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Modeling (not so) rare developmental disorders associated with mutations in the protein-tyrosine phosphatase SHP2

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Src homology region 2 (SH2)-containing protein tyrosine phosphatase 2 (SHP2) is a highly conserved protein tyrosine phosphatase (PTP), which is encoded by *PTPN11* and is indispensable during embryonic development. Mutations in *PTPN11* in human patients cause aberrant signaling of SHP2, resulting in multiple rare hereditary diseases, including Noonan Syndrome (NS), Noonan Syndrome with Multiple Lentigines (NSML), Juvenile Myelomonocytic Leukemia (JMML) and Metachondromatosis (MC). Somatic mutations in *PTPN11* have been found to cause cancer. Here, we focus on the role of SHP2 variants in rare diseases and advances in the understanding of its pathogenesis using model systems.

KEYWORDS

SHP2, Noonan syndrome, Noonan syndrome with multiple lentigines, metachondromatosis, modeling, fruitfly, zebrafish, mouse

1 SHP2 protein, its activation and downstream signaling

The SH2 domain-containing tyrosine phosphatase 2 (SHP2) is a broadly expressed non-receptor protein tyrosine phosphatase, encoded by the human *PTPN11* gene mapped at chromosomal location 12q24.1 (Freeman et al., 1992; Feng et al., 1993; Vogel et al., 1993). The *PTPN11* gene is comprised of 16 exons producing a 7 kb transcript that contains a 1.779 bp open reading frame which translates into a protein of 593 amino acids (68 kDa) (Grossmann et al., 2010; Tajan et al., 2015a) (Figure 1).

SHP2 protein is built of two tandem SH2 domains (N-SH2 and C-SH2), a catalytic protein tyrosine phosphatase domain (PTP) and a proline rich C-terminal tail all connected *via* flexible polypeptide linkers (Hof et al., 1998). The crystal structure of SHP2 protein (PDB:2SHP) displays a similar structure for N-SH2 and C-SH2 containing a central four-stranded β -Sheet enclosing two α -helices. Like all classical PTPs, the structure of the PTP domain includes catalytic loops: P-loop, WPD-loop, K-loop, Q-loop, and pYr-

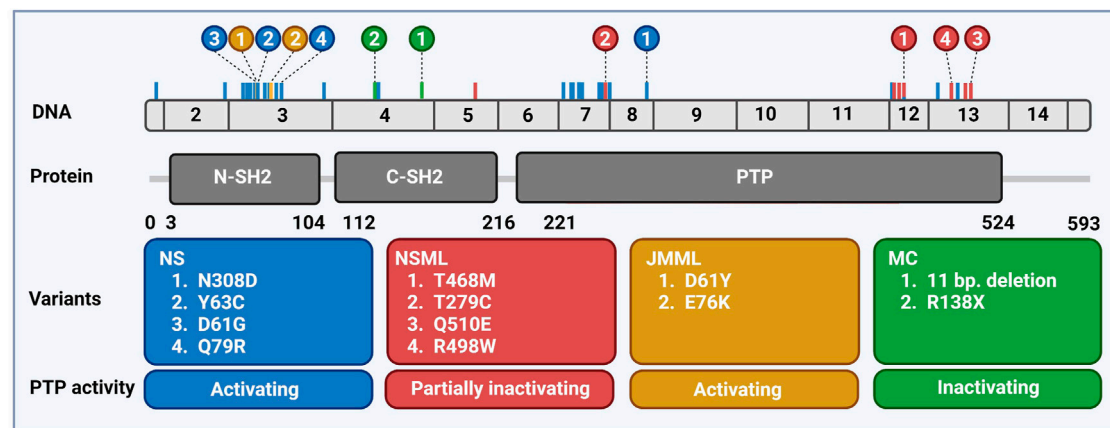


FIGURE 1

Distribution of PTPN11 genetic variants associated with rare diseases. Schematic overview of the exonic structure of *PTPN11*, consisting of 15 exons (above) and the encoded protein SHP2 (below) with two SH2 domains and the catalytic PTP domain. Frequently occurring mutations are indicated color-coded corresponding to the boxes with the 2–4 most frequently occurring mutations highlighted. Created using BioRender (BioRender.com).

loop, which are involved in substrate binding, formation of the phosphoenzyme complex and release of the phosphate group after the hydrolysis (Barford and Neel, 1998; Hof et al., 1998; Zhang, 2003). The P-loop includes the PTP signature motif [C459(X)₅R465] enclosing key residues for catalysis. The substrate-enzyme complex is stabilized by the R465 residue. The catalytic cysteine residue, C459, then provokes a nucleophilic attack causing the transfer of the phosphate group from the substrate to C459. The generated phosphocysteinyli moiety is hydrolyzed and the phosphate is released with the help of residues from the WPD loop (N425) and the Q loop (Q506/510) (Barford & Neel, 1998; Hof et al., 1998).

SHP2 activity is regulated through an allosteric mechanism: SHP2 undertakes either a closed conformation, representing the inactive, auto-inhibited state, or an open conformation, representing the active state. The inactive state is established by intra-molecular interaction of the N-SH2 (D'E loop) with the catalytic pocket of the PTP domain, which is then occluded from binding substrates. The C-SH2 domain is not involved in the interface between N-SH2 and PTP, however it supplies binding energy and specificity (Hof et al., 1998). Upon signaling activation SHP2 binds phosphotyrosine motifs on its upstream partners through the N-SH2 and C-SH2 domains through monopartite (only N-SH2 domain) or bipartite phosphotyrosine binding (Marasco et al., 2021). This triggers SHP2 to adopt the open conformational state, releasing the N-SH2 domain from the PTP domain and exposing the catalytic site to its substrates. Distinct SHP2 activators are known, such as receptor tyrosine kinases, insulin receptor substrate 1 (IRS1) and GRB2-associated binding protein 1 (GAB1) (Noguchi et al., 1994; Cunnick et al., 2001; Ekman

et al., 2002). Additional mechanisms of SHP2 activation have been proposed. The C-terminal tail of the SHP2 protein includes tyrosine phosphorylation sites Tyr⁵⁴² and Tyr⁵⁸⁰, of which the role is under debate. They are suggested to interact and recruit adaptor proteins, such as GRB2, but have also been proposed to bind SH2 domains of SHP2 and regulate SHP2 activity (Sun et al., 2013).

The downstream signaling of SHP2 is rather complex (Figure 2). Upon activation, SHP2 can act on several signaling pathways, mainly promoting signaling. Its role in RAS/MAPK, AKT, and JAK/STAT pathways is best established (Tajan et al., 2015b; Asmamaw et al., 2022). SHP2 signaling is cell type-, developmental stage- and stimulus-dependent. SHP2 is known to dephosphorylate proteins involved in regulation of RAS activity, but may itself act as docking protein as well (Guo & Xu, 2020). RAS activity can be induced by dephosphorylating proteins containing RASA/p120 binding sites, such as GAB1 (Agazie & Hayman, 2003; Montagner et al., 2005; Bard-Chapeau et al., 2006). Dephosphorylation of Sprouty (SPRY) by SHP2 induces release of SPRY from SOS/GRB2 complex, which is then available to activate RAS (Hanafusa et al., 2004; Jarvis et al., 2006). It binds to GAB1/GRB2/SOS1 protein complex as a docking protein at the plasma membrane to facilitate activation of RAS (Bennett et al., 1994; Li et al., 1994). Recruitment of GAB proteins established SHP2's role in activation of PI3K signaling (Asmamaw et al., 2022). The direct activation of RAS by dephosphorylation of Tyr³² on RAS has also been reported (Bunda et al., 2015). An alternative way to activate RAS is through direct or indirect dephosphorylation of the C-terminal Tyr in Src Family Kinases (SFKs), which activates SFKs that finally mediate RAS activation (Ren et al., 2004; Zhang

