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Biochemical Dissection of SHP-2 and C-CBL in Juvenile Myelomonocytic Leukemia

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Biochemical dissection of SHP-2 and C-CBL in juvenile myelomonocytic leukemia

by

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DISSERTATION

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For from him and through him and to him are all things.
To him be glory forever.
Amen.
(Romans 11:36 ESV)

Contributions to presented work:

Chapter 4 of this dissertation contains previously published material:

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* These authors contributed equally to this work.

DHS performed laboratory assays including cloning of *CBL* mutant expression constructs and shRNAs, proliferation assays, colony assays, and western blots.

C.M.N. coordinated and collected clinical data from the subjects with EWOG-MDS and wrote the manuscript; M.W.K. collected clinical data, performed laboratory assays including sequencing, proliferation assays and prepared figures; I.F. collected clinical data; M.E. collected subject samples and performed mutational analysis on highly purified populations of blood cells; N.J.B. contributed subject samples; S.B. performed ubiquitin assays; J.Z.F. and K.M.S. contributed subject samples; T.A.G. performed RNA isolation and cDNA sequencing; P.M. contributed subject samples; I.S., G.K., S.C., P.J.L., C.K. and P.G.S. contributed subject samples; A.H. provided age-matched control samples from children with asthma; M.S. performed mutational analysis; J.S., M.M.v.H., H.H. and F.L. contributed subject samples and collected clinical data; D.S. collected clinical data; S.A. performed colony assays and performed cDNA sequencing; L.C. collected clinical data; R.C.R. and S.S.S. performed ubiquitylation assays; M.O. supervised the ubiquitylation assays and wrote the manuscript; B.S.B. oversaw the shRNA and cell proliferative experiments and wrote the manuscript; C.F. collected clinical data and performed sequencing; M.L.L. coordinated and collected clinical and laboratory data from the USA, oversaw all of the laboratory work, coordinated the data and wrote the manuscript.

Abstract

Biochemical Dissection of SHP-2 and C-CBL

in Juvenile Myelomonocytic Leukemia

Danielle Shin

Juvenile myelomonocytic leukemia (JMML) is a rare childhood myeloproliferative neoplasm/myelodysplastic syndrome associated with mutations in the Ras/mitogen activated protein kinase (MAPK) signaling pathway. *PTPN11* is the most commonly mutated gene in JMML, accounting for 35% of cases, followed by gain-of-function mutations in *KRAS* and *NRAS* and loss-of-function mutations in *NF1*. The protein tyrosine phosphatase (PTP) SHP-2 encoded by the gene *PTPN11* is known to play a critical role in hematopoiesis and development, and studies in *Drosophila* and *C. elegans* have identified the SHP-2 homologs *corkscrew* and *ptp-2* as crucial mediators of Ras and growth factor receptor signaling. SHP-2 is an SH2-domain containing non-receptor PTP whose activity is basally auto-inhibited by the occlusion of its catalytic cleft by its N-SH2 domain. The most frequently identified *PTPN11* mutation results in an E76K substitution that is predicted to interfere with the N-SH2-PTP domain interface, thereby disrupting the auto-inhibited conformation. SHP-2 is unusual for a PTP in that it positively regulates growth factor signaling, and the mutations identified in JMML are gain-of-function mutations resulting in a basal increase in catalytic activity. A hallmark feature of JMML is hypersensitivity of hematopoietic progenitors to granulocyte-macrophage colony-stimulating factor (GM-CSF) in an in vitro colony-forming assay (CFU-GM). It

has been demonstrated that the catalytic activity of SHP-2 is required for its transforming ability, as substitution of critical catalytic residues abolishes E76K-mediated CFU-GM hypersensitivity. Utilizing PTP substrate-trapping technology, we have used tandem affinity purification and mass spectrometry to identify several putative substrates of SHP-2 E76K, including the protein parafibromin. Examination of changes in global phosphotyrosine mediated by SHP-2 E76K revealed a role for Src family kinases (SFK) in SHP-2 signaling downstream of the GM-CSF receptor, although CFU-GM transformation assays using hematopoietic progenitors from SFK-null murine models suggested that this relationship was not essential for leukemogenesis. Instead, high overexpression of SHP-2 in the human TF-1 leukemia cell line demonstrated a potential role for SHP-2 in promoting megakaryocytic differentiation via inhibition of the SFK Lyn. Lastly, the identification of mutations in *C-CBL* in a number of hematologic malignancies led us to discover and characterize the role of similar mutations in JMML.

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Chapter 1:

Introduction

Classifications of Leukemia

Hematopoiesis is a tightly regulated process by which immature, multipotent progenitors undergo a series of reprogramming steps resulting in their proliferation and differentiation in order to provide the necessary blood functions such as carrying oxygen, fighting infection, and clotting. Cancer, classically defined as a disease of uncontrolled cell growth, can be more precisely defined biologically by criteria like Hanahan and Weinberg's oft-referenced 'hallmarks,' which in addition to features like tissue invasion, metastasis, and angiogenesis, elaborate 'uncontrolled' to mean both self-sufficiency in growth signals and insensitivity to anti-growth signals and 'cell growth' to mean both limitless replicative potential and evasion of apoptosis (Hanahan and Weinberg, 2011). Cancer occurring within the hematopoietic system, or leukemia, can occur at any point along the differentiation tree and thus manifest in markedly distinct symptoms, lab findings, and importantly, prognoses, depending on the lineage(s) affected (Fig. 1). As modes of classification have improved, treatment strategies have been tailored accordingly in order to maximize efficacy within each distinct disease entity.

For all cancers, classification and staging are crucial to the proper management of the disease. Although genetic and biochemical markers are playing an increasingly important role in the diagnosis and treatment of solid tumors, leukemias have historically been most dependent on this type of molecular characterization as the standard 'TNM' (tumor-node-metastasis) staging for solid tumors which uses, roughly speaking, size and spread, as measures of aggressiveness, is obviously not applicable to a malignancy of circulating blood cells.

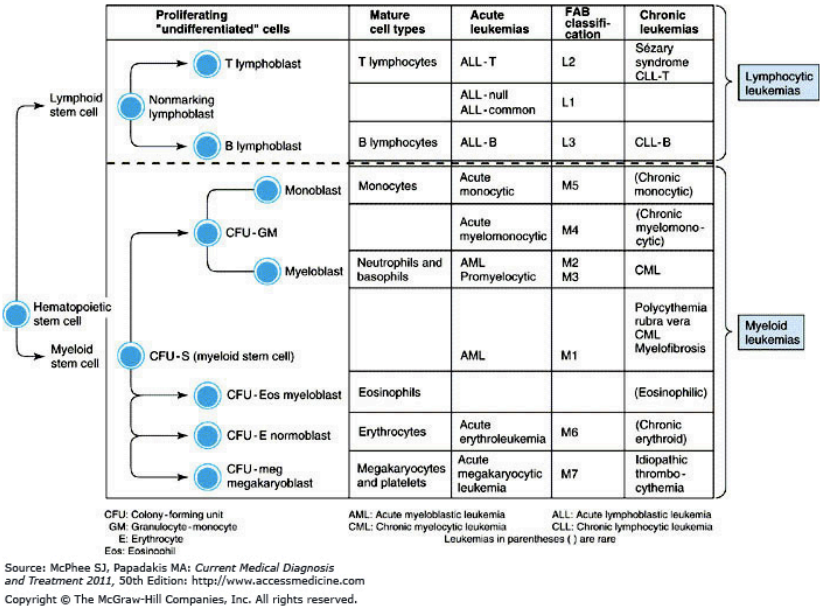

Figure 1. Classification of leukemias according to cell type and lineage. Adapted from Current Medical Diagnosis and Treatment (Linker CA, 2012).

Instead, morphology and immunophenotyping are used to determine lineage and maturation stage, while sequencing and cytogenetics are used to determine genetic and chromosomal abnormalities, thus resulting in classification matrices like the French-American-British (FAB) system that make it possible for clinicians to evaluate the efficacy of specific treatments on cohorts of patients with similar disease features. The broadest distinctions, and most clinically meaningful, are drawn between the less differentiated 'acute' leukemias and the more differentiated 'chronic' leukemias, and between leukemias of the myeloid and the lymphoid lineages.

In 2001, the World Health Organization (WHO) published a new classification for myeloid and lymphoid malignancies based on morphologic, genetic, immunophenotypic and clinical features (Vardiman et al., 2002) . As part of

their report, the WHO committee for the classification of myeloid disorders published a set of criteria that recognized four categories of chronic disease: chronic myeloproliferative disease (CMPD), myelodysplastic disease (MDS), MPD/MDS, and mast cell disease (Table 1). The CMPD category, which included the 'classic' MPDs: chronic myeloid leukemia, polycythemia vera, essential thrombocytosis, and primary myelofibrosis, was defined by effective clonal myeloproliferation without evidence of dyserythropoiesis, granulocytic dysplasia, or monocytosis. Presence of any of the latter features would warrant placement in the MDS or MPD/MDS categories. The MDS category was defined by dysplasia in one or more myeloid cell lineage associated with ineffective hematopoiesis. The MPD/MDS category, as its name implies, referred to a clinical presentation that combined features of CMPD and MDS with effective myeloproliferation, usually leukocytosis or monocytosis, in addition to evidence of dysplasia in the erythroid or granulocytic lineages. The

1. Acute myeloid leukemia
2. Myelodysplastic syndromes (MDS)
3. Myeloproliferative neoplasms (MPN)
3.1 Chronic myelogenous leukemia
3.2 Polycythemia vera
3.3 Essential thrombocythemia
3.4 Primary myelofibrosis
3.5 Chronic neutrophilic leukemia
3.6 Chronic eosinophilic leukemia, not otherwise categorized
3.7 Hypereosinophilic syndrome
3.8 Mast cell disease
3.9 MPNs, unclassifiable
4. MDS/MPN
4.1 Chronic myelomonocytic leukemia
4.2 Juvenile myelomonocytic leukemia
4.3 Atypical chronic myeloid leukemia
4.4 MDS/MPN, unclassifiable
5. Myeloid neoplasms associated with eosinophilia and abnormalities of PDGFRA, PDGFRB, or FGFR1
5.1 Myeloid neoplasms associated with PDGFRA rearrangement
5.2 Myeloid neoplasms associated with PDGFRB rearrangement
5.3 Myeloid neoplasms associated with FGFR1 rearrangement (8p11 myeloproliferative syndrome)

Table 1. 2008 WHO Classification scheme for myeloid neoplasms. Adapted from Tefferi and Vardiman (Vardiman et al., 2009).

2008 WHO update to these criteria replaced the 'disease' in CMPD and MPD/MDS with 'neoplasm' in order to reflect the clonal nature of these diseases (Vardiman et al., 2009).

Juvenile Myelomonocytic Leukemia

Juvenile myelomonocytic leukemia (JMML), according to the 2008 WHO criteria, is classified as an MPN/MDS because it displays the clonal hyperproliferation without differentiation arrest characteristic of MPN in addition to the dysplasia and ineffective hematopoiesis characteristic of MDS. Absolute monocytosis is a defining feature of the JMML, a prerequisite for diagnosis, but overall white blood cell (WBC) count is generally only modestly elevated and bone marrow findings can be nonspecific. Peripheral blood smears demonstrate monocytosis with immature and dysplastic forms in addition to circulating immature granulocytes and nucleated red cells. Blasts may be present but peripheral blast counts average 2% and rarely exceed 10-15%. Chromosomal abnormalities are seen in roughly one-third of patients, with monosomy 7 being present in 25% of cases. Megakaryocytes are reduced in roughly two-thirds of cases and thrombocytopenia and anemia are often present. Early descriptions referred to JMML as subacute or chronic myelomonocytic leukemia (S and CMMOL), JCML or JCMML, denoting a juvenile form of the adult diseases CML and CMML, but the identification of specific genetic abnormalities associated with the adult forms, specifically the t(9;22) translocation and BCR-ABL fusion in CML and receptor tyrosine kinase fusions like TEL-PDGFRβ in CMML, and the absence of these

molecular lesions in JMML suggested that this childhood disease was in fact a unique entity.

The minimal diagnostic criteria adopted by the International JMML Working Group and European Working Group on MDS in Children (EWOG-MDS) included both clinical and laboratory features of the disease (Emanuel, 2008; Hasle et al., 2003). The clinical features largely stem from the aggressive infiltration of immature and mature myelomonocytic cells into hematopoietic and non-hematopoietic tissues, which is responsible for the majority of the morbidity and mortality associated with this disease (Table 2). Patients typically present with hepatosplenomegaly and lymphadenopathy in addition to evidence of infiltration into the skin or lungs. The laboratory features involve a peripheral blood monocytosis in the absence of high blast count and BCR-ABL, thus ruling out the more common AML and CML diagnoses. Additionally, patients must demonstrate two or more other findings commonly associated with JMML such as monosomy 7, elevated fetal hemoglobin, or hypersensitivity of myeloid progenitors to stimulation with granulocyte-macrophage colony-stimulating factor (GM-CSF) in an 'colony

Minimal Diagnostic Criteria for JMML (adopted by the International JMML Working Group and by EWOG-MDS)[17]	Suggestive clinical features: Hepatosplenomegaly Lymphadenopathy Pallor Fever Skin Rash
	Laboratory criteria (all three must be met): 1. Persistent peripheral blood monocytosis (>1 × 10^9/l) 2. No Philadelphia chromosome or *BCR–ABL* fusion gene 3. <20% myeloblasts or monoblasts in the marrow
	Further criteria to be met (need to fulfill at least two): 1. Increased hemoglobin F (corrected for age) 2. Immature myeloid precursors on the peripheral blood smear 3. Peripheral blood white blood cell count >1 × 10^{10}/l 4. Clonal cytogenetic abnormalities (including monosomy 7) 5. GM-CSF hypersensitivity of myeloid progenitors (*in vitro* test)

Table 2. Diagnostic criteria for JMML. Adapted from PD Emanuel (Emanuel, 2008).

forming unit-GM', or CFU-GM, assay (Emanuel et al., 1991). Although not strictly required for the diagnosis, GM-CSF hypersensitivity is considered to be an in vitro hallmark of JMML and has been an invaluable tool in interrogating the biology of this disease. Given the varied presentation and lab findings associated with the disease, classification and diagnosis based on these features alone has been difficult, but this has been greatly aided by advances in our understanding of the genetics of JMML, as will be discussed.

JMML Epidemiology, Prognosis, and Treatment

JMML is a rare pediatric malignancy with an incidence of roughly 1.2 per million children of 0-14 years of age (Hasle et al., 2003). Although JMML accounts for less than 2% of all childhood leukemias, it represents the most common myeloproliferative disease among children. It is almost exclusively a disease of infancy and early childhood, affecting primarily children under the age of 5 with a mean age at diagnosis of 2 years. There is a male predominance with a male-to-female ratio of 2.5 to 1, and most patients are diagnosed at between 3 to 12 months of age. Poor prognostic factors include low platelet count, age >2 years, high fetal hemoglobin levels, and high marrow blast count at diagnosis (Castro-Malaspina et al., 1984). Progression to blast crisis is infrequent, but mortality is high due to aggressive infiltration of monocytic cells into non-hematopoietic organs such as the lungs and intestines causing bleeding, infection, and progressive organ and respiratory failure. Untreated, this disease is rapidly fatal with a median survival of approximately 1 year and 80% of patients surviving less than 3 years.

