glutamine motif is predicted to change the spatial orientation of the helix B side - chains, thus destabilizing the interdomain interactions. Molecular dynamics simulations (Figure 2e - l), showed that, in both mutants, the interdomain interaction is substituted by an intradomain salt bridge with Arg498 (PTP Q - loop). Two amino acid substitutions involving Gln256 (p.Gln256Lys and p.Gln256Arg; N= 7/1216, NSEuroNet database) have been reported in NS. Enhanced sampling replica exchange MD (REMD) simulations on wild - type and mutant proteins composed of the isolated PTP domain preceded by the C - SH2 domain, mimicking the active state of the protein and used in phosphatase assays indicated that the substitution leads to the formation of an additional salt bridge between Arg256 and Asp106, a residue located in the linker joining the two SH2 domains (Figure 2k). Helix B is adjacent to the catalytic site, and the mutation was observed to cause a perturbation of the mobility of the loops involved in substrates recognition. Based on these findings, substitutions at codon 256 are likely to affect the selectivity of the PTP domain.

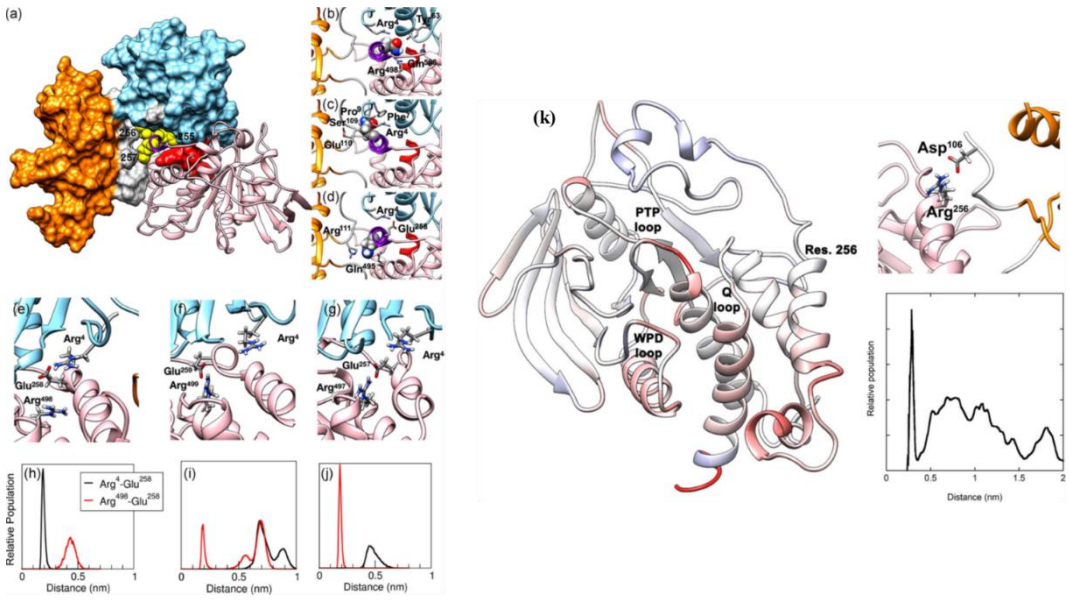

Figure 2. Structural characterization of SHP2's mutants. (a) Location of residues Gln255, Gln256 and Gln257 (reported in space - filling representation and colored in yellow) in the auto - inhibited structure of SHP2 (pdb code

2SHP). The N - SH2 domain (residues 3 - 104), C - SH2 domain (residues 112 - 216) and PTP domain (residues 221 - 524) are shown in light blue, orange and pink, respectively. The Q loop (residues 505 - 513) is colored in red and the helix B of the PTP domain (residues 247 - 261) is reported in purple. (b–d) Close up views of the regions surrounding Gln255, Gln256 and Gln257, respectively. (e–g) Ion - pair interactions involving the glutamic acid following the tri - glutamine motif in the wild - type (Glu258), p.Gln257dup (Glu259) and p.Gln257del (Glu257) proteins, as obtained from MD simulations. (h–l) Distributions of distances between Glu258, or the corresponding residue in the mutant proteins, and Arg4 (black) or Arg498 (red), in the wild - type, p.Gln257dup and p.Gln257del proteins, respectively. (k) Molecular dynamics simulations of the PTP domain of SHP2 carrying the Gln256Arg amino acid substitution.

Phosphatase activity of SHP2 mutants

Under basal conditions only the SHP2^{Gln257dup} mutant exhibited an increased basal activity ($p < .05$, Student's t test; Figure 3a). Conversely, following BTAM peptide stimulation, both mutants displayed enhanced catalytic activation ($p < .001$, in all comparisons), indicating that changes in the number of glutamine residues perturb the SHP2 autoinhibited conformation.

SHP2's mutants intrinsic phosphatase activities demonstrated no significant changes compared to the wild - type enzyme (Figure 3b), further supporting a major effect of deletion and duplication on the stability of the N - SH2/PTP interface. Unexpectedly, the NS-associated lesions at codon 256 did not affect catalysis at all, neither in basal conditions nor following stimulation (Figure 3c), with the intrinsic catalytic activity of the corresponding mutants being comparable to that of the wild - type protein (Figure 3d). Conversely, SHP2^{Gln256Glu} and SHP2^{Gln256Pro}, which have never been identified in subjects with NS, behaved as relatively mild gain - of - function mutants indicating that increased phosphatase activity does not automatically imply a disease - causing role for the relative missense variant.

Malachite green assay demonstrated that amino acid changes at codon 256 presented an enhanced phosphate release against two peptides encompassing stretches of GAB1 and EGFR containing a phosphorylated tyrosine residue (Tyr657 and Tyr1016, respectively) (Figure 3e) indicating that pathogenic variants at Gln256 impact substrate selectivity.