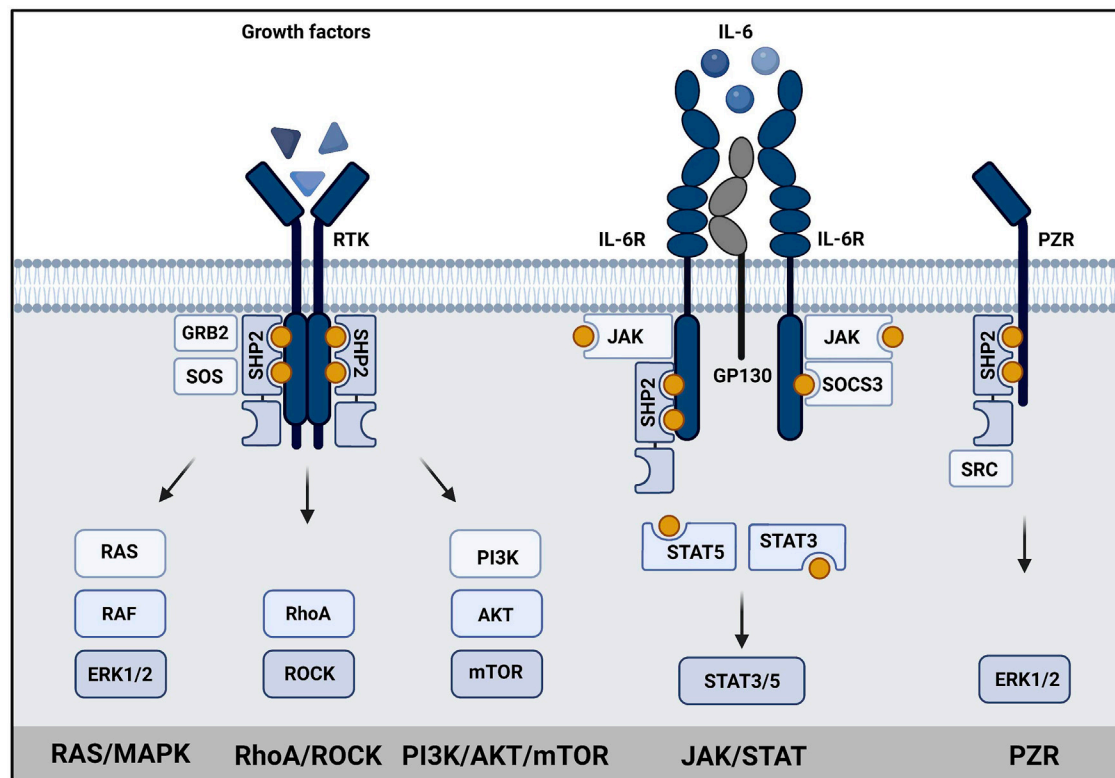


FIGURE 2

SHP2 has a central role in signaling. Schematic representation of SHP2 signaling in (left) growth factor signaling, (middle) JAK/STAT signaling and (right) PZR signaling. SHP2 binds to phosphotyrosine residues (yellow dots) in transmembrane growth factor receptors, cytokine receptors or PZR via its SH2 domains. Subsequently, RAS/MAPK signaling, PI3K/AKT signaling, STAT3/5 signaling or signaling via SRC to ERK1/2/MAPK is activated as indicated. Created using BioRender ([BioRender.com](https://www.biorender.com)).

et al., 2004). Recent efforts in understanding SHP2 signaling, such as large scale phospho-proteomic studies led to identification of several putative SHP2 phosphatase targets (Batth et al., 2018; Vemulapalli et al., 2021).

2 SHP2 is involved in distinct diseases

2.1 SHP2 in cancer

SHP2 is commonly misregulated in cancer either by overexpression or by somatic mutations and has emerged as a prominent cancer drug target. The roles of SHP2 in cancers have been extensively reviewed elsewhere (Zhang et al., 2015; Vainonen et al., 2021). Somatic SHP2 GOF variants are the most common variants (>30%) found in juvenile myelomonocytic leukemia (JMML) (Kratz et al., 2005), a rare but highly aggressive pediatric cancer affecting young children. JMML is characterized by expansion of monocytic and granulocytic blood lineage and hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF) (Emanuel

et al., 1991; Niemeyer & Flotho, 2019). Typical symptoms presented by JMML patients are hepatosplenomegaly, monocytosis, skin rash, lymphadenopathy and respiratory failure. The only treatment is hematopoietic stem cell transplantation (HSCT) with a high relapse rate (50%). SHP2 variants are also present in some other blood cancers, such as myelodysplastic syndrome (10%) and B cell acute lymphoblastic leukemia myelodysplastic syndrome (10%), and seldomly in solid tumors (Kanumuri et al., 2022).

2.2 SHP2 in developmental disorders

Given its role in crucial cellular processes, misregulation of SHP2 leads to various pathophysiological conditions. Germline SHP2 variants are found in developmental disorders: GOF mutations are found in 50% of the individuals with Noonan syndrome (NS) and 90% with Noonan syndrome with multiple lentiginos (NSML) (Gelb & Tartaglia, 1993; Roberts et al., 2013). NS and NSML together with Costello syndrome, Mazzanti syndrome, Legius syndrome, Neurofibromatosis Type 1,

Cardiofaciocutaneous syndrome and CBL (Casitas B-lineage lymphoma) syndrome form the RASopathies group (Tajan et al., 2018a). These syndromes are caused by mutations in components of the RAS/MAPK signaling pathway and share common features. Additionally, germline LOF mutations are found in a rare bone disorder metachondromatosis (MC) (Yang & Neel, 2013).

2.3 Noonan syndrome

NS (OMIM 163950) is the most common RASopathy affecting 1:1,000–1:2,500 individuals described for the first time by Jacqueline A. Noonan in 1968 (Noonan, 1968; Van Der Burgt, 2007; Roberts et al., 2013). Noonan syndrome is an autosomal dominant disorder with a vast genetic heterogeneity. In 2001 *PTPN11* variants were discovered as the first causative gene alterations associated to NS (Tartaglia et al., 2001). *PTPN11* variants are present in 50% of NS patients. A growing list of NS-causing variants in other genes exists including *SOS1*, *RAF1*, *RIT1*, *LZTR1*, *KRAS*, and *NRAS*, while for approximately 20% of NS patients the genetic cause is still unclear (El Bouchikhi et al., 2016). The clinical picture of NS patients is heterogeneous, both in regard to a large variety of symptoms and the severity of the disease. Some of the most common features of NS patients are short stature, craniofacial and cardiac defects (Van Der Burgt, 2007; Romano et al., 2010; Zenker et al., 2022). Typical craniofacial defects present in NS are wide-set and down-slanting eyes with palpebral ptosis, low-set posteriorly rotated ears and short webbed neck. These features become less noticeable in adulthood. The short stature is present in 70% of patients, and it has been more prevalent in patients with SHP2 variants (Binder et al., 2005; Grant et al., 2018). Short stature has been associated with growth hormone (GH) insensitivity due to the decreased insulin-like growth factor 1 (IGF-1) levels (Noonan & Kappelgaard, 2015). Cardiac defects are present in 80% of NS patients, of which pulmonary valve stenosis (PVS) is the most common defect (50%–60% of patients), and it is often associated with the occurrence of dysplastic valve and atrium septum defect (ASD) (Grant et al., 2018; Linglart & Gelb, 2020). Hypertrophic cardiomyopathy (HCM) occurs in 20% of NS patients, however it is prevalent in patients with *RAF1* variants, whereas SHP2 variants more commonly associate with PVS (Grant et al., 2018; Leoni et al., 2022). Other defects can also be present in NS, such as failure to thrive, chest deformities, including *pectus carinatum* and other skeletal deformities, undescended testicles, delayed puberty, deafness, cutaneous, metabolic, neurocognitive, lymphatic, and bleeding defects (Van Der Burgt, 2007; Yart & Edouard, 2018; Bellio et al., 2019; Gao et al., 2021; Sleutjes et al., 2022). Furthermore, 8-fold higher predisposition to develop cancers has been determined for NS (Kratz et al., 2011). An increased predisposition to develop JMML-like myeloproliferative

neoplasm (MPN), a hematological malignancy which rarely progresses to JMML is associated only with specific NS variants of SHP2. Unlike the highly aggressive sporadic JMML, the JMML-like phenotype in NS often intriguingly resolves on its own (Strullu et al., 2014; Niemeyer, 2018).

2.4 Noonan syndrome with multiple lentigines

NSML (OMIM 151100) is a dominant disorder formerly known as Leopard Syndrome (LS, Lentigines, Electrocardiographic conduction defects, Ocular hypertelorism, Pulmonary valve stenosis, Abnormalities of genitalia, Retardation of growth and Deafness). NSML is an autosomal dominant disorder, with 85% of patients bearing a *PTPN11* variant, while *BRAF*, *MAP2K1*, or *RAF1* variants are less common. It is a rare disorder, with estimated frequency of 1:3,500. Craniofacial features present in NSML and NS patients overlap, however they occur to be less prominent in NSML patients. Similarly is observed for the short stature, which is common in NS patients, is present in <50% of NSML patients. Cardiac defects are present in 70%–90% of NSML patients but, unlike in NS, HCM is predominant (60%–70%), not PVS, which is common in NS (Grant et al., 2018). Cutaneous features, such as lentigines and “Café-au-lait” spots are common in NSML. Lentigines are present in 90% of cases and are, besides HCM, a cardinal NSML feature. They appear mainly on the hands, neck, face and the upper body and their number increases throughout puberty (Sarkozy et al., 2008). Similarly to NS, NSML patients can present other features, such as chest deformities, delayed puberty, deafness and learning problems (Gelb & Tartaglia, 1993; Sarkozy et al., 2008; Gao et al., 2021).