Unlike other childhood leukemias, JMML is largely refractory to chemotherapy or radiation. Intensive chemotherapy regimens derived from AML treatment protocols have induced remission in a small fraction of cases, but with high incidence of treatment-related death, low rate of durable remissions, and long-term survival less than 10% (Hasle, 2007). Allogeneic hematopoietic stem cell transplant (HSCT) remains the only curative therapy for children with JMML, and HSCT shortly after diagnosis is generally recommended. Without HSCT, the survival rate at 10 years is only 5%, irrespective of chemotherapy. Unfortunately in all cases, disease recurrence is the major cause of treatment failure, and the relapse rate following HSCT remains unacceptably high at 30-40%. Median time to relapse is 4-6 months with most patients relapsing within a year after transplantation. Interestingly, a significant number of patients respond to a second HSCT after relapse, suggesting that the reduced intensity and duration of immunosuppressive prophylaxis during the second procedure leads to a stronger graft-versus-leukemia effect. Consistent with this hypothesis, both acute and chronic graft-versus-host disease are associated with a lower risk of relapse. Nearly half of relapsed children achieve a sustained remission by means of a second transplant, but still current regimens only succeed in achieving long-term event-free survival in about 50% of children.

JMML Genetics and Inherited Predisposition Syndromes

Despite the sometimes ambiguous clinical presentation of the disease, the genetics of JMML is astonishingly well-characterized and has greatly aided both its

diagnosis and our understanding of its molecular pathogenesis. These advances have been made in large part due to the close association of this disease with two inherited developmental disorders, neurofibromatosis type 1 (NF1) and Noonan syndrome (NS). NF1 is an inherited genetic disorder that occurs in 1 in 4,000 live births and is characterized by benign peripheral nerve sheath tumors, or neurofibromas, and pigmentation abnormalities such as axillary/inguinal freckling, multiple café au lait spots (light brown macules) and Lisch nodules (freckling of the iris). NF1 is caused by a germline loss-of-function of one allele of the gene *NF1*, which encodes the Ras GTPase activating protein (GAP) neurofibromin. Children with NF1 are at an increased risk of developing certain malignancies, including a 250- to 300-fold increased risk of JMML (Stiller et al., 1994). A diagnosis of constitutional NF1 can be made in 11% of patients with JMML, and loss of the normal *NF1* allele is common in myeloid progenitor cells from these patients (Flotho et al., 2007b). Interestingly, similar losses have been observed in cases of isolated JMML, which led to the discovery that an additional 10-15% of JMML patients harbored *NF1* gene mutations without evidence of clinical NF1 (Side et al., 1998).

Of similar note was the observation that infants with a different inherited genetic disorder, Noonan syndrome (NS), would sometimes present with symptoms of a myeloproliferative disease nearly indistinguishable from JMML. This 'JMML-like' syndrome, occurring in roughly 10% of patients with NS, was usually self-limiting and would resolve spontaneously, similar to a transient MPD seen in newborns with Down syndrome, but in rare cases would progress to full-blown JMML (Bader-Meunier et al., 1997). Noonan syndrome is an autosomal dominant

condition that occurs in 1 in 1,000 to 2,500 live births and is characterized by short stature, cardiac and skeletal defects, developmental delay, and dysmorphic facial features. Half of all patients with NS carry germline gain-of-function mutations in *PTPN11,* which encodes the protein tyrosine phosphatase SHP-2 (Tartaglia et al., 2001). This observation led investigators to search for *PTPN11* mutations in leukemic cells arising from sporadic cases of JMML. In 2003, Tartaglia et al. reported that somatic mutations in *PTPN11* accounted for approximately 35% of non-syndromic JMML, making it the most common molecular abnormality identified in JMML (Tartaglia et al., 2003b). JMML-associated *PTPN11* mutations are somatic and usually more potent than those identified in NS (Kratz et al., 2005).

The functional characterization of NF1 as a Ras GAP and the known role of SHP-2 as a positive effector of Ras/MAPK signaling suggested that JMML, as well as these associated genetic disorders, might be primarily diseases of hyperactive Ras signaling. Accordingly, Kalra et al. in 1994 identified activating mutations in *KRAS* and *NRAS* in 20-25% of cases, and as predicted, these mutations were mutually exclusive with *NF1* inactivation, confirming they were likely acting within the same pathway (Kalra et al., 1994). Just as the identification of *PTPN11* mutations in NS encouraged studies on somatic mutations in JMML, the identification of *KRAS* mutations in JMML led to the identification of germline *KRAS* mutations in NS. Most recently, as will be discussed, mutations in *C-CBL* were found to account for an additional 15% of cases of JMML (Niemeyer et al., 2010). These mutations are homozygous and in their heterozygous state appear to confer a noticeable Noonan-like inherited phenotype, suggesting a similar paradigm to that seen in NF1. Thus,

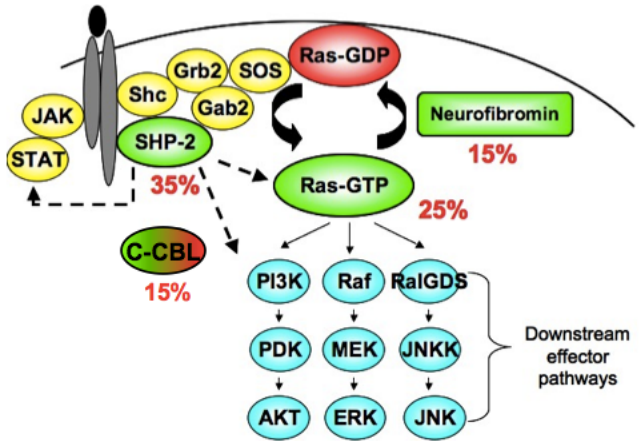

Figure 2. Overview of JMML genes. Percentage values refer to the percent of JMML cases accounted for by mutations in the respective genes. Green and red colors refer to gain- and loss-of-function mutations, respectively, with the C-CBL mutations resulting in loss of E3 ligase function but gain of positive signaling effect.

to date, approximately 90% of JMML patients have identifiable genetic lesions in one of five genes: *PTPN11, KRAS, NRAS, NF1,* and *C-CBL* (Fig. 2). Although uncommon, JMML has been of particular interest and importance in the field of cancer biology because its well-characterized spectrum of causative mutations directly link it to the canonical oncogenic signaling pathway mediated by the Ras family of proteins.

RAS Signaling

The *RAS* genes encode a family of monomeric G-proteins that serve to transmit signals from cell surface receptors to intracellular effector pathways. In humans, three *RAS* genes encode four distinct isoforms: H-Ras, N-Ras, K-Ras4A and K-Ras4B. Ras is the most commonly mutated dominant oncogene in human cancer and responsible for up to 30% of myeloid malignancies (Bos, 1989) (Table 3). Although *NRAS, KRAS,* and *HRAS* are all found to be mutated in human cancers, *KRAS* is the most commonly affected. These four proteins are very closely related, with

85% amino acid sequence identity, but mice lacking both H-Ras and N-Ras are viable, whereas mice lacking K-Ras are not (Rodriguez-Viciana et al., 2005). The first half of these three proteins, which include the switch 1 and 2 domains that contain the Ras effector binding domain (RBD), are identical, but they differ at the carboxyl terminus, the region involved in membrane localization. Accordingly, these Ras isoforms have been shown to localize to different microdomains of the plasma membrane and different endomembrane compartments. Although the biological consequences of this differential localization is unclear, this may account for the distribution of mutations identified in human cancer. The importance of these post-translational modifications for proper localization has also become the subject of intense scrutiny as a potential therapeutic target, as evidenced by the clinical trials of the farnesyl transferase inhibitor Zarnestra in myeloid malignancies (Lancet et al., 2002).

The Ras family of proteins is part of a larger superfamily of low molecular weight GTP-binding proteins that act as molecular switches by cycling between an active guanosine triphosphate (GTP)-bound form and an inactive guanosine diphosphate (GDP)-bound form. Under normal physiological conditions, this cycling is tightly regulated by the activity of GTPase activating proteins like NF1 and guanine nucleotide exchange factors like SOS1. The GEFs displace GDP and thereby indirectly promotes the formation of Ras-GTP due to the higher concentration of GTP in the cell. Although Ras possesses intrinsic GTPase activity and is capable of hydrolyzing GTP to GDP to terminate signaling, GAPs like neurofibromin and p120 dramatically accelerate this process and serve as key negative regulators of Ras

Tissue	*HRAS**	*KRAS**	*NRAS**	Incidence rate‡	Mortality rate‡
Endocrine	3% (535)	0% (670)	5% (570)	0.7	0.3
Biliary tract	0% (153)	31% (1,679)	1% (287)	NA§	NA
Bone	2% (199)	1% (252)	0% (207)	0.9	0.4
Breast	1% (716)	4% (782)	2% (504)	124	24
Central nervous system	0% (964)	1% (1,054)	1% (1,017)	6.5	4.3
Cervix	9% (264)	7% (637)	2% (132)	8.1	2.4
Endometrium	1% (291)	14% (2,251)	0% (314)	23.9	4.1
Eye	0% (33)	4% (90)	1% (106)	0.8	0.1
Haematopoietic and lymphoid tissue	0% (3,076)	5% (5,978)	10% (8,753)	35.2	14.5
Kidney	0% (273)	1% (704)	0% (435)	14.6	4.1
Large intestine	0% (617)	33% (34,013)	2% (1,570)	47.2	17.6
Liver	0% (270)	5% (461)	3% (310)	7.3	5.2
Lung	0% (2,091)	17% (16,348)	1% (3,081)	62	52.5
Oesophagus	1% (161)	4% (375)	0% (161)	4.5	4.4
Ovary	0% (152)	14% (3,181)	5% (191)	12.8	8.6
Pancreas	0% (278)	57% (5,329)	2% (305)	12	10.7
Pleura	0% (19)	0% (45)	0% (30)	NA	NA
Prostate	6% (558)	8% (1,184)	2% (588)	156	24.7
Salivary gland	15% (161)	3% (170)	0% (45)	NA	NA
Skin	6% (2,100)	3% (1,462)	18% (4,956)	22.7	3.5
Small intestine	0% (5)	20% (316)	0% (5)	2	0.4
Stomach	4% (384)	6% (2,793)	2% (215)	7.7	3.8
Testis	4% (130)	4% (432)	3% (283)	5.5	0.2
Thymus	2% (46)	2% (186)	0% (46)	NA	NA
Thyroid	3% (4,137)	2% (5,166)	8% (4,662)	11	0.5
Upper aerodigestive tract	9% (1,083)	3% (1,582)	3% (836)	14	3.7
Urinary tract	11% (1,765)	5% (1,099)	2% (873)	21.1	4.3

NA, not available. *Data from the COSMIC database (see Further information). Numbers in parentheses indicate total unique samples sequenced. ‡Data from the US National Cancer Institute SEER Cancer Statistics Review (see Further information). Rates are shown as per 100,000 people per year. §Tumour subtype for which data are unavailable in the SEER database.

Table 3. Frequency of RAS mutations in human cancer. Adapted from Pylayeva-Gupta et al (Pylayeva-Gupta et al., 2011).

signaling. In fact, the inactivation of the RAS protein's ability to hydrolyze GTP, and thereby its ability to turn 'off,' is the primary target of the somatic RAS mutations identified in cancer. Somatic Ras point mutations that introduce amino acid substitutions at codons 12, 13, and 61 are found in ~30% of human cancers (Pylayeva-Gupta et al., 2011). Oncogenic mutations of the catalytic glutamine, Q61, impair GTP hydrolysis by preventing the formation of a crucial hydrogen bond with an arginine residue in GAP known as the 'arginine finger,' which stabilizes the transition state and allows the nucleophilic attack of a water molecule on the gamma-phosphate of GTP. Similarly, mutations of the G12 and G13 residues, located near the 'finger loop' of the GAPs, create steric hindrance that disrupts the interface between Ras and GAP and prevents the correct positioning of Q61. All the substitutions, therefore, result in a constitutively 'on' GTP-bound Ras that continues

to transmit signals to its downstream effector pathways to promote survival and proliferation irrespective of extracellular signals.

Activation of membrane receptors by extracellular stimuli results in the recruitment of adaptor molecules like Grb2 and Shc, which help bring GEFs like Sos1 in the vicinity of membrane-localized RAS. Activated GTP-bound Ras initiates signaling through a number of downstream effector pathways that lead to transcriptional events in the nucleus affecting various cell processes including proliferation, differentiation and apoptosis. Perhaps the best characterized of these effector pathways are the mitogen activated protein kinase (MAPK) pathway and the phosphatidylinositol-3'-kinase (PI3K) pathway. The canonical MAPK pathway consists of three hierarchical protein kinases: Raf (MAPKKK), Mek (MAPKK), and Erk (MAPK). Raf is a serine/threonine kinase that upon binding to RAS-GTP initiates a series of sequential phosphorylation events down the MAPK axis that result in the activation of the transcription factors like myc, c-fos, jun, and members of the ETS family that regulate cell-cycle progression like elk1. Ras also interacts with the p110 catalytic subunit of PI3K to generate lipid second messengers that activate numerous proteins including AKT, which provides strong anti-apoptotic signals by modulating mTOR and negatively regulating Raf, Bad, and the forkhead family of transcription factors (Fig. 3).