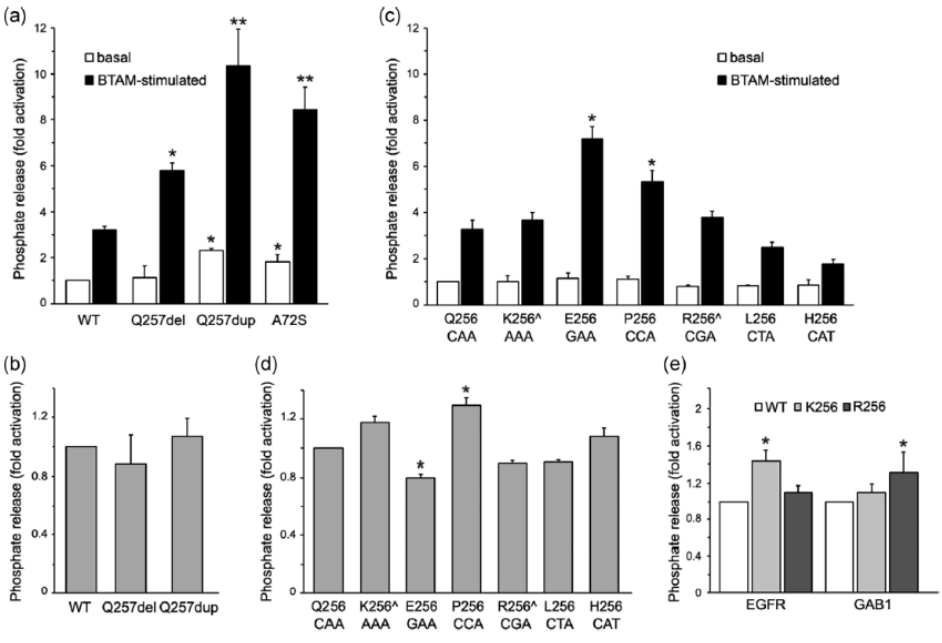

Figure 3. *In vitro* phosphatase assays. (a) Catalytic activities of the p.Gln257del and p.Gln257dup SHP2 mutants compared with that of the wild-type (WT) protein. (b) Catalytic activities of the WT, p.Gln257del and p.Gln257dup

SHP2Δ104 proteins corresponding to the catalytic PTP domain preceded by the C- SH2 domain. (c) Catalytic activities of the SHP2 mutants arising from single nucleotide substitutions at codon 256. (d) Catalytic activities of the SHP2Δ104 mutants affecting codon 256 compared with the WT protein. (e) Change in substrate specificity of SHP2Δ104 p.Gln256Lys and p.Gln256Arg recombinant proteins assessed by malachite green assay, using the GAB1Y657 and EGFRY1016 peptides as substrates.

ERK phosphorylation assay

SHP2 is a positive regulator of RAS and the MAPK cascade (Neel *et al.*, 2003, *Trends. Biochem. Sci. 28:284–293*), and *PTPN11* mutations underlying NS have been established to enhance signal flow through this pathway (Fragale *et al.*, 2004, *Hum. Mutat. 23:267–277*). To test whether the newly identified p.Gln257del and p.Gln257dup variants and the two previously identified missense changes affecting Gln256 were activating, their impact on ERK phosphorylation was assessed in transient transfection experiments. Ectopic expression of the V5-tagged SHP2^{Gln257del} and SHP2^{Gln257dup} proteins in HEK293T cells was found to promote enhanced ERK phosphorylation compared to cells overexpressing the wild-type protein, upon 30 ng/ml EGF stimulation, proving their activating effect on the MAPK cascade (Figure 4). Of note, overexpression of SHP2^{Gln256Lys} and SHP2^{Gln256Arg} also caused a variable stimulus-dependent enhanced ERK phosphorylation, demonstrating further their hyperactive behavior.

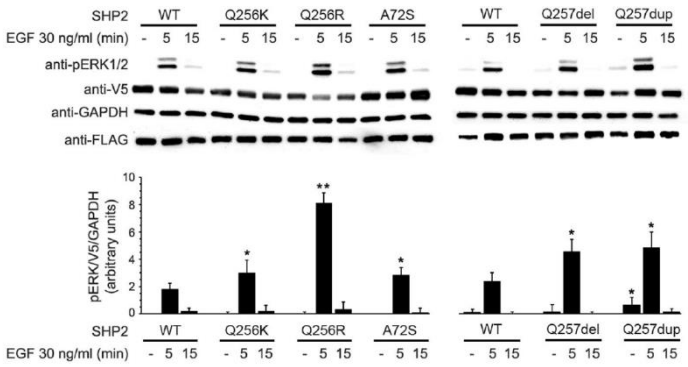

Figure 4. ERK phosphorylation assays. NS-causing mutations affecting the poly-glutamine stretch promote variably enhanced ERK phosphorylation.

Conclusions

Overall, the present findings support the role of helix B in the functional regulation of the phosphatase. In line with that, Chen and coworkers have recently identified a novel class of small molecules that stabilize SHP2 in its closed, inactive conformation by binding the enzyme in close proximity to the helix B (Chen *et al.*, 2016, *Nature, 535:148–152*). Given the central role of SHP2 in a wide spectrum of signaling pathways implicated in developmental disorders and oncogenesis (Mohi and Neel, 2007, Curr. Opin. Genet. Dev. 17:23–30), a deep comprehension of the molecular mechanism underlying SHP2's allosteric regulation and function represents a prerequisite for considering approaches targeting SHP2 in human disease.