2.5 Metachondromatosis

MC (OMIM 156250) is an autosomal dominant rare bone disorder inherited by incomplete penetrance. MC patients carry heterozygous LOF *PTPN11* variants, including nonsense mutations, frameshifts, splice-site mutations and an 11 base pair deletion (Bowen et al., 2011; Yang & Neel, 2013). During the first decade of life, MC induces benign bone tumors (exostoses) primarily in the hands and feet, as well as enchondromas on long bone metaphyses and iliac crests. Exostoses and enchondromas are usually asymptomatic and they stop occurring after skeletal maturity is reached. MC usually resolves itself spontaneously in adulthood (Yang & Neel, 2013). While heterozygous *Ptpn11* knock-out mouse exhibit no bone deformities, conditional biallelic inactivation of the *Ptpn11* gene within chondrocytes and perichondral cells of heterozygous *Ptpn11* knock-out mice, led to enchondromas in vertebrae and ribs as well as exostoses in the limbs (Kim et al.,

2013, 2014; Yang et al., 2013). This suggests that loss of heterozygosity is required for the tumor development, according to Knudson's two-hit hypothesis. Exostoses observed in the mouse model originate from the *Ptpn11*-null perichondrial cells which fail to differentiate into chondrocytes (Yang et al., 2013; Kim et al., 2014). Whereas the enchondromas derived from *Ptpn11*-null chondrocytes located at the growth plate, seem to originate from unsuccessful transition from cartilage to bone tissue. In the exostoses, decreased pERK1/2 levels were observed (Yang et al., 2013; Kim et al., 2014).

3 SHP2 activity is affected in NS and NSML pathogenic variants

Pathogenic SHP2 variants found in NS, NSML, JMML, and MC are largely mutually exclusive for these diseases and the most common variants for each disease are shown in Figure 1. In MC, LOF variants of SHP2 are found (Yang & Neel, 2013), whereas in all three other diseases most of the variants perturb the molecular switch mechanism, inducing a more opened conformation (Yu et al., 2013, 2014; Qiu et al., 2014; LaRoche et al., 2018; Tao et al., 2021). Based on the NSEuronet database, in NS a large number of pathogenic variants is known to date (>100), with some of them being hot spot variants (e.g., N308D, D61G, Y63C). NS mutations are clustered in exon 3, 8, and 13, and most of them affect the residues involved in the interface between the N-SH2 and the PTP domain (Barford & Neel, 1998). The NS SHP2 variants constitutively adopt the active conformation and display enhanced phosphatase activity, which activates downstream signaling pathways, acting as gain of function (GOF) variants (Keilhack et al., 2005; Kontaridis et al., 2006; Tartaglia et al., 2006). Some variants do not disturb the autoinhibition directly, such as T42A, which has rather drastically increased binding to GAB1, making it more easily activated (Toto et al., 2020).

Several studies addressed whether there is a specific phenotype associated to a specific genotype, however large variations among phenotypes were observed including phenotypical variations among the patients with the same pathogenic SHP2 variant (Tartaglia et al., 2002; Zenker et al., 2004; Shoji et al., 2019). The presence of enhancers and/or suppressors of the phenotype in the genetic background of patients may contribute significantly to the observed phenotypical variation among patients. The only established genotype-phenotype correlation is known for higher predisposition to JMML-like MPN in patients with D61G and T73I variants (Strullu et al., 2014). In sporadic JMML, pathogenic SHP2 variants affect specific residues in exon 3 and 13, increasing the activity of SHP2 in the same way as NS mutations (Tartaglia et al., 2003). However, sporadic JMML variants induce stronger activation of SHP2 than NS variants. It is suggested that the stronger activity of sporadic JMML variants makes them

incompatible with embryonic development, which may explain why these mutations are never observed in NS patients (Mason-Suares et al., 2017).

To date, 11 different SHP2 variants have been found in NSML patients, affecting residues in the PTP domain involved in catalysis, leading to a partial loss of the phosphatase activity (Kontaridis et al., 2006; Yu et al., 2013; Qiu et al., 2014). The most common NSML SHP2 variants T468M and Y279C, affect catalysis by provoking rearrangement in the catalytic loops or by inducing loss of substrate affinity, respectively (Qiu et al., 2014). However, upon EGF stimulation of cells, binding to its upstream interacting proteins, such as GAB1, is increased and sustained over time (Kontaridis et al., 2006; Yu et al., 2014). Although NS and NSML SHP2 variants induce opposite effects on the phosphatase activity of SHP2, NS and NSML patients display multiple overlapping phenotypic traits including short stature and facial dysmorphic features observed in NS, although these characteristics are usually milder in NSML patients (Grant et al., 2018). The observed SHP2 activity/patient phenotype discrepancy remains one of the biggest controversies in the field. It was initially suggested that NSML variants act as LOF mutations in the RAS/MAPK signaling pathway upon EGF stimulation in cells (Kontaridis et al., 2006), but also in zebrafish (Stewart et al., 2010) and the hearts of the SHP2-Y279C mouse model (Marin et al., 2011). In contrast, multiple studies reported increased RAS/MAPK signaling in cellular systems (Yu et al., 2013, 2014), fruit-fly (Oishi et al., 2009; Das et al., 2021), zebrafish during gastrulation (Bonetti et al., 2014a), mouse at least in some conditions, such as insulin stimulation (Tajan et al., 2014) and in induced pluripotent stem cells (iPSCs) generated from NSML patients (Carvajal-Vergara et al., 2010). Furthermore, knock-out of one allele of *Ptpn11* has no effects on mouse development, whereas complete knock-out is embryonically lethal (Yang et al., 2006). In addition, the complete LOF SHP2-R465M variant is never found in patients. Based on these findings, it was proposed that activation of RAS/MAPK signaling is established by NSML SHP2 variants, through remaining phosphatase activity and increased binding to its interacting proteins (Yu et al., 2013). Recently, an alternative explanation was proposed, according to which NS and NSML variants have increased liquid-liquid phase separation (LLPS) behavior in common. NS and NSML SHP2 localize to droplets through their well-folded PTP domain. NS and NSML SHP2 variants recruit wild type SHP2 to these droplets as well, which results in strong increases in pERK1/2 levels. Thus, NSML SHP2 variants, despite having reduced PTP activity themselves, promote pERK1/2 signaling through recruitment of wild type SHP2 to the droplets (Zhu et al., 2020). Although many of the NS and NSML symptoms are overlapping, others are specific for the disease and reflect unique mechanisms of NS and NSML SHP2 variant pathogenesis, which could be dependent on the cell type and developmental time window. While both NS and

NSML patients develop congenital heart defects, the proportion of patients with a particular heart defect is distinct: in NSML, the majority (85%) of patients presents with HCM (Martínez-Quintana & Rodríguez-González, 2012), whereas in NS PVS is predominant (Tartaglia et al., 2002). Multiple studies suggested that the HCM phenotype is accomplished through activation of the AKT pathway in cardiomyocytes by NSML variants in particular, and its role in HCM pathogenesis was further emphasized by improvement of heart failure in one NSML patient upon treatment with rapamycin (Hahn et al., 2015; Yi et al., 2020). The phenotypes triggered by NS and NSML SHP2 variants can also be completely opposite, as demonstrated by decreased platelet function in NS and increased function in NSML SHP2 variant mice and patients. This was accomplished through opposite effect of NS and NSML variants on the glycoprotein VI (GPVI) ITAM signaling by modulating the phosphorylation of downstream signaling proteins, such as SYK, LAT, AKT, and PLC γ 2, which was decreased in NS and increased in NSML platelets (Bellio et al., 2019).

Taken together, more than 100 variants in SHP2 have been found to be associated with different developmental disorders with overlapping and distinct symptoms. Some variants display enhanced catalytic activity and others reduced activity, but catalytic activity by itself does not fully explain the functional differences between the variants. Additional effects of the variants on signaling, which have not been elucidated definitively, must be involved as well.