In addition to its role in human cancer, the Ras family of proteins and its downstream effectors have been clearly implicated in a spectrum of inherited developmental disorders now termed 'RASopathies,' all of which are characterized by cardiac and skeletal abnormalities, dysmorphic craniofacial features, and

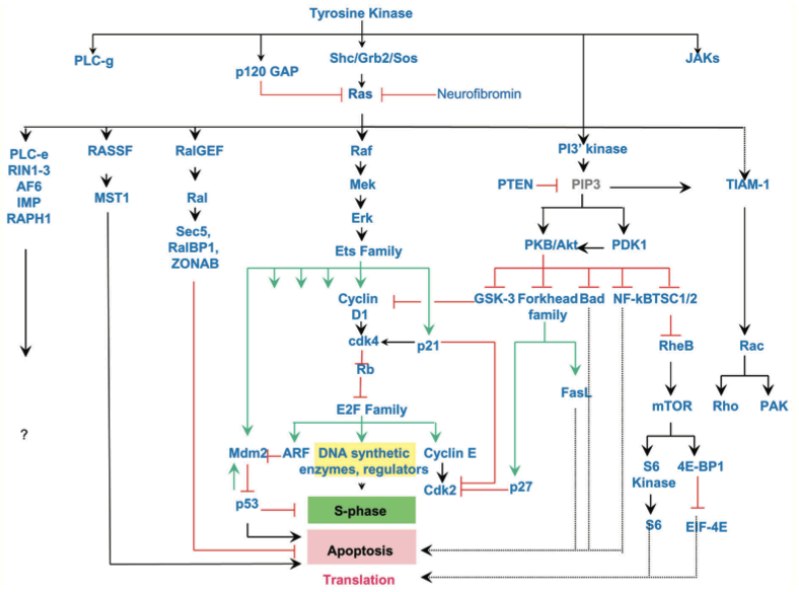

Figure 3. Overview of Ras signaling pathways. Adapted from Rodriguez-Viciana et al (Rodriguez-Viciana et al., 2005).

developmental delay (Table 4). NF1 and NS are the most prevalent within this group but bear phenotypic similarities to the much rarer Costello syndrome, which is caused by activating germline mutations in *HRAS* (Aoki et al., 2005), and cardio-facial-cutaneous (CFC) syndrome, which is caused in nearly 80% of cases by mutations in *BRAF* and in another 13% by mutations in *MEK1* and *MEK2* (Rodriguez-Viciana et al., 2006). Mutations in *BRAF* and *RAF1* have also been identified in 10% of cases of another RASopathy known as LEOPARD syndrome (Pandit et al., 2007). The other 90% of cases are caused by mutations in *PTPN11* (Digilio et al., 2002), the same gene mutated in 35% of JMML patients and 50% of NS cases. While germline *KRAS* mutations account for a small percentage of NS patients, as already discussed, germline gain-of-function mutations in *RAF1* (17%) alterations in *PTPN11* and the *RAS* genes overlap. The centrality of the Ras/MAPK signaling pathway in these disorders and the identification of *PTPN11* mutations in

Disorder	Causative gene(s)	Associated tumours and cancers	Comments
Neurofibromatosis type 1	*NF1*	Neurofibromas, astrocytoma, phaeochromocytoma, juvenile myelomonocytic leukaemia (JMML), malignant peripheral nerve-sheath tumours	Familial cancer syndrome caused by loss-of-function mutations affecting neurofibromin (NF1). Hallmark features include hyperpigmented skin lesions and benign neurofibromas. Learning disabilities are common and vascular abnormalities occur in some patients. Malignancies frequently show loss of normal *NF1* allele
Noonan syndrome	*PTPN11*, *KRAS*, *SOS1*	JMML	Mutations encode gain-of-function proteins in upstream components of the Ras–Raf–mitogen-activated and extracellular-signal regulated kinase kinase (MEK)–extracellular signal-regulated kinase (ERK) pathway. Clinical features include short stature, facial dysmorphism, skeletal abnormalities, cardiac defects, learning disabilities and a high incidence of pigmented skin lesions
LEOPARD syndrome	*PTPN11*	Neuroblastoma, myeloid leukaemia	Mutations encode SHP2 proteins with defective phosphatase activity and dominant-negative properties. Clinical features include multiple lentigines, electrocardiographic conduction abnormalities, ocular hypertelorism, pulmonic stenosis, abnormal genitalia, growth retardation and deafness
Costello syndrome	*HRAS*	Rhabdomyosarcoma, neuroblastoma, ganglioneuroblastoma, bladder cancer	Mutations encode strong gain-of-function proteins and overlap with somatic mutations found in cancer. Loss of wild-type *HRAS* allele reported in malignancies of individuals with Costello syndrome
Cardio-facio-cutaneous syndrome	*KRAS*, *BRAF*, *MEK1*, *MEK2*	None	Mutations occur in downstream components of the Ras–Raf–MEK–ERK pathway. Most mutant proteins show biochemical gain of function

Table 4. Developmental disorders associated with aberrant Ras signaling. Adapted from Schubbert et al (Schubbert et al., 2007).

a significant proportion of these cases together indicate an important role for its gene product, SHP-2, in regulating signaling through the Ras/MAPK pathway.

SHP-2 Epistasis in Lower Organisms

PTPN11 encodes the non-receptor protein tyrosine phosphatase SHP-2, which belongs to a class of evolutionarily conserved signaling molecules that interact with and regulate pathways activated by receptor tyrosine kinases (RTKs), many of which transduce their signals to the nucleus via the Ras/MAPK pathway. RTKs are essential for the proper regulation of cell proliferation, differentiation, development, and cancer. Src homology-2 (SH2) domain-containing protein tyrosine phosphatases, or SHPs, have been identified as positive and negative regulators of signaling events downstream of RTKs. Structurally, in addition to a conserved protein tyrosine phosphatase (PTP) catalytic domain, these proteins

contain two SH2 domains at their amino terminus and usually one or more phosphotyrosine-containing motifs at the carboxyl terminus. Lower eukaryotes only express a single SHP, encoded by the gene *corkscrew* (*csw*) in *Drosophila melanogaster* and *ptp-2* in *Caenorhabditis elegans*. Of the mammalian SHPs, only SHP-2 is capable of complementing *csw* mutations, confirming that it is the mammalian *csw* homolog (Perkins et al., 1996). Multiple lines of genetic evidence suggest that SHP-2 and its homologs function primarily as positive transducers of RTK signaling, and the close association between SHP-2 and RAS seen in human disease is mirrored by their epistatic relationship in development.

In *C. elegans*, genetic screens have led to the identification of two RTKs, LET-23 and EGL-15 that play critical roles in development (Aroian et al., 1990; DeVore et al., 1995). LET-23, homolog of the mammalian epidermal growth factor receptor (EGFR), is important for several developmental functions, including vulval induction, oocyte activation, and male tail development. Gutch et al. identified *ptp-2*, a 668-amino acid SHP containing N-terminal SH2 domains and a PTP domain, by designing degenerate PCR primers to conserved motifs present in all PTP catalytic domains (Gutch et al., 1998). Using transposon-mediated mutagenesis, they identified a recessive *ptp-2* loss-of-function allele, *op194*, in which the PTP domain had been deleted. Homozygous *ptp-2(op194*) hermaphrodites exhibited a completely penetrant zygotic semisterile/maternal-effect lethal phenotype, indicating that PTP-2 activity was essential for proper oogenesis. Interestingly, gain of function *let-60 ras* alleles rescued the defects associated with *ptp-2(op194)*, suggesting that LET-60 Ras acts downstream of, or, in parallel to, PTP-2 during

oogenesis. Conversely, however, the *ptp-2(op194)* allele decreased the penetrance of the multivulva phenotype caused by gain-of-function mutations in *let-23* or *let-60 ras* and increased the penetrance of the vulvaless phenotype caused by a weak loss of function allele of *sem-5*, a GRB2 homolog, suggesting that while not strictly required, PTP-2 could also function downstream of *let-23* and perhaps also *let-60 ras*.

Perhaps the most revealing studies with regard to SHP-2 signaling have come from genetic and biochemical analyses of the *Drosophila* homolog, *corkscrew*. In *Drosophila* as in *C. elegans*, RTKs play an important role in development. Corkscrew (CSW) was first identified in relation to the RTK Torso, a homolog of the mammalian platelet-derived growth factor receptor (PDGFR) that is required for proper cell fate determination at the embryo termini. The maternally expressed 'terminal' genes acts upstream of the gene *polehole,* later renamed *D-raf*, to initiate a phosphorylation cascade that results in expression of the zygotic gap genes like *tailless* and *huckebein* at the embryonic poles. Perkins et al. identified CSW as a member of this terminal class signal transduction pathway and found it to act as a positive transducer of the Torso terminal signal (Perkins, Larsen et al. 1992). Genetically null *csw* mutant embryos showed defects at their termini and misexpression of zygotic genes, and loss of *csw* activity was able to suppress a dominant activated *tor* mutant phenotype, placing it downstream of the receptor.

Interestingly, although *D-raf* null embryos display a more severe defect in terminal gene than the *csw* mutants, embryos mutant for both a *csw* null allele and a hypomorphic *D-raf* allele had a more severe phenotype than the *D-raf* hypomorph

alone, suggesting that these might act synergistically downstream of torso activation. Furthermore, *csw* mutations, unlike *tor* mutations, are associated with zygotic lethality, suggesting that *csw* also plays a role in other signaling pathways essential for development. A closer look at the *csw* mutant phenotype in comparison to the known mutant phenotypes of RTKs other than *torso* revealed defects in processes mediated by the epidermal growth factor receptor (EGFR) homolog DER, including embryonic ventral cell and dorsal follicle cell fate determination and proper development of embryonic CNS and trachea and adult cuticular structures (Perkins et al., 1996). Consistent with this phenocopy evidence, they found that a *csw* hypomorph greatly enhanced the loss-of-function phenotype of a *DER* hypomorph, suggesting that csw was indeed a positive signal transducer downstream of DER.

Another well characterized RTK pathway in *Drosophila* is mediated by the gene *sevenless,* which encodes an RTK that is required for R7 photoreceptor cell differentiation during compound eye development (Simon et al., 1992). Ras1 protein and the associated proteins Sos1, the Grb2 homolog Drk, Raf, MEK and MAPK were identified by mutagenesis screening as being critical for sev-mediated photoreceptor development, as activated alleles of Ras1, Raf or MAPK were able to bypass the requirement for sev activation in the R7 precursor cell (Fortini et al., 1992) (Biggs et al., 1994; Dickson et al., 1992). Using a similar screen, Allard et al. found that one of the previously identified *Enhancers of sevenless (E(sev))* mutations was a dominant negative allele of *csw* (Allard et al., 1996). They demonstrated that *csw* was required for the development of all photoreceptors including the R7 cell

and that expression of a membrane-targeted form of csw could bypass the need for sev activity during R7 photoreceptor development. A dominant negative Ras allele (Ras1N17) completely blocked the ability of the dominant csw allele to induce R7 development, indicating that Ras1 was required for csw activity, but a dominant negative csw allele also strongly suppressed the induction of R7 cells by activated Ras1 or Raf alleles. Thus, as with *ptp-2* and *let-60 ras* in *C. elegans*, these epistasis experiments placed *csw* both upstream and downstream of Ras, suggesting a role for corkscrew either downstream or parallel to Ras/Raf in regulating R7 photoreceptor development.

To date, two mammalian SHPs, SHP-1 and SHP-2 have been cloned (Freeman et al., 1992; Shen et al., 1991). The SHP-1, SHP-2, and csw PTP domains are more similar to one another (~60%) than to any other PTP (<45% identity), as are their SH2 domains (50-75%) compared with any other SH2-domain containing protein (<40%), but SHP-2 is more similar to csw than to SHP-1, consistent with their homologous nature. Although they share significant sequence identity, SHP-1 is expressed predominantly in hematopoietic cells while SHP-2 is expressed ubiquitously during embryogenesis, much like its *Drosophila* counterpart *csw*. Furthermore, while SHP-2 has been found, like *csw*, to be a positive effector of RTK signaling, SHP-1 has been well-characterized as a negative regulator of multiple signaling pathways. Their differential expression patterns and opposing roles in signaling are best illustrated by the vastly differing phenotypes of murine models of SHP-1 and SHP-2 deficiency.

The requirement of SHP-1 for hematopoiesis was illustrated by the finding

that mutations in SHP-1 were responsible for the lethal *motheaten (me)* mouse phenotype, an autosomal recessive disorder arising spontaneously in C57BL/6J mice and characterized by multiple hematologic abnormalities (Shultz et al., 1993; Tsui et al., 1993). Two alleles, *me* and *me viable (me^v)* have been described, with homozygous *me* mice living for only 2-3 weeks while *me^v /me^v* mice live for several months. Both alleles affect splicing of the SHP-1 transcript, with the more severe phenotype of the *me* mutant caused by a 1 base pair deletion in the primary transcript which results in activation of a cryptic splice site, deletion of 101 bp, and a frameshift within the first SH2 domain that results in little to no protein expression. The *me^v* allele, by contrast, contains a point mutation in a normal 5' splice donor site within the PTP domain resulting in the production of aberrant transcripts from two cryptic splice sites that encode one mutant protein product with a 5 amino acid deletion and another with a 33 amino acid insertion within a highly conserved region of the PTP domain. These mice demonstrate an 80% decrease in SHP-1 PTP activity, with the residual function likely accounting for the decreased severity of the phenotype.

The *motheaten* mice primarily display features of a severe combined immunodeficiency disease**,** with a panoply of immune cell abnormalities including defective homing of pre-T cells, impaired T cell receptor (TCR) signaling, decreased NK cell function, and depletion of B cell precursors (Green and Shultz, 1975; Shultz and Green, 1976). However they also demonstrate macrophage hyperproliferation, hypersensitivity of erythroid precursors to EPO stimulation, and plasma cell accumulation, resulting in chronic inflammation and systemic autoimmunity. The

'motheaten' appearance of their coats is attributable to sterile subcutaneous accumulation of macrophages and granulocytes, and despite the severity of their immunodeficiency, *me/me* mice invariably die from a hemorrhagic pneumonitis induced by macrophage infiltration into the lungs. Subsequent studies have demonstrated that SHP-1 loss results in increased JAK family activity, resulting in sustained phosphorylation of IL-3 and EPO cytokine receptors (Klingmuller et al., 1995). SHP-1 has also been found to be responsible for the signal inhibition mediated by co-ligation of the Fcγ and B cell receptors (D'Ambrosio et al., 1995). These phenotypic and signaling data strongly indicate that SHP-1 functions as a negative regulator of hematopoietic signaling.

Unlike the primarily hematopoietic effects of SHP-1 deficiency, loss of full-length SHP-2 protein in mice is embryonic lethal in mice, just as *csw* null embryos are nonviable. Several different models of SHP-2 loss have been generated, with slight variations. Saxton et al. generated a SHP-2 Δ46-110 allele containing a deletion of 65 amino acids within the N-SH2 domain (Saxton et al., 1997). The resulting 57 kD protein, expressed at ~25% of the wild type SHP-2 protein levels, does not act as a dominant negative, as heterozygous animals have no apparent phenotype, but homozygosity was found to result in embryonic lethality prior to embryonic day E11.5. E9.5 mutant embryos of this 'Ex3$^{-/-}$' genotype were smaller than littermates and exhibited severe developmental abnormalities suggestive of defects in gastrulation and embryonic patterning, as well as an improperly formed yolk sac vasculature. Fibroblast-like cells isolated from the embryos demonstrated attenuation of FGFR-mediated activation of the MAPK pathway. Interestingly,

despite the severely compromised the ability of SHP2 to bind PDGFR, the deletion also appeared to potentiate PDGFR-mediated stimulation of the same pathway, suggesting a different relationship between this RTK and MAPK. Of note, however, though the authors speculate that, in light of the severity of the observed phenotype, this deletion creates a severe loss of function allele that 'may be tantamount to a genetic null,' the possibility remains that the remaining 25% expressed mutant protein with its missing N-SH2 domain could have hypo-, hyper-, or even neomorphic capabilities.