4 Understanding SHP2-variant associated NS and NSML pathogenesis using animal models

Models used to study pathogenesis of SHP2 variant-associated symptoms range from invertebrate fruitfly and nematodes to vertebrates, including frog, zebrafish and mouse, and patient-derived (iPSCs). Here, we summarize the models that are being used to study the function of SHP2 variants (see Figure 3 for an overview).

4.1 Fruit-fly models

Transgenic *Drosophila melanogaster* (fruitfly) expressing the NS, NSML and JMML mutants in corkscrew (*csw*), the fruitfly homologue of *PTPN11*, have been established using the UAS-Gal4 system (Oishi et al., 2006; Pagani et al., 2009; Ruzzi et al., 2020; Das et al., 2021). Whereas fruit-flies ubiquitously expressing NS mutant A72S and JMML mutant E76K are lethal, fruit-flies expressing NSML mutants Y279C and T468M or NS mutant N308D develop ectopic veins on their wings. SHP2 has a central role in RAS/MAPK signaling and since the wing veins in fruitfly emerge at

the sites of enhanced RAS/MAPK signaling (Karim & Rubin, 1998), the observed vein phenotype is perhaps not surprising. Fruit-flies expressing the Y279C mutant also develop a rough eye phenotype, another hallmark of enhanced RTK signaling. Both NS and NSML mutants cause the same phenotype through activation of RAS/MAPK signaling, indicated by genetic interaction studies and increased ERK1/2 activation. The wing phenotype depends on phosphatase activity of the mutants, since the Y279C/R465M double mutant containing the phosphatase-dead R465M mutation develops normal wing veins (Oishi et al., 2009). In addition to RAS/MAPK signaling, there is a genetic interaction with the NOTCH, BMP and JAK/STAT signaling pathways (Oishi et al., 2006). Expressing NS and JMML (D61Y, A72S, T73I, E76K, I282V, and N308D), but not NSML (T468M) *csw* variants in mushroom body neurons, which are essential for the formation of memory in fruitfly, leads to reduction in long term memory in response to spaced training. During spaced training, the resting periods between each repetitive training are prolonged. Presumably, mutant *csw* induces elevated ERK1/2 activation during the resting period, which reduces long term memory (Pagani et al., 2009). Another study showed that expression of the T73I variant in the fat body reduces the life span of fruitfly, which may be due to altered metabolism presumably by modulating insulin-like growth factor signaling (Ruzzi et al., 2020). Finally, in an integrated approach using a platform of 13 transgenic fruit-flies expressing human RASopathies variants in *CRAF*, *BRAF*, *KRAS* and *PTPN11* the phenotypic differences by distinct variants were identified, as well as the responses to a wide range of therapeutic treatments and differences in downstream signaling (Das et al., 2021). All *PTPN11* mutant lines induce the formation of ectopic wing veins, specifically cross veins. The pattern of activated signaling pathways is unique for specific *PTPN11* alleles and distinct from the *RAF* and *KRAS* mutants. Activation of the RAS/MAPK pathway is transient for *PTPN11* variants, while the response in the JNK and Hippo pathways is more robust. Similarly, there are large differences in response to different therapeutics between distinct genes and even between distinct variants of the same gene. However, HDAC inhibitors and statins have an effect on most of the transgenic lines.

Although the expression of RASopathies-associated SHP2 variants in fruitfly induces phenotypes that are not directly translatable to NS or NSML in human patients, these studies serve not only as a prominent tool to understand basic pathogenesis principles of RASopathies-associated SHP2 variants, but also to perform drug screens and understand variant-specific phenotypes, downstream signaling differences and drug response, as demonstrated recently for the first time by Das et al. (Das et al., 2021).

4.2 Zebrafish and frog models

Danio rerio (zebrafish), a complex vertebrate model system with rapid ex-utero embryonic development, represents an





Model system	Expression	Variant(s)	Phenotypes	References
Fruitfly  ✓ easy manipulation ✓ amenable for high-throughput drug screening / genetic studies ✗ phenotypes are not directly translatable	Ubiquitous (<i>tub-GAL4</i>)	A72S, T73I, E76K T468M, N308D Y279C	Lethal Ectopic wing veins Ectopic wing veins and rough eye	Oishi et al. 2009; Ruzzi et al., 2020
	Mushroom body neurons (<i>OK107</i> and <i>247-GAL4</i>)	D61Y, A72S, T73I, E76K, I282V, N308D	Reduced long term memory	Pagani et al., 2009
	Neurons (<i>elavC155-GAL4</i>), muscle (<i>DJ757-GAL4</i>), fat body (<i>FB4-GAL4</i>)	T73I	Reduced life span when expressed in fat body	Ruzzi et al., 2020
	Wing disc (<i>ptc-GAL4</i> , <i>Bx-GAL4</i>), Uniform wing epithelium (<i>765-GAL4</i>)	D61G, T73I, Y279C, N308D, R498W, Q510P, Q510E	Ectopic wing veins	Das et al., 2021; Ruzzi et al., 2020
Zebrafish  ✓ following developmental defects from initial stages ✓ organogenesis similar to human ✓ amenability for high-throughput drug screens ✗ anatomical and physiological differences from human	Transient by microinjection of synthetic mRNA at the one-cell stage	D61G, T73I, A462T, G465A A462T G60A, P491A	Oval embryos, defective C&E movements during gastrulation, edema, reduced body axis length, craniofacial defects, 'hammerhead' appearance, defective heart looping, lower heart rate, defective ciliogenesis in Kupffer's vesicle and L-R symmetry Neural crest specification and migration Decrease in hair cells and supporting cells in neuromast	Bonetti et al., 2014; Jopling et al., 2007; Paardkoop Overman, Preisinger et al., 2014; Paardkoop Overman, Yi, et al., 2014; Stewart et al., 2010 Stewart et al., 2010 Gao et al., 2021
	Knock-in / germline	D61G	Reduced body axis length, craniofacial and heart function defects, hematopoietic defect resembling JMML-like MPN	Solman et al., 2022
Frog	Transient (mRNA injection)	N308D, Q79R, D61G	Smaller hearts, impaired cardiac looping and chamber formation	Langdon et al., 2012
Mouse  ✓ mammalian system ✓ preclinical trials ✗ studying prenatal development is difficult	Knock-in / germline	D61G	Embryonic lethality (strain dependent), smaller, craniofacial defects, triangular faces, mild MPD, splenomegaly, cardiac defects, cell type imbalance in central nervous system, spatial learning and memory deficits, reduced adiposity, insulin resistance, bleeding anomalies	Araki et al., 2004; Araki et al., 2009, Yi et al., 2016; Xu et al., 2010; Dong et al., 2018; Gauthier et al., 2007; Lee et al., 2014; De Rocca Serra-Nedelec et al., 2012; Tajan et al., 2018; Paccoud et al., 2021; Bellio et al., 2019
		N308D	Similar to D61G (above), but less prominent	Araki et al., 2009; Lee et al. 2014
		Y279C	Smaller, craniofacial, skeletal/chest abnormalities, abnormal genitalia, cardiac defects	Marin et al., 2011; Lauriol et al., 2016
		T468M	Similar to Y279C (above), metabolic defects, increased platelet activity	Tajan et al., 2014; Bellio et al., 2019
	Knock-in / endothelial cells (<i>Tie2-Cre</i> promoter)	D61Y	Embryonic lethality, embryonic cardiac defects	Araki et al., 2009
	Knock-in / myocardial cells and fetal atria myocardium (<i>aMHC-Cre</i> promoter)		No cardiac defects	Araki et al., 2009
	Knock-in / excitatory neurons and astrocytes (<i>Emx1-Cre</i> promoter)		Defects in exploratory activity and memory specificity	Altmüller et al., 2017
	Knock-in / neural crest cells (<i>Wnt1-Cre</i> promoter)		Defects in craniofacial development	Araki et al., 2009
	Knock-in / excitatory neurons (<i>αCaMKII-Cre</i>)	D61G	Impaired long-term synaptic plasticity	Ryu et al., 2019
	Knock-in / excitatory neurons (<i>αCaMKII-Cre</i>)	D61A	Increased energy expenditure and insulin sensitivity, resistance to high fat diet induced obesity	He et al., 2012
	Knock-in / endothelial cells (<i>Tie2-Cre</i> promoter)	Q79R	Embryonic cardiac defects	Krenz et al., 2008
	Knock-in / fetal ventricular cardiomyocytes (<i>bMHC-Cre</i> promoter)		Embryonic cardiac defects	Nakamura et al., 2007
	Knock-in / oligodendrocytes (<i>Olig2-Cre</i> promoter)		Increase in oligodendrocytes precursor cells and reduction of myelination of axons	Ehrman et al., 2014
	Knock-in / neural crest cells (<i>Wnt1-Cre</i> promoter)		Defective craniofacial development	Nakamura et al., 2009
	Knock-in / endothelial cells (<i>Tie2-Cre</i> promoter)	Y279C	Embryonic cardiac defects, postnatal HCM	Lauriol et al., 2016
	Knock-in / myocardium (<i>cTnT-Cre</i> promoter)		Embryonic cardiac defects	Lauriol et al., 2016
	Knock-in / neural crest cells (<i>Wnt1-Cre</i> promoter)		Defective craniofacial development	Lauriol et al., 2016
	Knock-in / endothelial cells (<i>Tie2-Cre</i> promoter)	Q510E	Embryonic cardiac defects	Edwards et al., 2015
	Knock-in / fetal ventricular cardiomyocytes (<i>bMHC-Cre</i> promoter)		Postnatal HCM	Schramm et al., 2012
Patients-derived iPSCs  ✓ patient-derived system potential for drug screening ✗ in-vitro cellular system ✗ limited genetic variation	not applicable	T468M	Differentiated into cardiomyocytes - HCM features	Carvajal-Vergara et al., 2010
		N308D	Differentiated into cardiomyocytes - cellular hyperplasia	Meijer et al., 2021
		Y63C, E76D, D61H, G503R	Differentiated into hematopoietic lineages - recapitulated JMML features for iPSCs derived from NS/JMML patients	Mulero-Navarro et al., 2015
		E76K	Differentiated into hematopoietic lineages - recapitulated JMML features	Gandre-Babbe et al., 2013; Shigemura et al., 2019
		N308D, D61N	Differentiated into neural lineages - Impaired early neuroectodermal differentiation and differentiation of neural cells and cerebral organoids	Ju et al., 2020