Arrandale et al. instead generated a protein null allele by replacing exon 2 and flanking intron sequences with a neomycin sequence (Arrandale et al., 1996). Homozygous 'Ex2$^{-/-}$' embryos died prior to day 10.5 of development, suggesting a slightly more severe phenotype than the previous model. Heterozygous animals did not display any gross alterations of phenotype compared with wild type littermates, even though SHP- levels were decreased by 40-50% in all tissue examined. Given the embryonic lethality associated with SHP-2 deletion, Fornaro et al. generated a conditional null by introducing loxP sites on either side of exon 11, which contains the sequence encoding the catalytic motif (Fornaro et al., 2006). Homozygous *Ptpn11 fl/fl* mice expressing a muscle-specific MCK-Cre transgene displayed normal skeletal muscle development, as Cre-mediated excision in this model occurs postnatally, but decreased myofiber size, myonuclear number, and IL-4 expression reminiscent of that observed in NFAT1-deficient animals. The authors propose a model in which NFAT activation requires SHP-2 catalytic activity to stimulate c-Src activation downstream of integrin signals provided by the extracellular matrix in

order to promote skeletal muscle growth. In addition, the authors observed that the MCK-Cre transgene caused significant deletion of SHP-2 in cardiomyocytes, resulting a dilated cardiomyopathy in which cardiomyocytes demonstrated a decrease in ERK activation but hyperactivation of the RhoA signaling pathway, both of which were found to contribute to the phenotype (Kontaridis et al., 2008). Chimeric animals with tissue-specific deficiencies in SHP-2 also demonstrated a role for this PTP in limb development and hematopoiesis. (Qu et al., 1997; Saxton et al., 2000). More recently, Yang et al. demonstrated that even the $Ex2^{-/-}$ mice still encodes an N-terminal truncation mutant of SHP-2 similar to the $Ex3^{-/-}$ model and generated a new conditional knockout model with a strong splice acceptor site introduced before the beta-galactosidase gene in order to 'capture' all initiated transcripts and result in a true protein null allele (Yang et al., 2006). Though similarly embryonic lethal, embryos homozygous for this new allele ($Ex2^{*-/-}$) were found to die peri-implantation before E6.5, significantly earlier than observed in either of the previous models, due to induction of apoptosis in trophoblast stem cells.

Across models, the effects of SHP-2 deletion are remarkably consistent, with constitutive homozygous deletion resulting in embryonic lethality and chimeric and conditional models pointing to a critical role for SHP-2 in multiple different tissue types. The SHP-2 null phenotype is striking not only for how greatly it contrasts with the restricted defects caused by loss of its structurally highly related family member, SHP-1, but also in the surprising scope and severity of phenotype

generated by loss of a single protein tyrosine phosphatase, thus highlighting the biological importance of these molecules in signaling.

Protein Tyrosine Phosphatases

Protein tyrosyl phosphorylation is a key component of intracellular signaling in response to extracellular stimuli. The binding of growth factors and cytokines to cell surface receptors initiates a cascade of phosphorylation events that regulate diverse cellular processes including growth, proliferation, differentiation, and cell death. The phosphorylation states of these signaling molecules are determined by the opposing activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs), a balance that is often perturbed in cancer. PTKs most often function as positive regulators of growth signaling, as many signaling molecules require phosphorylation at specific tyrosine residues for activation. Gain-of-function PTK mutations have been identified in a number of cancers, including EGFR in lung cancer, Her2/Neu in breast cancer, and BCR-ABL in chronic myelogenous leukemia (CML). This has led to the development of molecular targeted therapies like gefitinib (Iressa), trastuzumab (Herceptin), and imatinib (Gleevec), respectively, which are now used routinely in the clinic (Sawyers, 2004). Conversely, PTPs most often function as negative regulators of signaling by catalyzing the removal of 'activating' phosphates and thereby terminating signal transduction. Overall there are approximately 107 human PTP genes compared to 90 PTK genes, but 11 PTPs are catalytically inactive as compared with only 5 PTKs. Both classes exhibit comparable tissue distribution patterns, with individual cells expressing 30-60% of

the entire complement of PTPs and PTKs (Alonso et al., 2004). Although PTKs have received historically received more attention as the drivers of both physiologic and pathologic processes, PTPs are becoming increasingly recognized as important modulators of these signaling pathways.

Unlike the protein kinases which are derived from a common ancestor, the 107 PTPs in the human genome have evolved in four separate families that are structurally and mechanistically distinct and can be subdivided based on the amino acid sequences of their catalytic domains (Tonks, 2006). The largest of these, the class I cysteine-based PTP family, contains all 38 of the 'classical' tyrosine-specific PTPs as well as the majority of the dual-specificity phosphatases (DSPs), which can accommodate the dephosphorylation of tyrosine, serine, and threonine, in addition to inositol phospholipids, in their active site (Barr et al., 2009). The 'classical' PTPs, which can be further subdivided into transmembrane, receptor-like enzymes (RPTPs) and intracellular, nonreceptor PTPs (NRPTPs), are notable in that most of them consist of a combination of modular domains, including extracellular fibronectin-type or immunoglobulin repeats for RPTPs and SH2 domains for intracellular NRPTPs (Denu et al., 1996). The presence of motifs characteristic of cell-adhesion molecules in the extracellular domains of many RPTPs have led to their implication in cell-to-cell or cell-to-extracellular matrix signaling, whereas the NRPTPs are characterized by regulatory domains that flank the catalytic domain and regulate its activity either directly by interfering with the active site or indirectly by mediating regulatory processes like subcellular localization and post-translational modification. This modular structure is thought to contribute to the striking degree

of substrate specificity exhibited by PTPs in vivo, in addition to the specificity conferred by the catalytic domain itself. Despite this marked structural diversity, PTPs share >30% sequence identity within the catalytic domain, as compared with the less than 5% sequence identity between the PTPs and the DSPs, a figure only slightly better than that predicted by random chance for two unrelated proteins.

PTP Catalytic Mechanism

'Classical' PTPs are defined by the active-site signature motif HC(x5)R(S/T), in which the cysteine performs the nucleophilic attack required for catalysis. The PTP catalytic domain spans approximately 280 residues and consists of the signature motif, a phosphotyrosine recognition loop, and a mobile 'WPD' loop that positions the conserved aspartate residue, which functions as a general acid in the cleavage of the phosphate ester bond. The PTPs consist of a single alpha+beta type domain where four parallel beta-strands form a core sandwiched by alpha-helices. The residues within the signature motif are found between the beta-strand (HC) and the alpha-helix (R(S/T)) and form the phosphate binding pocket for the substrate. The catalytic cysteine nucleophile is located at the base of the active site cleft and the guanidinium group of the arginine is pointed toward the active site to assist in phosphate binding. The catalytically important aspartate residue is located 30-40 amino acids amino-terminal to the cysteine as part of the flexible WPD loop, which undergoes dramatic conformational change upon substrate binding. In brief, catalysis proceeds through the formation of a phosphoryl-cysteine intermediate. The aspartate must be protonated to perform its function as the general acid and the

cysteine must be unprotonated to provide the nucleophilic thiolate anion for phosphoryl transfer to the enzyme. The phosphotyrosine substrate is bound to the center of the active-site motif with the dianion coordinated by the nitrogens of the arginine guanidinium group and the amide groups of the active site motif and the scissile oxygen positioned within hydrogen bonding distance of the protonated aspartate residue. The thiolate anion is thus positioned for nucleophilic attack, directly in line with the P-O bond for efficient expulsion of the leaving group. The WPD loop, normally flipped away from the active site, folds over the active site upon binding of the enzyme to a phosphorylated substrate, thereby bringing the aspartic acid into position to protonate the leaving group phenolic oxygen and facilitating its expulsion. This phosphoenzyme intermediate then undergoes hydrolysis by a water molecule likely activated by proton abstraction by the same aspartate residue, now acting as a general base.

SHP-2 Structure and Function

SHP-2 is marked structurally by the presence of two N-terminal SH2 domains, denoted N-SH2 and C-SH2, upstream of the phosphatase domain (Fig. 4a). On its C-terminal end, SHP2 has two tyrosyl phosphorylation sites (Y542 and Y580) joined by proline-rich region. SHP-2 can bind via its SH2 domains to phosphotyrosine residues on variety of RTKs and adaptor proteins, as well as via its C-terminal phosphotyrosine residues to the SH2 domains of other signaling molecules. Its basal catalytic activity is low, but the addition of mono- or bisphosphorylated tyrosine-containing peptides results in a 10-fold or 100-fold

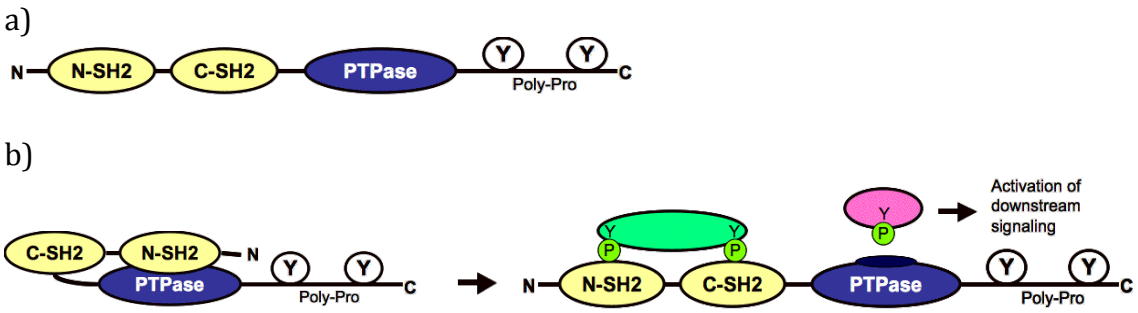

Figure 4. SHP-2 structure and function. a) Structural domains of Shp2. b) Mechanism of basal auto-inhibition of Shp2

increase in activity, respectively (Barford and Neel, 1998) (Fig. 4b). Crystallization of the protein revealed an auto-inhibitory mechanism by which the catalytic domain, in the absence of an appropriate phosphotyrosine-containing ligand for its N-SH2 domain, is kept inactive in the basal state due to folding over of the 'backside loop' of the N-SH2 domain into the catalytic cleft, blocking substrate access. The phosphotyrosine binding site of the N-SH2 domain remains accessible, however, and binding of appropriate phosphotyrosine residues to the N- and C-SH2 domains prevent the N-SH2 domain from binding to the PTP domain, thereby relieving the auto-inhibition and activating the enzyme. The C-SH2 domain does not directly contribute to PTP activation, but may play a role in binding specificity or binding energy by increasing local concentration of phosphotyrosine-containing ligand (Hof et al., 1998).

Several studies have been conducted to identify the sequence determinants of SH2 domain specificity, with comparisons between SHP-2 and SHP-1 being of particular importance in light of their very similar structural organization and high degree of sequence identity. The first N-SH2 binding sequence for SHP-2 was derived from the region surrounding PDGFR Y1009, a known N-SH2 domain binding

partner, with the minimum required amino acid sequence VL(pY)TAV determined to be sufficient for high-affinity binding (Huyer et al., 1995). A more comprehensive approach using a combinatorial library of synthetic phosphotyrosine-containing peptides resulted in the identification of four different classes of consensus sequence that retained high affinity binding to the N-SH2 domain of SHP-2: (I/L/V/m)XpY(T/V/A)X(I/V/L/f) (class I), W(M/T/v/)pY(y/r)(I/L)X (class II), (I/V)XpY(L/M/T)Y(A/P/T/S/g) (class III), and (I/V/L)XpY(F/M)XP (class IV), where the lower case letters represent less frequently selected residues and X is any amino acid (Sweeney et al., 2005). The class I ligands correspond to the ITIM motifs found on many cell surface receptors which are known to bind SHP-1 and SHP-2 in vivo, and class III and IV motifs have been found on SHP-1/2-binding proteins like c-Kit (YVpYIDP), prolactin receptor (LDpYLDP) and STAT3 (LVpYLYP), but no class II motif has been found to mediate SHP-2 binding in vitro thus far. The SHP-2 C-SH2 had a single consensus sequence: (T/V/I/y)XpY(A/s/t/v)X(I/v/l).

Interestingly class I ligands were pulled out most frequently, but class IV ligands demonstrated higher affinity by an order of magnitude, suggesting that a better 'fit' required more selective choice of binding partner. Consistent with this, while most of the class I and II peptides that bound to the N-SH2 domain of SHP-2 also bound SHP-1 with similar affinity, the class III and VI peptides showed greater selectivity for SHP-2. A more recent study found that specificity for the N-SH2 domain of SHP-2 over that of SHP-1 was also determined by residues at the pY+4 and +5 positions, suggesting that these sequences, which are already somewhat degenerate and not altogether specific, also reside in a much broader span than

previously thought (Imhof et al., 2006). Interestingly, crystallization of the SH2 domains of SHP-2 revealed that selectivity might reside not only in their individual sequence specificities but in the combinatorial requirements of the tandem SH2 domains. Eck et al. found that the N- and C-SH2 domains maintain a fixed relative orientation stabilized by a disulfide bond and a small hydrophobic patch within the interface, such that phosphotyrosine binding sites are widely separated (~40A) and run almost antiparallel to one another, thus adding the element of spatial constraint to their choice of bisphosphorylated ligand (Eck et al., 1996).

SHP-2 is also known to be phosphorylated on its C-terminus by PDGFR, EGFR, and other RTKs (Feng et al., 1993). Phosphorylation of one or both of its C-terminal tyrosine residues can promote the adaptor function of SHP-2, but several studies have also suggested a potential role for C-terminal phosphorylation in directly regulating PTP activity. Sugimoto et al. found that limited tryptic cleavage within the C terminus resulted in a 27-fold activation of PTP activity against a peptide substrate derived from IRS2 (Sugimoto et al., 1994). The activity of the tryptic fragment was not further augmented by peptide binding to the SH2 domains, suggesting that SH2-mediated modulation of PTP activity could be dependent on this C-terminal region. In a different set of experiments, Wei et al. used 'expressed protein ligation' to introduce a nonhydrolyzable phosphotyrosine analog, phosphonomethylene phenylalanine (Pmp), at Y542 or Y580 and found that these phosphonates interacted intramolecularly with the N- and C-SH2 domains, respectively (Lu et al., 2003). Either modification was found to result in increased phosphatase activity against the artificial substrates tyrosyl-phosphorylated para-

nitrophenylphosphate (pNPP) and reduced, carboxymethylated lysozyme (RCML), with expression of the dual phosphonate demonstrated additive effects on PTP activity, giving rise to overall 9-fold increase in PTP activation, 30-50% of the value associated with deletion of the SH2 domains. Microinjection of REF-52 cells with a solution containing the SHP-2 with unmodified Y542 or the Pmp-modified 542P demonstrated activation of a CAT-tagged serum response element (SRE) reporter construct, which they used as a readout of MAPK activation, with 542P being more efficient than 542Y, suggesting that phosphorylation of Y542 was sufficient to activate MAPK. Interestingly the mutation of the catalytic cysteine in the phosphonate-modified context abolished CAT expression, suggesting that C-terminal phosphorylation is tied directly to PTP activity, rather than adaptor function (Lu et al., 2001). Despite this compelling evidence, C-terminal phosphorylation has been shown to be dispensable in other contexts, and no model as yet exists for how this intramolecular interaction might promote the active conformation of the PTP domain. Further insights into the intricate structure-function relationship underlying SHP-2 regulation have been provided by the identification of gain-of-function mutations in human disease and the mechanisms by which they promote catalytic activity.

SHP-2 Mutations in Cancer

Given the identification of numerous PTKs as proto-oncogenes, PTPs appear well-poised to act as tumor suppressors, much like the lipid phosphatase PTEN, which directly antagonizes PI3K activity and is mutated or lost in a significant

percentage of human cancers. However, the first PTP-encoding gene found to be mutated in human cancer was, in fact, a proto-oncogene. In 2003, Tartaglia et al. reported mutations in the gene *PTPN11*, which encodes SHP-2, in 35% of sporadic cases of juvenile myelomonocytic leukemia (JMML) (Tartaglia et al., 2003a). In addition to their causal role in 50% of cases of Noonan syndrome (NS), SHP-2 mutations have also been identified at lower frequency in pediatric AML and MDS (Loh et al., 2004a), 10% of pediatric B cell acute lymphoblastic leukemia (B-ALL) (Tartaglia et al., 2004a), 5% of adult AML and a small percentage of solid tumors (Bentires-Alj et al., 2004; Loh et al., 2004b). All of these *PTPN11* mutations introduce amino acid substitutions, with no nonsense, frameshift, or splicing defects observed to date, suggesting that haploinsufficiency is not responsible for the disease phenotype. Furthermore, in JMML where *PTPN11* is the most commonly affected gene, gain-of-function alleles of *NRAS* and *KRAS* and loss-of-function alleles of the tumor suppressor *NF1* together account for nearly 40% of cases, strongly implicating hyperactivation of the Ras pathway in this disease (Flotho et al., 2007a). Given the known positive role of SHP-2 in Ras/MAPK signaling and the fact that mutations in these genes are largely mutually exclusive, the genetics of JMML strongly suggest that these lesions are gain-of-function in effect, a hypothesis supported by numerous biochemical analyses.

The majority of mutations identified in JMML fall in the regions of the N-SH2 and phosphatase domains that are involved in mediating the basal auto-inhibition, the disruption of which results in increased phosphatase activity (Tartaglia et al., 2003b). Interestingly with both *PTPN11* and *KRAS* mutations in JMML and NS, a

strong genotype-phenotype correlation has been noted, with NS-associated mutations generally conferring a weaker gain-of-function than those identified in leukemia. This can be attributed both to the locations of the affected residues as well the nature of the substitutions. In NS, defects in exons 4,7, and 8 account for approximately 50% of cases, with the N308D substitution accounting for a one-third of all lesions, whereas 98% of the mutations identified in JMML, AML, and MDS cluster in exon 3, with lesions altering E76 accounting for 35% of defects (Tartaglia et al., 2004b). Keilhack et al. modeled the activity of the various disease-associated SHP2 mutants as a function of phosphotyrosine-containing peptide concentration and found that that the SH2 domain mutants fell into two classes (Keilhack et al., 2005). There were those activated the enzyme by shifting the basal equilibrium constant toward the active state (E76K, D61Y, T73I), which were primarily identified in JMML and tended to fall in the region of the N-SH2 domain that interacts with the PTP domain. Then there were those that mainly acted by increasing the affinity of the pY peptide for the open (active) versus closed (inactive) state (T42A, D106A, E139D). These tended to be identified in NS and fell in regions likely to affect the affinity of the SH2 domains for pY peptide or interactions between the N- and C-SH2 domains. Kinetic analysis of the PTP domain mutants identified in NS (N308S and Q506P) showed apparent inhibition of catalytic activity at lower pY concentration than WT, suggesting that these mutants may affect the substrate specificity of the catalytic site rather than the equilibrium between open and closed states.

In cases where the JMML- and NS-associated mutations affect the same

residues, the JMML-associated mutations tend to be less conservative in their effects. The most common mutants in JMML, D61Y and E76K, display the highest levels of basal activity against pNPP and RCML, with no gain in activity upon pY peptide addition. In contrast, the weaker gain-of-function alleles affecting the same residues, D61G and E76D, are only observed in NS and display increased basal activity compared with wild-type but less than their JMML counterparts. Both D61 and E76 participate in a hydrogen bond network with water molecules in the active site, with D61 surrounded by several positively charged side chains and E76 located adjacent to an arginine residue. The substitution of a noncharged amino acid for D61 likely disrupts the N-SH2/PTP domain interface, with the effect depending on the size of the substituted residue, hence the greater activity of D61Y as compared with D61G. The full basal activation of E76K is likely attributable to charge effects, whereas the more conservative NS-associated E76D mutant preserves the charge/charge interaction. Together these data suggest that low levels of SHP-2 activation result in NS while higher levels of activity result in leukemia, with the latter effect not being tolerated in the germline. This is consistent with the observation that the JMML-like myeloproliferative disease seen in NS patients is usually self-limiting and resolves on its own, a result that was recapitulated in a knock-in mouse model of the NS mutant D61G, which exhibited the major features of NS and developed an MPD that, unlike the MPDs caused by *KRAS* activation (Braun et al., 2004) or homozygous *NF1* loss (Bollag et al., 1996; Largaespada et al., 1996; Le et al., 2004), was not fatal and never progressed to an acute leukemia. To date no mutations affecting residues essential for PTP activity have been identified,

confirming that the mechanism of oncogenesis relies on specific disruption of the N-SH2/PTP interaction in such a way as to destabilize the inactive conformation without altering catalytic ability.

Prior to the identification of E76K as the most common SHP-2 mutant identified in JMML patients, O'Reilly et al. predicted that a substitution at this site would disrupt the basal auto-inhibitory mechanism and result in an activated phosphatase (O'Reilly et al., 2000). In light of the crystal structure of SHP-2, they hypothesized that mutations affecting the N-SH2 residues on the 'backside loop' that bind to the PTP domain, such that basal inhibition would be disrupted without affecting phosphotyrosyl peptide binding, would result in novel activated mutants. Two of these mutants, E76A and D61A, were basally activated in vitro, as predicted. D61A exhibited a 20-fold increase in activity against RCML that was further increased in a ligand concentration-dependent manner upon addition of a phosphotyrosine-containing ligand for the N-SH2 domain. E76A, in contrast, was found to be fully activated (~100 fold) in the absence of any ligand, consistent with E76K being the most activated of the SHP-2 mutants identified in JMML. Both D61A and E76A bound to a known N-SH2 ligand, SHPS-1, with approximately equal affinities compared with wild type, suggesting that neither mutation disrupted the N-SH2 phosphotyrosine binding pocket or ligand binding but led to enzymatic activation by weakening the interaction between the N-SH2 and PTP domain.

They further tested these mutants in a *Xenopus* animal cap assay and found them to result in a gain of biological function mimicking FGF stimulation and Ras activation. FGF signaling has been shown to be essential for mesodermal tissue

differentiation and gastrulation in *Xenopus laevis*, both of which can be studied ex vivo in ectodermal explants known as 'animal caps.' FGF family members induce a number of tissues derived from ventrolateral mesodermal including muscle, mesenchyme, and mesothelium, as well as elongation of the animal cap. Inhibition of members of the Ras/MAPK pathway prevents mesoderm induction by FGF, and expression of activated forms of these molecules induces mesodermal genes and tissue formation. Overexpression of wild type SHP2 in the absence of FGF did not evoke animal cap elongation, but expression of D61A or E76A induced substantial elongation in the absence of exogenous growth factors. The elongation induced by D61A was found to be proportional to expression level, whereas E76A induced full elongation (comparable to FGF stimulation) at most doses. Despite their efficacy in inducing elongation, E76A induced only low levels of mesodermal differentiation, whereas no mesodermally derived tissues were detected in D61A expressing caps. Expression of early mesodermal marker *Xbra* was stimulated by expression of an activated form of MEK, a downstream effector in the FGF pathway, but only by very high levels of expression of E76A. FGF stimulation results in rapid activation of MAPK, and while E76A was able to activate MAPK in a dose-dependent manner, activation of D61A resulted in minimal MAPK activation, suggesting that elongation could occur with minimal MAPK activation, potentially via a parallel pathway regulated preferentially by SHP-2. However, expression of a dominant negative Ras blocked both FGF-dependent elongation and MAPK activation induced by E76A, a result reminiscent of the complex epistatic relationship suggested by genetic experiments in *Drosophila* and *C. elegans*.

Models of SHP-2 Transformation

The original papers by Loh and Tartaglia identifying *PTPN11* mutations in JMML found that SHP2 E76K enhanced factor-independent growth in Ba/F3 cells, but surprisingly without any increase in pERK or pAKT (Loh et al., 2004b; Tartaglia et al., 2003b). Transient transfection of COS-7 cells resulted in prolonged EGF-dependent ERK activation, suggesting that the activated mutant still remained dependent upon ligand stimulation (Tartaglia et al., 2003b). Retroviral expression of SHP-2 E76K in the GM-CSF dependent human erythroleukemia TF-1 cell line was found to transform these cells to factor independence with constitutive activation of Ras and ERK1/2, though little effect on AKT. Cytokine-independent survival was found to be mediated by upregulation of Bcl-xl, which was suppressed by inhibition of MEK, but not AKT (Ren et al., 2007). Retroviral transduction of fetal liver or bone marrow cells reproduced the GM-CSF hypersensitivity in CFU-GM assays seen in JMML patients (Chan et al., 2005; Schubbert et al., 2005). Schubbert et al. demonstrated that this hypersensitivity was catalytic dependent and more pronounced in the JMML-associated E76K mutant as compared with the NS N308S mutant. Additionally they found that SHP-2 E76K expression resulted in increased erythroid (BFU-E) and immature myeloid progenitor (HPP-CFCs and LPP-CFCs) colony formation and increased proliferation and myelomonocytic differentiation in liquid culture. Chan et al. found that SHP-2 E76K expression resulted in increased proliferation of bone marrow-derived macrophages in response to GM-CSF but not M-CSF, and constitutive ERK activation that was sustained following GM-CSF stimulation. Chan et al. later demonstrated that bone marrow derived-macrophages

expressing E76K showed decrease apoptosis and an increased proportion of cells in S or G2 phase in response to low dose GM-CSF (Yang et al., 2008). These cells also demonstrated hyperphosphorylation of Erk and Akt and increased expression of Cyclin D1, Bcl1 and Bcl-xl, as well as decreased phosphorylation of p38 and decreased expression of p27, p21, and Bim.

Similar to previous groups, Mohi et al demonstrated that retrovirally-mediated expression of SHP-2 E76K in primary murine bone marrow cells with SHP-2 E76K resulted in GM-CSF hypersensitivity in CFU-GM assays and factor-independent colony formation that was primarily monocytic in nature (Mohi et al., 2005). Interestingly, adoptive transfer experiments with transduced bone marrow undertaken by Schubbert et al. in C57Bl/6 mice did not consistently result in MPD, although some anemia and splenic infiltration was observed. However, transduction/transplantation of Balb/c bone marrow by Mohi et al. resulted in a fatal JMML-like MPD with marked splenomegaly and tissue infiltration, as well as some T-cell lymphoblastic leukemia/lymphoma, while expression of wild-type SHP-2 had no effect. Bone marrow from the diseased recipient mice demonstrated the characteristic factor-independent colony formation and GM-CSF hypersensitivity as well as increased basal pERK and pAKT which was sustained in response to IL-3 stimulation in bone marrow-derived mast cells. In an unique cross-species experiment, they also demonstrated that expression of SHP-2 E76K caused a leukemia-like phenotype in *Drosophila*, with an increase in the number of myeloid-like plasmatocytes, similar to published effects of activated Ras or Raf mutants.

The first transgenic mouse model of SHP-2 activation was generated using a

knock-in allele of the NS mutant SHP-2 D61G (Araki et al., 2004). Homozygous expression of this allele was embryonic lethal, and heterozygotes also demonstrated decreased viability. Amongst the surviving heterozygous animals, a remarkably NS-like phenotype could be observed, with short stature, craniofacial abnormalities, and cardiac defects, as well as a chronic, non-progressing MPD reminiscent of the self-limiting JMML-like myeloproliferation seen in children with NS. In contrast, conditional expression of a knock-in allele encoding the D61Y substitution, the second most commonly identified SHP-2 mutant in JMML after E76K, in hematopoietic cells resulted in a fatal, non-transplantable MPD with progressive leukocytosis, splenomegaly, anemia, and factor independent colony formation by bone marrow progenitors (Chan et al., 2009). They also saw expansion of the LSK population, increased SCF-evoked colony formation, and enhanced basal and evoked ERK and AKT activation in hematopoietic stem cells and CMPs/ GMPs in response to SCF and GM-CSF, respectively. Consistent with the anemia phenotype, erythroid differentiation was found to be defective despite an increase in BFU-E formation. Most recently, a conditional knock-in model of SHP-2 E76K expression was generated. Unlike the D61G substitution, this allele was embryonic lethal even in a heterozygous state when expressed globally using an HPRT-Cre transgene was embryonic lethal. Like the D61Y conditional knock-in model, limited expression in the hematopoietic compartment resulted in a myeloproliferative disease that eventually progressed to acute leukemias of both myeloid and lymphoid lineages. These authors also observed genomic instability and localization of SHP-2 to centrosomes, which they suggest could contribute to the development of the

leukemia stem cell (Xu et al., 2011).

All of these studies demonstrate the sufficiency of activated SHP-2 mutants to induce transformation in hematopoietic cells, both in vitro and in vivo. Most of these studies support a model in which SHP-2 mediated transformation is acting via activation of the Ras/MAPK pathway, as evidenced by increased and/or sustained, basal and/or evoked activation of ERK. However, the mechanism by which this is accomplished is unclear. Given the complexity of SHP-2 structure and signaling, it has yet be determined which domains of the protein are critical for oncogenic activity, whether its catalytic activity accounts for all or just some of its transforming ability, and where it falls in the growth factor signaling pathway, whether upstream or downstream of Ras. The mechanism of oncogenic signaling should be informed by its physiologic role in growth factor signaling, but numerous experiments done both in mammalian cells and lower organisms suggest a complex array of cell type- and receptor-specific mechanisms of action.

Mechanisms of SHP-2 Signaling

Despite the detailed genetic characterization of c*orkscrew* in relationship to *ras1* in *Drosophila*, the proposed models for the RTK torso, which specifies embryonic terminal cell fate, and sevenless, which specifies R7 photoreceptor differentiation, are quite distinct. In the former case, Cleghon et al first demonstrated that csw bound to a phosphotyrosine site (Y630) on torso and was responsible for the known positive signaling effect of this residue (Cleghon et al., 1996). In a subsequent paper, the authors found that csw was a substrate of torso

RTK activity and phosphorylated at Y666 to create a binding site for the Grb2 homolog Drk (Cleghon et al., 1998). They described the recruitment of Drk-Sos to torso via phosphorylated csw as the 'first physical link between the torso receptor and ras activation' but were quick to note that if the sole function of csw was to serve as an adaptor molecule linking torso to drk, then a catalytically inactive C583S mutant, which they found was equally capable of binding to torso at Y630, being phosphorylated on Y666, and recruiting Drk, should be sufficient to transduce an RTK-mediated signal, which it is not. Consistent with this idea, homozygous female flies expressing a truncated csw protein that lacks Y666 survive and reproduce, indicating that its adaptor function is not essential for viability or fertility (Allard et al., 1998). In recognition of this incongruity, they proceed in the very same paper to describe a mechanism by which csw is not only a substrate of torso's RTK activity, but torso was a substrate of csw's phosphatase activity. In examining the role of the then newly identified D-RasGAP (Feldmann et al., 1999), the *Drosophila* homolog of p120-RasGAP, downstream of torso, they found that D-RasGAP was negatively regulated torso signaling and bound to a previously characterized negative regulatory phosphotyrosine site (Y918) on the receptor. They found that csw dephosphorylated torso at Y918 and that csw binding at pY630 on torso was required for dephosphorylation. Thus, in this model the csw dephosphorylates the receptor itself and promotes ras activation by preventing the activity of RasGAP. These parallel results suggest a redundant role for csw downstream of torso.