FIGURE 3

Models used to study SHP2 variants in NS, NSML and leukemia. Advantages and disadvantages of the models are listed in the first column, how the models were derived in the second column. The third column displays the different variants that were used, color-coded as in Figure 1. The observed phenotypes and original references are listed in columns 4 and 5, respectively. Created using BioRender (BioRender.com).

exciting system to study defects caused by Shp2 variants during early development. In zebrafish, due to a genome duplication event during evolution, two paralogues exist, *ptpn11a* and *ptpn11b*, of which *ptpn11a* is indispensable for development (Bonetti et al., 2014b). Most of the studies to elucidate the effect of Shp2 variants were performed by injecting synthetic mRNA encoding NS or NSML variants of Shp2a into embryos at the one cell stage, which results in widespread expression during the first 5 days of development. At bud stage at the end of epiboly, zebrafish embryos expressing NS Shp2-D61G or -T73I, and NSML Shp2-A462T or -G465A variants are more oval in shape compared to the round normal embryos. The cell fates do not seem to be affected, while the coordinated convergence and extension (C&E) cell movements during gastrulation are disturbed (Jopling et al., 2007). NS Shp2-D61G and NSML Shp2-A462T variants induce increased Erk1/2 signaling at bud stage and the oval phenotype is rescued using a MEK inhibitor during epiboly (Jopling et al., 2007; Stewart et al., 2010; Bonetti et al., 2014a). Besides Erk1/2 signaling, signaling through Src family kinases, Protein zero related (Pzr), Fer kinases and RhoA are suggested to be involved in the disturbed C&E cell movements during gastrulation (Jopling et al., 2007; Paardekooper Overman et al., 2014a; Paardekooper Overman, Yi, et al., 2014). Later during development, injected embryos are shorter in length, edemic, have craniofacial defects with “hammerhead” appearance and wider set eyes, and display defective cardiac looping (Jopling et al., 2007; Stewart et al., 2010). NS Shp2 variants (G60A and -P491A) decrease the hair cells and supporting cells of the neuromasts (Gao et al., 2021). Expression of NSML Shp2-A462T results in defects in neural crest lineages, including pigmentation defects. The NSML variant expression driven defects in neural crest specification and migration are due to phosphatase and Erk1/2 dependent function of Shp2, whereas the prevention of apoptosis is phosphatase and Erk1/2 independent (Stewart et al., 2010). NS Shp2-D61G and NSML Shp2-A462T variants induce randomized heart displacement in zebrafish embryos, which is associated with overall disruption of left/right asymmetry due to impaired Erk1/2 dependent ciliogenesis and cilia function in Kupffer’s vesicle (Bonetti et al., 2014a).

Expression of Shp2 variants N308D, Q79R or D61G in *Xenopus laevis* embryos leads to the development of smaller hearts. These hearts show impaired cardiac looping and chamber formation as a result of cardiac precursor cell cycle arrest and their inability to incorporate in the developing heart tube. This seems to be due to disruption of the formation of cardiac actin fibers and their polarity, presumably due to defective ROCK signaling (Langdon et al., 2012).

Ectopic expression of Shp2 variants by microinjection is far from ideal: the amount of the Shp2 variant protein varies from embryo to embryo and is not at the physiological level, tissue and developmental stage specific control of expression is lacking and the expression is transient. With the advent of CRISPR gene

editing technologies, a genetic zebrafish model carrying the patient-associated single point mutation Shp2-D61G was recently established in our laboratory (Solman et al., 2022). The Shp2-D61G zebrafish are shorter than wild type siblings and have craniofacial and cardiac function defects. Phenotypes are rather mild and both heterozygous and homozygous mutants are viable. In human patients, the SHP2-D61G variant is known to be associated with higher predisposition to JMML (-like MPN)) and clinical evidence suggests that JMML pathogenesis is related to prenatal stages of blood ontogeny (Behnert et al., 2021). The blood ontogeny is conserved in zebrafish. In addition, embryos are transparent and transgenic fluorescent lines are available that allow direct live observations of distinct blood lineages at single cell resolution in developing embryos, which makes the zebrafish an excellent model to study embryonic hematopoiesis (Gore et al., 2018). Mutant Shp2-D61G zebrafish embryos display expansion of the myeloid lineage, which is due to hyperproliferation of hematopoietic stem and progenitor cells (HSPCs). The single cell transcriptomes of HSPCs derived from zebrafish embryos reveal expansion of monocyte/macrophage progenitor cells, which have a striking proinflammatory gene signature (Solman et al., 2022). Transcriptomes of HSPCs from bone marrow of human JMML patients with activating mutations in SHP2, reveal a similar proinflammatory gene expression pattern. Treating zebrafish embryos with the anti-inflammatory drug Dexamethasone reverts the blood phenotype, suggesting anti-inflammatory treatment as an exciting future JMML-(like MPN) drug target at least in JMML patients with activating mutations in SHP2.

Development of zebrafish is a rapid process, which can be followed directly in living embryos. Nonetheless it recapitulates closely human development. NS and NSML Shp2 variant expression in zebrafish induces phenotypes that closely resemble symptoms in NS and NSML patients. Findings derived from the zebrafish model broadens our understanding of the NS and NSML pathogenesis during early development (Jopling et al., 2007; Stewart et al., 2010; Bonetti et al., 2014a; Solman et al., 2022). The technological advances and recent establishment of the first knock-in Shp2-D61G NS zebrafish model (Solman et al., 2022) paves the way for development of further genetic models for distinct SHP2 variants. These will allow to study genotype-phenotype correlations, but also to perform high throughput drug screens.

4.3 Mouse models

4.3.1 General phenotypes

Currently, two NS (SHP2-D61G and SHP2-N308D) and two NSML (SHP2-Y279C and SHP2-T468M) knock-in *Mus musculus* (mouse) models expressing SHP2 variants exist (Araki et al., 2004, 2009; Marin et al., 2011; Tajan et al.,

2014). Both D61G and N308D mice display typical NS phenotypes: they are smaller in size and weight; have characteristic craniofacial features, such as greater length/width skull ratio, increased inner canthal distance and typical triangular faces; develop mild myeloproliferative disease and splenomegaly after 6 weeks of age; and develop cardiac defects prenatally, including ventricular septal defects (VSD), double-outlet right ventricle (DORV), enlarged valve primordia and thinned myocardium (Araki et al., 2004; Araki et al., 2009). However, the phenotypes in N308D mice are less severe, presumably reflecting the lower level of SHP2 activation of the N308D compared to the D61G allele (Araki et al., 2009). Furthermore, phenotype penetrance is dependent on the mouse strain, likely due to the presence of genetic modifiers. D61G/wt mice show 50% embryonic lethality on the mixed 129S4/SvJae X C57BL6/J background and normal viability on the 129S6/SvEv background. D61G/wt embryos show almost complete lethality on the C57BL/6 background with cardiac defects, edemas, hemorrhages and decreased liver size (Araki et al., 2009). On the C57BL/6NTac X 129S6/SvEvTac background, D61G/wt mice are fully viable and, unlike other genetic backgrounds, developed HCM postnatally with declining cardiac function (Yi et al., 2016).

The Y279C and T468M mice recapitulate features of NSML: they are smaller in size, have abnormal craniofacial characteristics, skeletal/chest abnormalities, mainly *pectus carinatum*, abnormal genitalia, macrophage accumulation in the inner ear, and HCM evident from 12 weeks of age (Marin et al., 2011; Tajan et al., 2014). Hearts from NSML mice are characterized by elevated heart weight to body weight ratio, thickening of the left ventricular wall and septum and increase in cardiomyocyte cell size. With age HCM evolves to dilated cardiomyopathy with decreased cardiac function (Marin et al., 2011; Tajan et al., 2014). In addition to the knock-in NS and NSML mouse models, several mouse models expressing NS and NSML SHP2 variants under distinct promoters were used to study cell-type specific effects, in order to scrutinize the pathogenesis of specific phenotypes.

4.3.2 Cardiac defects

Cardiac defects in D61G and N308D NS mouse models originate in the endothelial cells and they are dependent on ERK1/2 signaling. Araki et al. (2009) demonstrated that expression of the D61Y mutant induced by Tie2-Cre (endothelial cells), and not α MHC-Cre (adult myocardial cells and fetal atria myocardium) or Wnt1-Cre (neural crest), give rise to enlarged endocardial cushions, VSD, DORV and thinned myocardium. This finding is further supported by a model in which the Q79R variant is expressed under the control of Tie2-Cre (Krenz et al., 2008). Valvulogenesis happens during embryonic development when cushion endothelial cells undergo epithelial-mesenchymal transition (EMT) and mesenchymal cells proliferate. Enlarged cushions in the NS mice are formed due to ERK1/2-driven prolonged EMT and

mesenchymal proliferation (Krenz et al., 2008; Araki et al., 2009). The increased pERK1/2 seems to be controlled by ErbB family of RTKs, as ErbB family inhibitors decrease the number of mesenchymal cells in the D61G cushion explants. In contrast, a study in which expression of the Q79R mutant is controlled by β MHC-Cre (fetal ventricular cardiomyocytes), pERK1/2 dependent ventricular noncompaction, VSDs and abnormal anatomy of the interventricular groove are observed, suggesting that myocardial cells also contribute to the development of the NS cardiac defects (Nakamura et al., 2007). The NSML mouse SHP2 Y279C knock-in displays phenotypically similar cardiac defects during prenatal development: enlarged valves, septal defects and myocardial wall thinning (Lauriol et al., 2016). Moreover, expression of the Y279C mutant specifically in the endocardium by Tie2-Cre or myocardium by cTnT-Cre, shows the endothelial origin of these defects and partial contribution of myocardium. The effect of Q510E variant expression under the control of Tie2-Cre on the valves confirms this notion, and enhanced EMT underlies the valve enlargement (Edwards et al., 2015). Endothelial expression is required for postnatal development of HCM, which is suggested to be due to perturbing the endocardial-to-myocardial crosstalk during development. Unlike in NS mouse, rather than ERK1/2 activation, AKT signaling is increased and downstream signaling of FOXF1/FGF and NOTCH1/EPHB2 pathways is decreased (Lauriol et al., 2016). However, the expression of Q510E mutation under the control of β MHC, also leads to postnatal development of HCM, which is dependent on AKT/mTOR signaling (Schramm et al., 2012).

Although distinct signaling pathways are recognized to be upregulated, the central role of AKT/mTOR signaling in driving the NSML mice associated HCM is established (Marin et al., 2011; Schramm et al., 2012; Yi et al., 2020). Blocking AKT signaling by the mTOR inhibitor rapamycin or the AKT inhibitor ARQ 092 inhibits the development and progression of HCM in NSML mice, suggesting AKT signaling is an exciting therapeutic target (Marin et al., 2011; Wang et al., 2017). Molecular mechanisms underlying pathways that hyperactivate AKT in NSML-associated HCM still remain poorly understood. The immunoreceptor PZR is hyperphosphorylated in heart lysates of both NS and NSML mice (Paardekooper Overman et al., 2014b). PZR contains two immunoreceptor tyrosine based inhibitory motifs (ITIM), which bind SHP2 once phosphorylated (Zhao & Zhao, 2000). The kinase c-SRC is a probable candidate to phosphorylate PZR, and it also binds more strongly to NS- and NSML-SHP2 mutants. Thus, this leads to enhanced c-SRC, SHP2, and PZR complex formation, and subsequent hyperphosphorylation of PZR and c-SRC activation (Paardekooper Overman et al., 2014b). Knock-out of PZR in the NSML mouse leads to decreased AKT signaling and prevents the development of HCM, indicating a role of PZR in AKT signaling and HCM pathogenesis (Yi et al., 2020). Additionally,

c-SRC signals to NF- κ B through IKK α phosphorylation and induces IL-6 expression and secretion, which in an autocrine fashion leads to JAK/STAT3 activation and promotion of fibrosis in NSML mouse hearts (Yi et al., 2020). PZR/SHP2 signaling seems to be targetable, since the treatment with low dose of the anticancer drug dasatinib, which is a tyrosine kinase inhibitor, leads to inhibition of PZR hyperphosphorylation and reverses the cardiomyopathy and fibrosis in NSML, as well as HCM in NS mice (Yi et al., 2016; Yi et al., 2021).

4.3.3 Cognitive defects

Cognitive defects are observed in 30%–50% of NS patients. In the SHP2-D61G mouse model, defective SHP2 signaling in brain results in a cell fate shift resulting in imbalance of cell types in central nervous system with more neurons and less astrocytes in the hippocampus and cortex (Gauthier et al., 2007). The SHP2-D61G and, to a lesser extent, SHP2-N308D mouse model display spatial learning and memory deficits, due to enhanced RAS/MAPK signaling, which enhances excitatory synaptic function and impairs hippocampal long-term potentiation (Lee et al., 2014). Related to impaired synaptic plasticity and increased RAS/MAPK signaling, glutamate AMPA and NMDA receptors are differentially expressed during neuronal maturation in SHP2-D61G mouse (Oh et al., 2017). Furthermore, expression of SHP2-D61G in α CaMKII⁺ excitatory, and not vGAT⁺ inhibitory neurons is sufficient to drive the hippocampus-associated memory formation defects and impaired synaptic plasticity (Ryu et al., 2019). The observed cell-type specific effect is caused by higher expression of components of the RAS/MAPK pathway, such as GAB1 and GRB2 in excitatory neurons. Phospho-Y1252 on the GluN2B subunit of NDMAR is a direct substrate of SHP2 and dephosphorylation leads to release of the actin-regulatory adaptor protein NCK2 from the NDMA receptor and hence defective NDMAR mediated neurotransmission in SHP2-D61G mouse (Levy et al., 2018). Expression of SHP2-D61Y in excitatory neurons and astrocytes of the forebrain and ventral pallidum under the control of Emx1-Cre induces defects in exploratory activity and memory specificity and impairs surface expression of AMPA and NMDA receptors. Basal level ERK1/2 signaling is increased, but dampened upon neuronal activity, which may be driven by compensatory mechanisms from differential JAK/STAT, PI3K, and mTOR signaling (Altmüller et al., 2017). SHP2 has a critical role in oligodendrocyte differentiation and maturation (Kuo et al., 2010; Liu et al., 2011). Expression of NS-mutant SHP2-Q79R under the control of Olig2-Cre in the ventral telencephalic progenitors and the entire oligodendrocyte lineage leads to a RAS/MAPK signaling-dependent increase in oligodendrocyte precursor cells and a reduction in myelination of axons in the white matter (Ehrman et al., 2014). Expression of JMML-associated GOF mutant SHP2-E76K in neuronal stem cells using Nestin-Cre results in hydrocephalus, due to a decreased

number of ependymal cells and abnormal ependymal cilia. Similar defects in ependymal cells, albeit no hydrocephalus, are observed in the NS SHP2-D61G mouse, whereas the NSML SHP2-Y79C mouse does not show such defects (Zheng et al., 2018).