In contrast, the model surrounding *sevenless* signaling center on the adapter molecule *daughter of sevenless*, or *dos*, a homolog of the mammalian Gab family of

proteins. Given the importance of csw's catalytic function in receptor signaling, Herbst et al. used genetic and biochemical approaches to identify a substrate downstream of sevenless (Herbst et al., 1996). Using immunoprecipitation with a catalytically inactive csw mutant C583S followed by anti-phosphotyrosine immunoblotting, they identified a 115 kD protein, which by peptide analysis and genetic complementation was found to be encoded by the recently cloned *dos* gene (Raabe et al., 1996). dos was found to be tyrosine phosphorylated in response to constitutively activated sevenless and capable of interacting with the SH2 domains of csw. Inactivating mutations in dos were found to suppress the effects of a dominant csw or sev allele and enhance the phenotype of a csw hypomorph, indicating an essential role in csw activity downstream of sev. Herbst et al. later identified Y801 and Y854 on dos as the sites phosphorylated by sev and required for binding to csw SH2 domains. Y801F or Y854F mutants are unable to function during signaling by sev and other RTKs, whereas a mutant in which all phosphotyrosine sites except 801 and 854 have been removed is able to effectively provide dos function during sev signaling and rescue the lethality associated with dos los of function mutations (Herbst et al., 1999). These results indicate that dos functions in sev signaling primarily by recruiting csw.

Unlike torso, csw does not interact with a phosphotyrosine in the sev receptor nor has it been found to be phosphorylated in response to sev activation (Allard et al., 1996). Consistent with these observations, it was later found that although the SH2 domains of csw were required for sev signaling (with either domain being sufficient to confer function), the interaction between csw and sev did

not require the SH2 domains or tyrosine phosphorylation of sev, suggesting that the primary role of csw's SH2 domain were to bind dos (Allard et al., 1998). Furthermore, deletion of the entire c terminus, including drk/grb2 sh2 domain binding sequence, did not abolish csw function. Unlike torso, the sev receptor interacts directly with Drk, with the Drk binding sites on sev being required for signaling, much like the SH2 domains of csw (Raabe et al., 1995). Feller et al later demonstrated that Drk could also use its C-terminal SH3 domain to bind and recruit dos (Feller et al., 2002). Together these data make it unlikely that csw plays an adaptor role in this pathway. What remains unclear, however, is what csw recruitment to the sev receptor and dos dephosphorylation accomplishes to promote sev signaling. To further complicate matters, although PTP activity was required for full csw activity, a catalytic inactive csw was capable of providing partial downstream of the sev receptor, suggesting that csw plays an adaptor role of some importance as well.

This redundance of catalytic and non-catalytic roles for Shp-2 downstream of growth factor receptors is recapitulated in the mammalian settling. In mammalian cells, Shp2 can bind directly to the growth factor receptor, as is the case with PDGFR (Klinghoffer and Kazlauskas, 1995), or indirectly via adaptor molecules like Grb2 and Shc. Shp2 can regulate signaling as an adaptor molecule, by using its SH2 and phosphotyrosine motifs to recruit other signaling proteins, and/or through its catalytic function, by directly dephosphorylating and thereby regulating important signaling molecules. As an adaptor, Shp2 has been suggested to bind Grb2, which associates with the GEF Sos, thereby helping to recruit Sos to the receptor in

proximity to Ras. Other potential adaptor-type interactors include the family of Grb-associated binding proteins, or Gabs, and the structurally related IRS (Sugimoto et al., 1994) and FRS proteins (Kontaridis et al., 2002), all of which have been noted to resemble the *Drosophila* protein dos. As a phosphatase, Shp2 can either dephosphorylate and inactivate negative regulators of signaling, or dephosphorylate and thereby activate positive regulators. In support of the former model, Shp2 has been found to dephosphorylate RasGAP binding sites on Gab1/2 (Y317) (Montagner et al., 2005) and EGFR (Y992) (Agazie and Hayman, 2003), thus preventing localization of GAPs to the membrane to terminate Ras signaling. Klinghoffer et al. also demonstrated that SHP2 dephosphorylates a RasGAP binding site at Y771 on PDGFR, which is consistent with the mechanism by which csw regulates its homolog torso (Klinghoffer and Kazlauskas, 1995). Shp2 has also been proposed to dephosphorylate Sprouty, thereby preventing it from sequestering Grb2/Sos and freeing Sos to help activate Ras (Hanafusa et al., 2004). In support of the latter model, Shp2 has been proposed to remove an inhibitory phosphate on Src family kinase (SFK) or alternatively dephosphorylate the SFK-related proteins paxillin and Cbp/PAG so that they are no longer able to recruit the inhibitory kinase Csk to the vicinity of SFK (Zhang et al., 2004). Their ubiquitous expression pattern and known importance in growth factor signaling and transformation make the SFKs attractive candidates for regulation of SHP-2 and Ras/MAPK signaling.

Src family kinases

While SHP-2 was the first PTP to be identified as a proto-oncogene, Src was

the first PTK and first proto-oncogene ever discovered. The Src family kinases (SFKs) are the evolutionarily conserved normal cellular homologues of the Rous sarcoma virus oncogene, p60v-src. There are 8 known mammalian SFKs: Src, Fyn, Yes, Fgr, Lyn, Hck, Lck, and Blk, with one additional member, Yrk, which was identified from a chicken cDNA library and still lacks a mammalian counterpart. Some are expressed in a wide variety of tissues, while others like Lck (lymphoid cell kinase) and Hck (hematopoietic cell kinase) are more restricted. They share a common structural organization from which the Src-homology (SH) domain designations were derived, with SH1 referring to the catalytic kinase domain, SH4 referring to a myristylation sequence, and the already described SH2 and SH3 domains possessing phosphotyrosine-binding and proline rich region-binding properties, respectively. The SH4 myristylation sequence directs membrane localization, while the SH2 and SH3 domains are critical for targeting the Src kinases to their appropriate cellular targets. In addition to these SH domains, the SFKs also possess a C-terminal tail containing a tyrosine residue, and in this way markedly resemble SHP-2 in the combination of an N-terminal SH2 domain with a C-terminal phosphotyrosine motif. While the function of this tyrosine phosphorylation is not entirely clear in the context of SHP-2, it is critical to the function of the SFKs. Early mutagenesis studies on viral and cellular SFKs demonstrated that alterations in the SH2 domain could activate the transforming potential of c-Src, suggesting simple autoinhibitory model in which the SH2 domain bound to a phosphotyrosine residue in the C-terminus of the protein and thereby blocked the active site of the kinase. This binding interaction was later confirmed by crystallization of c-Src and

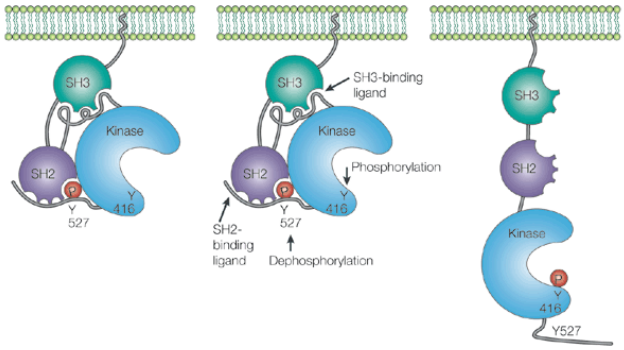

Figure 5. Mechanism of Src family kinase activation. Adapted from Martin et al (Martin, 2001).

Hck (Sicheri et al., 1997; Xu et al., 1997) in the closed, inactive state, though not the way models had predicted. Surprisingly both SH2 and SH3 domains were located at the back of the kinase, and though the tyrosine-phosphorylated C-terminus did in fact complex with the SH2 domain, this interface was surprisingly located ~40A away from the active site. In an unexpected departure from previously proposed models, the SH3 domain was found to dock onto an internal polyproline helix between the SH2 and kinase domains, and while these extensive intramolecular contacts did not block the ATP or peptide substrate binding sites, they effectively sequestered the binding surfaces of the SH2 and SH3 domains and rendered the enzyme inactive by disrupting the orientation of the N and C lobes of the kinase domain (Fig. 5). The SH2 domain-phosphotyrosine interaction was found to be critical for the correct positioning of the SH3 domain, thus providing a basis for the inhibition of Src activity by phosphorylation of this conserved tyrosine (Y527) by c-Src kinase (Csk) as well as the constitutive, oncogenic activity induced by mutation of this tyrosine to phenylalanine. In the 'open' state, phosphorylation of Y416 in the kinase domain further enhances catalytic activity and is often used as a marker of

SFK activation.

Of the known mammalian SFKs, four (Src, Fyn, Yes, and Lyn) are expressed broadly across tissue types and during embryonic development, with especially high levels in hematopoietic and neural cells, while the other four (Hck, Fgr, Lck, and Blk) are restricted in their expression to the hematopoietic compartment. The first knockout mouse model targeted Src itself and resulted in osteopetrosis, a disease in which defective osteoclast function results in excess bone accumulation, also seen in mice deficient in CSF1 (Soriano et al., 1991). Fyn mutant mice, in contrast, show defects in hippocampal development and learning (Grant et al., 1992). However, apart from these two models, knockout of the other SFKs either alone in combination result either in no phenotype or in a variety of hematologic abnormalities, reflecting the importance of this family of signaling molecules in hematopoiesis. The role of SFKs in T- and B-cell development and signaling has been well characterized, with these cell types displaying overlapping patterns of SFK expression: T cells express both Fyn and Lck, while B cells express Lyn, Blk, Fyn, Hck, and Fgr. Lck and Fyn play critical roles in proximal TCR signaling, with Lck knockout mice displaying a more severe phenotype (al-Ramadi et al., 1998). Lck deficient mice display thymic atrophy with a 90% reduction in thymocyte number, in addition to defective TCR signaling in the peripheral T cells that remain. Fyn deficient mice demonstrate decreased responsiveness to TCR activation in immature single-positive population, but peripheral T cells are unaffected, and thus no defect in T-cell development or immune response is observed (Appleby et al., 1992). Defects in Fyn, Blk and Fgr have little effect on B-cell function, but Lyn

deficient mice display a 50-65% reduction in peripheral B cells (Hibbs et al., 1995; Nishizumi et al., 1995). Myeloid cells predominantly express Hck, Fgr, and Lyn, the importance of which will be described in a later chapter.

The first link between the SFKs and growth factor signaling was the discovery that that c-Src was recruited to and activated by PDGFR. The SH2 domains of c-Src bind to Y579 on the receptor (Mori et al., 1993), presumably resulting in activation by the freeing of the C-terminal tail, a model supported by in vitro experiments using a phosphopeptide derived from PDGFR. Most evidence suggests that c-Src is also required for the mitogenic activity of PDGF, as dominant negative SFKs, SFK-neutralizing antibodies and the SFK inhibitor SU6656 have been shown to inhibit PDGFR-stimulated DNA synthesis. However, a mutant PDGFR incapable of binding to SFKs was still sufficient to induce DNA synthesis, suggesting that a direct binding interaction may not be necessary for SFK activity in this pathway. Similar experiments have also shown SFKs to be important in EGF-mediated DNA synthesis, which is of clinical importance as both SFKs and EGFR have been shown to be overexpressed in breast cancer, as well as in DNA synthesis stimulated by CSF1R in myeloid cells and FGFR in fibroblasts (Roche et al., 1995b)). c-Kit, a member of the same subfamily of receptors as PDGFR and CSF-1R, has similarly been shown to bind and activate the SFK Lyn in response to its ligand, SCF, with inhibition of Lyn resulting in decreased SCF-induced proliferation, thus suggesting that the SFKs play a general role in RTK-mediated mitogenesis (Linnekin et al., 1997). PDGFR-mediated induction of c-myc in 3T3 cells has been shown to be SFK-dependent, and the inhibition of PDGFR-, EGF- and CSF-1 stimulated DNA

synthesis by expression of dominant negative SFKs has been shown to be overcome by exogenous expression of c-myc (Barone and Courtneidge, 1995), suggesting that the SFKs may regulate the G1/S transition and DNA synthesis via induction of c-myc. SFKs are also activated at the G2/M transition and can associate with the cyclin-dependent kinase Cdc2 (Resnick et al., 1997; Roche et al., 1995a), thus cementing its role as an important regulator of cell proliferation. Despite its original identification as the product of a transforming oncogene, mutations in SFKs were not identified until relatively recently, when c-Src mutations were identified in a subset of advanced human colon cancers (Irby et al., 1999). Although SFKs have been implicated in GM-CSF signaling (Perugini et al., 2010), no mutations in SFKs or in the GM-CSF receptor itself have been reported in JMML. However, the recent implication of the ubiquitin ligase CBL in various malignancies has highlighted the importance of more proximal signaling events such as those modulating the regulation and turnover of the RTKs themselves.

Casitas B-lineage lymphoma (CBL)

Cbl is the 120-kD product of the proto-oncogene *C-Cbl*, the cellular homolog of the transforming *v-cbl* oncogene of the Cas-NS-1 retrovirus, which causes pre-B lymphomas and myeloid leukemias in mice (Langdon et al., 1989). The Cbl family of proteins are RING-finger domain containing E3 ubiquitin ligases that play a significant role in receptor trafficking. There are three mammalian isoforms: Cbl, Cbl-b, and Cbl-c, the first two of which are expressed highly in hematopoietic cells. All Cbl family members contain the RING finger motif that enables them to

participate in the ubiquitination process and thus function as critical negative regulators of RTK-mediated signaling (Fig. 6a). The *C. elegans* homolog of Cbl, SLI-1, has been shown to be a negative regulator of LET-23 (Yoon et al., 1995) and mammalian Cbl has been shown to negatively regulate EGFR (Ueno et al., 1997). The Drosophila homolog D-cbl has also been shown to negatively regulate R7 photoreceptor development downstream of Sevenless (Meisner et al., 1997).