4.3.4 Other anomalies

The characteristic NS craniofacial deformities, including reduced skull lengths, greater inner canthal distances, taller frontal bone distances and reduced mandibular bone length, are induced by expressing SHP2-Q79R and SHP2-D61Y in mouse neural crest under the control of Wnt1-Cre (Araki et al., 2009; Nakamura et al., 2009). The defective craniofacial development is dependent on RAS/MAPK signaling, as treatment of pregnant mutant mice with MEK inhibitor abolishes the observed defects (Nakamura et al., 2009). Short stature in the NS SHP2-D61G mouse model is associated with decreased levels of insulin-like growth factor 1 (IGF-1). The low levels of IGF-1 upon GF stimulation are mediated by increased RAS/MAPK signaling, presumably through increased dephosphorylation of GAB1 in cell lines responsive to GH. Finally, treatment with MEK inhibitor leads to a partial growth increase (De Rocca Serra-Nédélec et al., 2012). Another study demonstrated that the growth plate of the SHP2-D61G mouse is shorter, which is due to a decrease in the length of the hypertrophic zone, which displays impaired chondrocyte differentiation during endochondral ossification. Treatment with MEK inhibitor, but not IGF-1, rescues the growth plate defects, indicating an endogenous effect on endochondral ossification through RAS/MAPK signaling (Tajan et al., 2018b).

SHP2's involvement in metabolism is complex. The lean phenotype in NSML SHP2-T468M mice is accompanied by reduced adipogenesis, increased energy expenditure and enhanced insulin sensitivity. The adipogenesis and weight are increased in response to a MEK inhibitor, but not to the mTOR inhibitor rapamycin. Furthermore, SHP2-T468M mice are resistant to weight-gain induced by high fat diet (Tajan et al., 2014). When the NS SHP2-D61A mutant is expressed in the forebrain neurons under the control of the CaMKII α promoter, specifically female mice exhibit resistance to high fat diet-induced obesity. These mice have increased energy expenditure, decreased food intake and improved insulin sensitivity mediated by cross talk of leptin and estrogen signaling (He et al., 2012). A recent study revealed that NS SHP2-D61G mice, although lean with reduced adiposity, are insulin resistant. Insulin resistance is not associated with altered lipid management, but rather with meta-inflammation of the tissues. The meta-inflamed state is triggered by macrophages, which have a pro-inflammatory state induced by SHP2 hyperactivation. Depleting macrophages or inhibiting SHP2 reduces meta-inflammation and leads to improved insulin sensitivity (Paccoud et al., 2021).

NS patients often suffer from bleeding anomalies. Analysis of the platelet function indicates an opposite effect in NS SHP2-D61G and NSML SHP2-T468M mouse models. Platelets from NS mice show delayed GPVI and C-type lectin-like receptor 2 (CLEC-2) induced aggregation and decreased thrombus growth on a collagen surface under arterial shear stress, whereas platelets from NSML mice show increased activity (Bellio et al., 2019).

4.3.5 Hematopoietic defects

NS patients with specific SHP2 variants have increased predisposition to develop JMML-like MPN, which can in some cases progress to highly aggressive JMML. NS SHP2-D61G mouse develops a mild myeloproliferative disease (MPD) and splenomegaly after 6 weeks of age. The JMML-defining properties, such as myeloproliferation and hypersensitivity to GM-CSF are recapitulated in the mutant mice (Araki et al., 2004; Xu et al., 2010). The HSCs from the SHP2-D61G mouse exhibit defective cycling and have increased short- and long-term repopulating abilities (Xu et al., 2010). They exhibit increased IL3-cytokine induced RAS/MAPK and PI3K signaling. The deletion of GAB2 in the *Ptpn11^{D61G/+}/Gab2^{-/-}* double mutant mice leads to partially diminished aberrant HSC activity and MPD (Xu et al., 2010). The conditional knock-in models of JMML-associated SHP2 variants E76K or D61Y in hematopoietic lineages reflected the more aggressive JMML phenotype observed in patients (Mohi et al., 2005; Chan et al., 2009; Xu et al., 2011). The inflammatory cytokine profile marrow is known to exist in the JMML patients (Bagby et al., 1988; Freedman et al., 1992; Dong et al., 2016). Although its role in the pathogenesis of JMML has been highlighted both in murine and zebrafish models, the underlying mechanisms remain unclear. A study by Dong et al. proposed that the observed MPD is driven by effects of mutant cells in the hematopoietic niche, in particular mesenchymal stem/progenitor cells and osteoprogenitors, which produce excessive amounts of CC chemokine CCL3 and induce recruitment of monocytes to the niche. Monocytes then hyperactivate HSCs through release of proinflammatory cytokines, such as interleukin 1 β (IL-1 β) (Dong et al., 2016). However, it is unclear how this is accomplished in sporadic JMML patients, where the niche cells are not mutated. SHP2-E76K expression driven in myeloid lineage by LysM-Cre induces overproduction of IL-1 β , which then activates the wild-type HSCs (Yan et al., 2022). The study performed by our laboratory in zebrafish indicates that proinflammatory signaling is induced already in the HSPCs during early differentiation of monocyte/macrophage progenitors and suggests that proinflammatory signaling may be an interesting drug target (Solman et al., 2022).

Knock-in and conditional knock-in mouse models have been essential for understanding of pathogenesis of various SHP2 variant associated NS- and NSML-phenotypes and the cells of origin responsible for these phenotypes. Furthermore, understanding of prenatal and postnatal effects of Shp2 variants,

effects of genetic background of mouse strains and genotype-phenotype correlations are being examined using these models. Finally, these models are essential in preclinical trials to identify therapeutic leads.

4.4 Induced pluripotent stem cells

As human model for NS and NSML, patient derived iPSCs containing SHP2 variants, are used to study cardiac, neuronal and hematopoietic defects (Carvajal-Vergara et al., 2010; Hamada et al., 2020; Li et al., 2019; Meier et al., 2021; Mulero-Navarro et al., 2015; Pearson et al., 2020). The first iPSCs with SHP2 variants were derived more than a decade ago from skin fibroblasts of two NSML patients carrying the SHP2-T468M mutation (Carvajal-Vergara et al., 2010). These NSML iPSCs display increased RAS/MAPK signaling, in that pMEK1 and pEGFR levels are increased as well as the basal level pERK1/2, which is not further induced by bFGF stimulation. When differentiated into cardiomyocytes these cells show a phenotype consistent with cardiac hypertrophy, with larger surface area compared to wt-iPSCs and HES2 cells, increased sarcomere assembly and nuclear localization of NFATc4. In contrast, both cardiac tissue from NS patients with CRAF and SHP2 variants, and iPSCs with SHP2-N308S variant, display cellular hyperplasia, rather than hypertrophy, which is presumably due to the increased cell cycle progression (Meier et al., 2021). However, hypertrophic phenotypes are observed in cardiomyocytes differentiated from NS-derived iPSCs with CRAF mutations (Jaffré et al., 2019; Nakhaei-Rad et al., 2022). The observed discrepancy may be related to the genetic background of the patients and/or the difference in the mutated gene itself: whereas NS-patients with a mutation in the CR2 domain of CRAF develop HCM in more than 90% of cases, this is the case for only 10% of NS-patients with *PTPN11* mutations. iPSCs from skin fibroblasts of two NS patients with SHP2-Y63C and E76D variants and two NS/JMML patients with SHP2-D61H and G503R variants show increased and sustained pERK1/2 levels after EGF stimulation compared to wt iPSCs. When differentiated to hematopoietic progenitors and precursor cells, the NS/JMML iPSCs are hypersensitive to GM-CSF and display hyperproliferation of myeloid lineages, recapitulating JMML features. The NS/JMML iPSCs have increased pSTAT5 signaling and elevated miR-223 and -15a expression levels, which is also observed in bone marrow mononuclear cells from 60% of JMML patients. Myeloid lineage expansion of NS/JMML iPSCs is normalized upon inhibition of miR-223's function (Mulero-Navarro et al., 2015). Proteomic analysis revealed association of TP53 and NF- κ B signaling with NS/JMML iPSCs-derived myeloid cells. The NF- κ B inhibitor CBL0137 has an effect specifically on clonogenic myeloid cells from JMML patients, but not on cells from healthy individuals (Pearson et al., 2020). iPSCs generated from two JMML patients

with somatic SHP2-E76K mutation upon differentiation into the myeloid lineage, show hyperproliferation, constitutive activation of GM-CSF and increased levels of pSTAT5. The GM-CSF independence is ameliorated by MEK inhibition (Gandre-Babbe et al., 2013). iPSCs generated from another JMML patients with SHP2-E76K produce hyperproliferated CD34⁺ cells and CD34⁺CD45⁺ cells during hematopoietic differentiation. Additional mutations in several genes are present, as revealed by whole exome sequencing. However, the isogenic control cells in which SHP2-E76K variant is reverted to wild type by zinc-finger nuclease-mediated homologous recombination show reversion of defective differentiation of CD34⁺ cells and CD34⁺CD45⁺ cells, indicating the essential role of this SHP2 variant in driving the hematopoietic abnormalities (Shigemura et al., 2019).