Ubiquitination is a multi-enzyme process that regulates the degradation and trafficking of many proteins. Ubiquitin moieties are transferred to target molecules via a three-step process: the ubiquitin molecule is first 'activated' by the formation of a thioester bond on one of two ubiquitin activating enzymes (E1); ubiquitin is then transferred from the E1 to a cysteine on one of more than 30 ubiquitin conjugating enzymes (E2); finally, RING-finger containing E3 ubiquitin ligases, such as Cbl, bind the E2 and catalyze the direct transfer of ubiquitin to a lysine on the substrate (Fig. 6b). The most well-characterized substrate of Cbl ubiquitin ligase activity is EGFR. Upon binding to Y1045 on EGFR via its TKB domain, Cbl can then promote its ubiquitination by interacting with the E2 UbcH7. Furthermore, Cbl contributes to internalization of EGFR by recruiting the Cbl-interacting molecule of 85 kD (CIN85) and initiating the early steps in clathrin-mediated endocytosis. Once internalized the EGFR complex can be targeted for lysosomal degradation or for recycling, thereby terminating signaling.

Unlike the E1 and E2 families of ubiquitin activating and conjugating enzymes, there are several hundred E3s, as these molecules are thought to determine the substrate specificity of the ubiquitination process. As such, Cbl

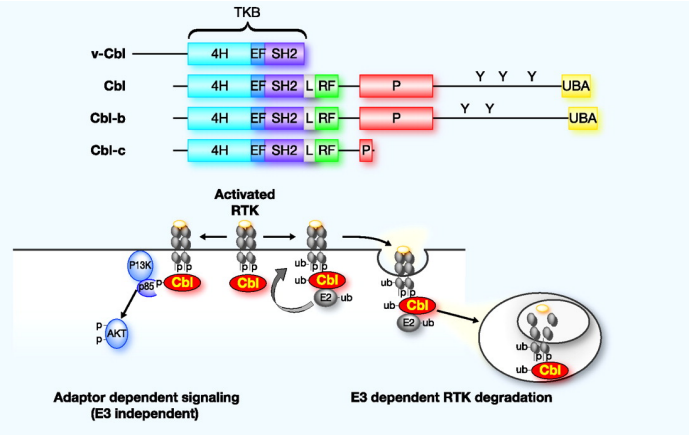

Figure 6. Structure and function of Cbl proteins. Adapted from Kales et al (Kales et al., 2010).

contains several protein-protein interaction domains that confer significant adaptor function as well. An amino terminal SH2-like phosphotyrosine interaction domain known as a tyrosine kinase binding domain (TKB) believed to be important for substrate targeting has been shown to bind the negative regulatory phosphorylation sites within the SH2 kinase linker of ZAP70 and Syk tyrosine kinases (Meng et al., 1999). A loss of function mutation (G315E) in the *C. elegans* gene *sli-1* maps to this domain, and the equivalent mutation (G306E) in mammalian cbl disrupts its binding to known phosphotyrosine-containing substrates (Yoon et al., 1995). This N-terminal TKB domain is followed by the RING finger, a proline rich region, and C-terminal ubiquitin-associated (UBA) domain overlapping with a leucine zipper motif (LZ). The proline-rich region is responsible for its constitutive association with the SH3 domains of a number of adaptor proteins like Grb2 and Nck, as well as Src family kinases. Cbl contains a number of conserved tyrosine residues that are rapidly and prominently phosphorylated upon stimulation of a number of different RTKs including growth factor receptors, immunoglobulin receptors, antigen and integrin receptors, resulting in its association with SH2 domain containing proteins

such as the p85 subunit of PI3K (Hartley et al., 1995).

The pleiotropic nature of CBL's role in signaling has been highlighted by dissection of known transforming alleles of CBL and their consequences. The retroviral oncogene v-cbl contains a large C-terminal truncation resulting in loss of the proline-rich region and RING finger domain. v-cbl associates with RTKs but cannot mediate receptor degradation, suggesting that v-cbl might act as a dominant negative protein by competing with endogenous Cbl for binding sites on activated receptor complexes. All other known Cbl mutants carry deletions or mutations in the RING finger domain and/or preceding linker region. The best characterized of these is the 70Z/3 allele, which was isolated from a mouse pre-B cell lymphoma cell line and carries a small internal deletion of 17 amino acids that includes the linker region and N terminus of the RING finger. Studies in 3T3 fibroblasts showed 70Z to be a more potent oncogene than v-cbl, suggesting that the C terminus of Cbl is important for its transforming potential. Interestingly, the 70Z mutant was found to result in ligand-independent activation of EGFR and increased signaling upon ligand binding, suggesting that Cbl has proto-oncogenic potential that is unmasked when its E3 function is lost.

Consistent with this hypothesis, there is no evidence for increased cancer susceptibility in mice that are null for any of the three Cbl genes, suggesting that loss of E3 activity is necessary but not sufficient for transformation. *c-Cbl* knockout mice demonstrate normal thymic development despite elevated TCR and CD3 levels on the surface of CD4/CD8 double positive thymocytes, increased levels of the Src family kinases Lck and Fyn, and increased activity of ZAP-70 (Murphy et al., 1998;

Naramura et al., 1998; Thien et al., 1999). However, mice expressing a full-length Cbl RING finger mutant (C379A) lacking E3 ubiquitin ligase activity display complete thymic deletion, despite similar increases in the intensity and duration of TCR signaling (Thien et al., 2001). Subsequent biochemical analysis revealed that the RING finger mutant thymocytes demonstrate prominent TCR-directed activation of AKT as compared with *c-Cbl*$^{-/-}$ thymocytes, underscoring the importance of CBL's non-ubiquitin ligase functions in cellular signaling. This seemingly dual function has been further highlighted by the recent identification of *CBL* mutations in human cancers, most notably in 5% of myeloid malignances, as will be discussed.

Conclusion

Murine models of JMML-associated mutations have confirmed the causal role of these genes in JMML via hyperactivation of the Ras pathway. Mice homozygous for NF1 deletion die in utero at gestational day 12-14, but hematopoietic progenitors derived from NF1$^{-/-}$fetal livers displayed GM-CSF hypersensitivity in a colony forming CFU-GM assay characteristic of leukemic cells isolated from patients. Adoptive transfer of fetal liver cells resulted in a JMML-like myeloproliferative disease in mice, as did a subsequent model using conditional deletion of NF1 in hematopoietic cells (Largaespada et al., 1996; Le et al., 2004). A similar conditional model using the interferon inducible Mx1-Cre transgene to express oncogenic K-Ras G12D from its endogenous locus resulted in a rapid onset fatal myeloproliferative disease that demonstrated infiltration of non-hematopoietic organs and GM-CSF hypersensitivity in vitro, thus recapitulating human disease (Braun et al., 2004). As

already discussed, retroviral transduction of murine bone marrow with the JMML-associated SHP-2 mutants E76K and D61Y induced GM-CSF hypersensitivity in a CFU-GM assay, hyperproliferation of macrophages derived from the transduced bone marrow, and constitutively elevated and sustained phospho-ERK levels following GM-CSF stimulation (Chan et al., 2005; Schubbert et al., 2005). Transplantation of bone marrow expressing E76K or D61Y resulted in a fatal myeloproliferative disease resembling JMML, as did more recent knock-in models using these same alleles (Chan et al., 2009; Mohi et al., 2005; Xu et al., 2010). The mutations in c-CBL being the most recently characterized, knock-in or transduction-transplantation models of JMML-associated mutations do not yet exist, but c-cbl$^{-/-}$ mice have been noted to develop a mild non-progressive MPD, while mice double null for c-cbl and cbl-b develop an early-onset rapidly fatal MPD (Naramura et al., 2010). Mice hemizygous for a single RING finger mutant of CBL (C379A/-) also develop an MPD that progresses to an aggressive, fatal leukemia (Rathinam et al., 2010).

Although these models confirm the sufficiency of these genetic lesions in initiating JMML-like myeloproliferative disease, they do not address the mechanism by which this transformation is accomplished. A more thorough understanding of the biology underlying this fatal childhood leukemia is necessary in order to develop better, more targeted agents for therapy and ultimately cure. In the following chapters, several attempts to address this question will be described. Using a variety of biochemical and genetic approaches, we have gained new insights into JMML pathogenesis including the identification of several putative substrates of

oncogenic SHP-2, the implication of SFKs in GM-CSF signaling SHP-2 signaling downstream of the GM-CSF receptor not in JMML but rather in a novel pathway mediating megakaryocytic differentiation, and the identification and characterization of a novel JMML gene, *C-CBL*.

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Chapter 2:

Identification of putative SHP-2 substrates in JMML

Introduction

The protein tyrosine phosphatase SHP-2 has been identified across organisms and cell types as being a critical positive effector of growth factor and oncogenic signaling. Gain-of-function mutations have been identified in a number of myeloid malignancies, most frequently in the pediatric myeloproliferative disease juvenile myelomonocytic leukemia (JMML). The most common JMML-associated mutation results in the amino acid substitution E76K, which lies within the N-SH2 domain and is thought to form key contacts with the PTP domain that are important for mediating inhibition of catalytic activity. It is not only the most common but also the most potent among the disease-associated SHP-2 mutants, demonstrating the highest fold increase in catalytic activity and the greatest transforming potential in cell lines and murine models. Although SHP-2 has been shown to play both catalytic and non-catalytic roles in signal transduction, deletion of the PTP domain has shown to abolish most, if not all, of its ability to function as a positive effector of RTK signaling in *Drosophila* and *C. elegans*.

In the context of JMML, evidence strongly suggests that the catalytic activity of SHP-2 is required for the transforming effect of SHP-2 E76K in the hematopoietic context. Schubbert et al. found that substitution of the catalytic cysteine abolished the GM-CSF hypersensitivity conferred by the oncogenic E76K SHP2 mutant in murine fetal liver-derived hematopoietic progenitors. Similarly, Mohi et al. found that substitution of the conserved arginine residue in the conserved phosphatase domain also eliminated transformation of bone marrow cells expressing E76K (Mohi et al., 2005). Of note, the latter group intentionally used the R465M

substitution rather than the C459S to avoid the potentially confounding effects of a substrate-trapping mutant, the subtleties of which will be discussed more in detail in subsequently and proved an important distinction for our purposes as well. In both cases, however, the PTP domain of SHP-2 was found to be required for myeloid transformation. Thus, the substrate question is crucial to our understanding of Shp2 in JMML. Contrary to expectations, PTPs have been found to exhibit a remarkable degree of substrate specificity in vivo, likely conferred both by the sequence of the catalytic domain as well as its regulatory domains.

The unique modular structure of SHP-2 lends itself to several different potential mechanisms of specificity. While the N-SH2 domain of SHP-2 is known to be critical for granting access to the catalytic cleft, the specificity of both the N- and C-SH2 domains are also likely to be important for targeting SHP-2 to the correct subcellular location as well as its specific substrates. This is supported by the finding that deletion of the N-SH2 in a murine model of SHP-2 deficiency was embryonic lethal, similar to the phenotype of true genetic null models developed later (Saxton et al., 1997). In the *Xenopus* animal cap assays performed by O'Reilly et al, the elongation evoked by the activating D61A mutant was abolished by mutation of the essential arginines in the conserved FLVRES motif of the SH2 domains, suggesting that their phosphotyrosine-binding function was required for its role in FGF-mediated signaling (O'Reilly and Neel, 1998; O'Reilly et al., 2000). In the *Drosophila* model where dos was identified as a substrate of csw in the Sevenless signaling pathway, substitution of residues R32 or R137 in the conserved FLVRES motif of the SH2 domains of csw negated the substrate trapping ability of

C583S mutant, suggesting the catalytic interaction was dependent on one or both csw SH2 domains (Herbst et al., 1996). Similarly, in the setting of JMML, Mohi et al. found that second site mutations in the FLVRES motif of either the N- or C- SH2 domains ablated factor-independent colony formation by SHP-2 E76K (Mohi et al., 2005).

Despite the importance of the SH2 domains, several important experiments using chimeras of SHP-1 and SHP-2 swapping their SH2 and catalytic domains have demonstrated that significant specificity resides in the catalytic domain itself. In the *Xenopus* animal cap assays, O'Reilly et al. found that elongation was abolished when the PTP domain of XSHP-2 was replaced with the PTP domain of SHP-1, despite 61% identity in this region (O'Reilly and Neel, 1998). Conversely, in looking at requirements of SHP-1 function in COS-7 cells, Tenev et al. found that chimeras constructed from SHP-1 and SHP-2 were able to dephosphorylate EGFR as long as they contained the catalytic domain of SHP-1, but lacked catalytic activity when then contained the catalytic domain of SHP-2, even though these latter molecules retained their ability to associate with the receptor and dephosphorylate an artificial phosphopeptide (Tenev et al., 1997). Initial studies to determine the substrate specificity of PTPs were conducted using a recombinant bacterial PTP from Yersinia and a recombinant mammalian PTP, rat PTP1, and synthetic phosphotyrosine-containing peptides corresponding to EGFR 988-998, which contains the autophosphorylation site at Y992. Sequential substitution of each residue in this peptide with alanine (Ala-scan) resulted in a 'consensus sequence' as DADEpYAAPA, with acidic residues on the N-terminal side of the pY being critical

for high-affinity binding and catalysis (Zhang et al., 1993). A subsequent study by this same group found that efficient binding and catalysis required 6 amino acids including the pY residue, 4 N-terminal and 1 C-terminal to the pY (DADEpYL in EGFR), with the predominant determinants of PTP specificity residing in the residues N-terminal to pY (Zhang et al., 1994). Screening of a combinatorial synthetic peptide library for determinants of PTP specificity found that unlike the catalytic domain of RPTPα, which had a very weak sequence specificity, and PTP1B, which demonstrated only a modest preference for acidic residues, SHP-1 and SHP-2 had much narrower substrate specificities, preferring two or more acidic residues on the N-terminal side and one or more acidic residues on the C-terminal side (Ren et al., 2011).

The ubiquitous expression of SHP-2 across cell types and the similarly ubiquitous usage of tyrosine phosphorylation as an activating (or inactivating) mark in signal transduction suggest that the highly specific nature of SHP-2 activity is likely the product of multiple overlapping mechanisms of regulation. That is to say, it is likely a combination of all these factors: the protein-binding capabilities of each individual domain, the sequence determinants of those binding interactions, the orientation of those domains, and the subcellular localization resulting from those interactions which in turn determines its proximity to potential binding partners, that contribute to a sort of concentric layering of requirements that narrow the pool of available substrates. When coupled with the inherent restriction of that pool based on cell type-specific differences in protein expression and receptor type-specific differences in signaling pathway components, the importance of attempting

substrate identification under circumstances as closely resembling the biological setting of interest as possible becomes apparent. To this end, despite the importance of evaluating each of these factors and their individual contributions to specificity, ultimately the identification of the SHP-2 substrates important for JMML pathogenesis will require the careful preservation of these determinants of physiological, and in this case pathological, relevance.