Differentiation of NS iPSCs into neural lineages differs from wild type at two time points: impaired early neuroectodermal differentiation of embryonic bodies in NS iPSCs, which is mediated by RAS-MAPK, BMP, and TGF- β signaling pathways; defective differentiation of neural cells and cerebral organoids, with preferred gliogenesis, shortened neurites and decreased neural activities in NS iPSCs, which is rescued by SHP2 inhibition (Ju et al., 2020).

The development of iPSC technologies allows us to study a spectrum of variants in cells derived from human patient material. iPSCs can be differentiated in many distinct cell types, allowing us to study pathogenesis in cell type related to the phenotype of interest. Finally, iPSCs have strong potential to be used in drug screening efforts.

5 Moving towards therapies

Since the discovery of *PTPN11* mutations as the cause of Noonan syndrome in 2001, a plethora of preclinical research has been performed in order to better define the pathological mechanisms of the disease and establish potential drug targets. SHP2 is an exciting cancer drug target and four classes of inhibitors are known [for recent reviews, see (Vainonen et al., 2021; Kong & Long, 2022; Song et al., 2022)]: orthosteric inhibitors are directed to the catalytic pocket and block phosphatase activity of SHP2, allosteric inhibitors act as a molecular glue and lock SHP2 in the autoinhibited state, PROTAC degraders target SHP2 for E3 mediated degradation, and interaction inhibitors abolish protein-protein interactions of SHP2 with its binding partners. The discovery of allosteric inhibitors by Novartis in 2016 boosted preclinical cancer studies (Chen et al., 2016), and such inhibitors are currently in clinical trials (Vainonen et al., 2021). Unfortunately, allosteric inhibitors are poorly efficient in affecting the activating NS SHP2 variants (LaRochelle et al., 2018). The recent finding that a peptide, which targets the N-terminal SH2 domain of SHP2, binds activating

SHP2 mutants with higher affinity than SHP2 wild-type, may pave the way for this type of inhibitor. Administration of this peptide to zebrafish embryos ameliorates the gastrulation defects and mortality in response to overexpression of Shp2-D61G in zebrafish embryos, providing evidence that his approach is feasible in whole organisms (Bobone et al., 2021). Other strategies for direct targeting of pathogenic SHP2 variants may also be used, including strategies using antisense oligonucleotides, which were used to selectively target the cancerogenic KRAS-G12C variant in whole organisms (Ross et al., 2017).

To date, treatment of SHP2-associated NS and NSML targeted the symptoms, rather than SHP2 itself. For instance, RASopathy patients are treated with recombinant human GF (rhGF) to overcome their short stature, although the effectiveness is not completely established. Treatment with rhGH for 1 year or until (near-) adulthood (Dahlgren & Noordam, 2022) leads to increased growth in the short term (Limal et al., 2006), but remains inconclusive in the long term (Noordam et al., 2008). It is noteworthy that the short term effect on patients with *PTPN11* mutations is less prominent than on patients with other RASopathies-associated mutations (Limal et al., 2006). Long term studies show a broad range of height gain with SD varying from 0.6–1.4 (Giacomozzi et al., 2015). The treatment duration and the start of the therapy prior to puberty seem to have an influence on the effectiveness of the therapy (Stagi et al., 2022).

Preclinical research in the NSML mouse indicates the importance of PI3K/AKT/mTOR signaling for the pathogenesis of HCM (Marin et al., 2011; Schramm et al., 2012; Yi et al., 2020). Furthermore, rapamycin or AKT inhibitor ARQ 092 prevent and revert HCM in NSML mice (Marin et al., 2011; Wang et al., 2017). The treatment of one NSML patient with SHP2-Q510E mutation and progressive HCM with the rapamycin analogue everolimus led to improvement, but not reversal of HCM, prior to heart transplantation. An earlier start of the therapy might be beneficial to prevent cardiac remodeling (Hahn et al., 2015).

Accumulating evidence from preclinical studies implies that targeting RAS/MAPK pathway drugs developed for anti-cancer therapies, such as MEK inhibitors or the farnesyltransferase inhibitor statin, may be beneficial in ameliorating distinct mutant-SHP2 associated phenotypes, such as short stature, cardiac, craniofacial and neurocognitive defects (Nakamura et al., 2007; Araki et al., 2009; De Rocca Serra-Nédélec et al., 2012; Bonetti et al., 2014b; Lee et al., 2014; Tajan et al., 2018b; Das et al., 2021). Intriguingly, a low dose of the MEK inhibitor trametinib drastically improved the clinical and cardiac status of two NS patients with HCM due to *RIT1* mutations (Andelfinger et al., 2019). Trametinib treatment in a NS patient with a *SOS1* mutation and associated severe lymphatic defects completely resolved the lymphatic symptoms after 8 weeks of treatment (Dori et al., 2020). Severe respiratory

distress related to lymphatic defects and multifocal atrial tachycardia (MAT) present in a NS patient with *SOS1* mutation (Lioncino et al., 2022), as well as MAT present in a NS patient with *RAF1* mutation (Meisner et al., 2021) were resolved upon trametinib treatment.

The 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase) inhibitor lovastatin decreases growth plate defects and cognitive deficits in NS SHP2-D61G mice (Tajan et al., 2018b). Cognitive deficits are also decreased in a mouse model for neurofibromatosis 1 in response to lovastatin (Lee et al., 2014; Li et al., 2005). However, in clinical trials lovastatin and simvastatin do not improve the cognitive dysfunction in NF1 patients (Ottenhoff et al., 2020). Simvastatin is being used in an ongoing clinical trial to determine the efficacy on growth and bone defects in NS patients (ClinicalTrials.gov identifier: NCT02713945).

Recent preclinical studies suggest other interesting potential therapies, such as the protein tyrosine kinase inhibitor dasatinib, which reverts HCM in both NS SHP2-D61G and NSML SHP2-Y279C mice (Yi et al., 2016; Yi et al., 2021), the anti-inflammatory corticosteroid Dexamethasone, which ameliorates the JMML-like MPN in NS Shp2-D61G zebrafish model (Solman et al., 2022), and HDAC inhibitors used in fruitfly (Das et al., 2021).

In conclusion, the preclinical and clinical findings clearly indicate the benefits of RAS/MAPK pathway targeting in NS patients with mutations in different genes, although not yet in patients with *PTPN11* variants. Alternative routes to target SHP2-variant induced NS and NSML have been suggested. The development of fruit-fly and zebrafish models, which facilitate whole organism drug screens, may further boost the future drug target discovery. However, translation of potential therapies identified in preclinical models into the clinic through clinical trials remains challenging. Particularly for the RASopathies this is challenging, because of the rarity of RASopathies, broad spectrum and severity of phenotypes, wide range of underlying genotypes and impact of the time when the therapy is initiated.

6 Conclusion

Animal models have greatly contributed towards better understanding of SHP2-associated human developmental disorders. Each model has its advantages and disadvantages. Nevertheless, it remains to be determined why different genetic variants in a single gene provoke such a range of distinct human disorders. Genotype-phenotype

correlations in animal models are starting to provide answers. Whereas there is no model that accurately recapitulates all symptoms observed in humans, we believe that zebrafish is the model of choice for genotype-phenotype analyses, because many complex and specific traits that are observed in human patients, including cardiovascular defects, hematological malignancies and neural crest anomalies are also observed in zebrafish. Moreover, the zebrafish is amenable for analysis of early development, because the embryos are easily accessible and many transgenic lines are available, facilitating analysis of cell and tissue fates in live embryos. Better insights into the genotype-phenotype correlation may help to define and/or design therapies for patients suffering from the consequences of expression of SHP2 variants.

Author contributions

MS and DW wrote the first draft of the manuscript. JdH supervised, acquired funding and revised the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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