In a recent review, Tiganis and Bennett examined the three predominant methods for defining PTP substrates: 1) the ability of a putative substrate to bind a substrate-trapping mutant, 2) the modulation of the tyrosine phosphorylation status of the putative substrate by gain- or loss-of-function of the PTP, and 3) the ability of the PTP to dephosphorylate the substrate in vitro (Tiganis and Bennett, 2007). The authors argue that each of these approaches suffers from key limitations that make them alone insufficient to identify a substrate. Moreover, they point out that ultimately the ability to define the role of a PTP in signaling depends upon being able to identify substrates that can account for its known biological function. Many putative substrates of SHP-2 have been reported in a variety of contexts, including EGFR, Cbp/PAG, Spry, MVP, Gab1, Stat5, and Spred1, but none of these studies have been conclusive in that they rely on a single approach and result in models that are highly cell- and growth factor/cytokine-specific (Agazie and Hayman, 2003; Chen et al., 2004; Jarvis et al., 2006; Kolli et al., 2004; Montagner et al., 2005; Quintanar-Audelo et al., 2011; Zhang et al., 2004). Given the diverse mechanisms by which SHP-2 regulates signaling depending on its cellular context, it is difficult to extend any one of these models to account for the mechanism of SHP-2 mediated

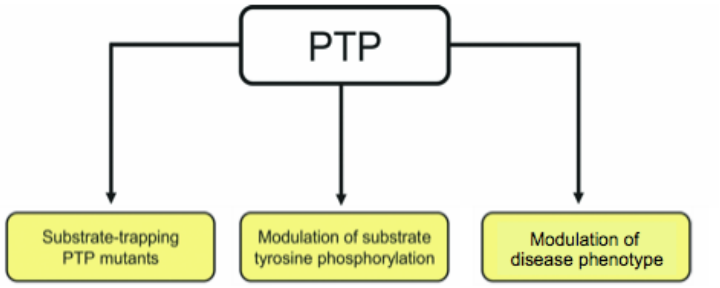


Figure 1. Methods of protein tyrosine phosphatase substrate identification. Adapted from Tiganis and Bennett.

oncogenesis, and none of these putative substrates have been identified or confirmed in the context of leukemia. Thus we believe that showing biological activity mimicking that of SHP-2 in leukemia is a critical criteria for determining substrate validity (Fig. 1).

Of the three approaches just described, the binding of a substrate-trapping mutant is thought to provide the highest degree of specificity because it retains the appropriate intracellular context, which given the importance of subcellular localization as a regulatory mechanism, is critical for demonstrating any identified protein of interest is physiologically relevant, and demonstrates a direct binding interaction, which given the 'cascade'-like organization of phosphorylation-mediated activation/inactivation of kinases and phosphatases in signaling pathways is critical for demonstrating that any observed modulation of phosphorylation is not an indirect effect of SHP-2 activity. The canonical 'substrate trapping' mutant involves substitution of the critical active-site cysteine residue to a serine, thus abolishing the enzymatic activity of the protein while leaving the rest of it intact and thereby allowing binding of the phosphotyrosine-containing substrate while preventing the nucleophilic attack necessary for phosphate release. This type of

PTP mutant has been used successfully for the identification of a number of putative PTP substrates, but the single substitution has not always proven sufficient.

Based on the crystal structure of the archetypal PTP, PTP1B, Flint et al. generated a series of novel substitutions predicated to differentially affect substrate affinity and catalytic capability. They identified several mutants possessing an affinity similar to or greater than that of the wild-type enzyme but with catalytic activity low enough to theoretically permit stable isolation of enzyme-substrate complex (Flint et al., 1997). Substitution of the critical aspartate residue in the WPD loop, in particular, resulted in a similar Km but greatly reduced V_{max} compared with the wild-type enzyme, suggesting that it possessed the ideal 'substrate trapping' qualities of an inactive enzyme that retains substrate binding capability (Fig. 2). Overexpression of this D/A mutant of PTP1B in serum- or EGF-stimulated COS and 293 cells resulted in the accumulation of tyrosine phosphorylated EGFR and unknown proteins of 120, 80 and 70 kD. An equivalent substitution in PTP-PEST by Garton et al. resulted in the identification of p130cas as a putative substrate in

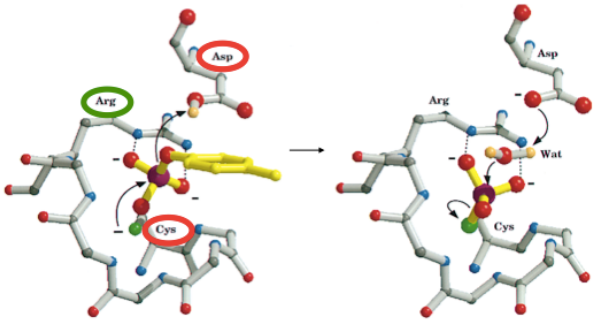


Table 1. Kinetic parameters of purified point mutants of 37-kDa PTP1B assessed with pTyr-RCML as substrate

Enzyme		V_{max}, nmol/min/mg	K_m, nM	k_{cat}, min^{-1}
Wild type		60,200	102	2244
Tyr-46 → Ser		4,120	1700	154
→ Leu		4,160	1700	155
Glu-115 → Ala		5,700	45	212
→ Asp		5,900	20	220
Lys-116 → Ala		68,600	150	2557
Lys-120 → Ala		19,000	80	708
→ Asp-181 → Ala	D/A	0.61	≤126	0.023
→ Glu		97	10	3.6
His-214 → Ala		700	20	26
→ Cys-215 → Ser	C/S	No detectable activity		
→ Arg-221 → Lys		11	80	0.41
→ Met	R/M	3.3	1060	0.12
Gln-262 → Ala		720	9	27

Figure 2. Effect of substitution of critical residues for substrate affinity and catalytic activity. Adapted from Flint et al (Flint et al., 1997).

pervanadate-treated HeLa and COS-7 cells (Garton et al., 1996). In contrast, Agazie et al. found that the D/A alone was not sufficient to confer substrate-trapping ability to SHP-2 (Agazie and Hayman, 2003). They found that a double mutant in which the D/A was combined with the canonical C/S substitution resulted in a more effective substrate trap than either mutant alone. Using this approach they identified EGFR, GAB1, and three other unknown proteins of 190, 150, and 90 kD as putative substrates in EGF-stimulated COS-1 and 293T cells.

Despite the specificity achieved by this method, oft-cited limitations of conventional substrate trapping approaches point to its reliance on overexpression, which can lead to mislocalization and/or nonspecific, nonphysiologic complex formation, and on antibody-guided detection, which is highly biased and not very robust because it requires that the substrate be sufficiently abundant to allow detection by immunoblotting and that investigators have sufficient prior knowledge of the signaling pathway to guess as to the identity of potentially relevant proteins that might correspond to the apparent molecular weight indicated by the gel band of interest. Thus, a protein with no known function, or a known protein lacking a commercially available antibody, or even a known protein that has a commercially available antibody but was previously unknown to be related to that signaling pathway, would all be missed by this standard. The difficulty this presents is reflected in the examples just discussed, where in addition to EGFR and Gab1, several of the putative substrates were simply designated by their molecular weight and left unidentified. The latter caveat has begun to be addressed by the use of more unbiased, high throughput protein identification methods such as yeast two

hybrid screening using a cDNA library whose products were phosphorylated by a constitutively active PTK (Kawachi et al., 2001) or mass spectrometry. One approach that potentially addresses both concerns is the tandem affinity purification (TAP) method first described by Rigaut et al., which utilizes high-affinity tags fused to a protein of interest to purify native protein complexes, the components of which are then identified by mass spectrometry (Rigaut et al., 1999).

Originally developed in yeast, the TAP strategy was designed with the idea that identification of the protein components of a heteromeric complex would require near-physiologic levels of expression, as overexpression could result in nonphysiological complexes, and that purification of proteins expressed at these lower levels would require a combination of high-affinity tags. This consideration makes it especially attractive for substrate-trapping purposes, as achieving near-physiologic expression levels of the 'bait' in order to preserve stoichiometry is also ideal for the isolation of an enzyme-substrate complex. After screening a number of tags including the FLAG tag, the Strep tag, the His tag, the chitin binding domain, two IgG-binding units of protein A of *Staphylococcus aureus*, and the calmodulin binding peptide (CBP), the authors found that only the latter two allowed efficient recovery (80% and 50%, respectively) of a fusion protein present at low concentration in a complex mixture. The original 'TAP' tag consisted of a cassette encoding the calmodulin-binding peptide (CBP), a cleavage site for the tobacco etch virus (TEV) protease, and two IgG-binding units of protein A of *S. aureus* (ProtA) (Fig. 3a). The TAP method involves fusing the TAP cassette to the target protein, introducing it into the target cell, and then recovering the fusion protein and associated binding

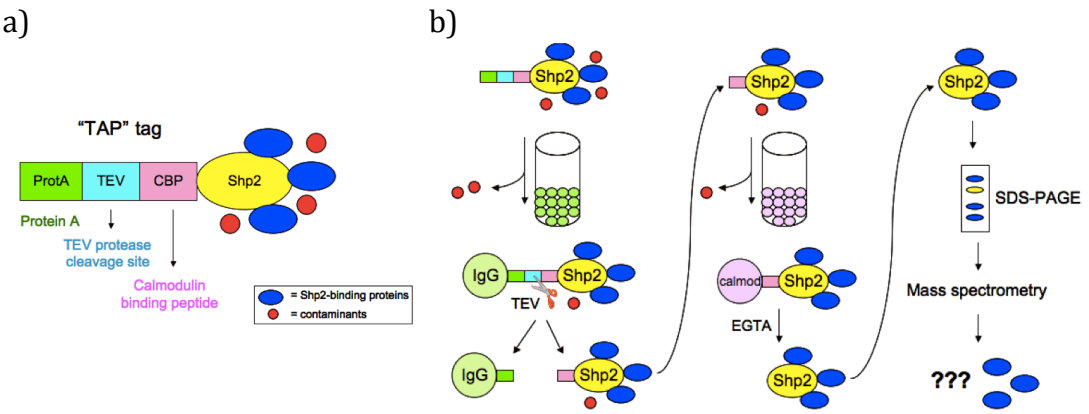


Figure 3. Overview of tandem affinity purification. a) Structure of the 'TAP' tag. b) Overview of 'TAP' procedure.

partners from cell extracts by affinity selection on an IgG matrix. This is followed by cleavage with the TEV protease to release the bound material, and then incubation with calmodulin-coated beads in the presence of calcium to remove the TEV protease and contaminants remaining after the first affinity selection. The bound material is then released with EGTA and subjected to mass spectrometry (Fig. 3b).

Although this technology has been applied in mammalian cells, Burckstummer et al. sought to further optimize this protocol for this particular application. The authors noted several limitations of the original tag, most notably the requirement for large initial quantities of cells (Burckstummer et al., 2006). They tested several different TAP fusion protein cassettes wherein they had streamlined the sequence by removing residues artificially derived from the original cloning junctions (and therefore unlikely to contribute to purification) and modified it to reflect human codon preferences and then swapped out the CBP moiety with the streptavidin-binding peptide (SBP) and the ProtA moiety with the two IgG binding units of protein G from *Streptococcus*. The resulting 'GS' tag led to an increase in expression as well as dramatic improvement in overall recovery of the

bait which the authors attributed to increased bait retrieval after TEV cleavage and increased capture of TEV-cleaved bait by the streptavidin resin. By thus optimizing the system for use in mammalian systems, they were able to obtain yields an order of magnitude higher than the original 'AC' tag. In an even more recent paper, Junttila et al. demonstrated the use of a single Strep-tag for affinity purification in mammalian cells to identify binding partners of the PP2A complex from the HT-1080 mammalian cancer cell line, citing speed and increased recovery as the primary reasons for undertaking this single-step purification approach.

As mutations in *PTPN11* are responsible for a significant proportion of cases of JMML, and multiple studies have shown that the catalytic activity of SHP-2 E76K is required for its transforming potential, we believe that the identification of the relevant substrate(s) in this particular context is crucial to understanding the pathogenesis of the disease and ultimately developing novel treatment strategies to improve survival. To that end, we devised a strategy combining the specificity of PTP substrate-trapping technology with the sensitivity of mass spectrometry-based protein identification and the stringency of tandem affinity purification in order to allow for the unbiased identification of putative substrates. This strategy was adopted specifically in the biological context of the JMML-associated mutant SHP-2 E76K and its effect on signaling in hematopoietic cells. Although ideal in theory, in practice we discovered that certain 'advantageous' aspects of each of these methods in fact worked against each other, and against the biology itself, requiring modifications be made along the way. Ultimately sacrifices in stringency in the interest of maximizing speed and protein recovery resulted in the identification of

several proteins of interest with potential biological relevance for SHP-2 signaling in hematopoietic cells. Further investigation will be required to determine their importance in the JMML phenotype and targetability for therapeutic purposes.

Results

Schubbert et al demonstrated that SHP-2 E76K could transform primary bone marrow cells in a colony forming (CFU-GM) assay and produce both factor-independent colonies and GM-CSF hypersensitivity characteristic of hematopoietic progenitors isolated from JMML patients (Schubbert et al., 2005). They further demonstrated that catalytic activity was required for this transforming ability, as mutation of the catalytic cysteine to serine caused colony-forming ability in response to GM-CSF to revert back to a dose-dependence akin to that of wild-type bone marrow. We sought to identify the critical substrate(s) responsible for the E76K-mediated transformation by combining the biochemical principles of substrate-trapping for PTPs with recent advances in immunoaffinity purification that utilize mass spectrometry rather than antibody-specific immunoblotting for protein identification.

Based on the kinetic data generated by Tonks et al. demonstrating the effects of different substitutions at conserved residues in the PTP domain and previous work by Agazie et al. demonstrating that substitution of the catalytic cysteine to serine (C/S) was not sufficient to generate a substrate-trapping mutant of SHP-2, we introduced mutations affecting both the catalytic cysteine (C/S) and the critical aspartate residue of the WPD loop (D/A) into a murine SHP-2 cDNA already

encoding the JMML-associated E76K substitution, thus generating a substrate-trapping oncogenic SHP-2 mutant hereafter referred to as 'KAS'. In the original experiment performed by Schubbert et al, a 'KS' mutant (E76K coupled with C/S) was found to display a wild-type phenotype in a CFU-GM assay. This, as mentioned previously, suggested that the transforming ability of SHP2 E76K was catalytic dependent, but it also suggested that the C/S version of the mutant protein did not function as a dominant negative, as might have been expected. This was somewhat surprising, as a catalytically inactive SHP-2 should in theory result in the sequestration of substrates, or at the very least binding partners important for localization or complex formation, but this was not in fact the case. This data was consistent with the finding by Agazie et al. that C/S alone did not confer substrate-trapping ability. We repeated this same CFU-GM assay with our 'KAS' mutant in murine fetal liver cells and found that this allele did, in fact, suppress colony formation below that of wild-type, suggesting that the addition of the D/A substitution improved the substrate-trapping ability of the C/S mutant, thus allowing it to exert a dominant negative effect (Fig. 4a).

In addition, in order to distinguish purely structural binding partners from catalytic substrates, we also generated an E76K clone bearing a substitution at a conserved arginine residue (R/M) known to be crucial for coordinating the negative charges of the phosphate anion. In the original experiment using PTP1B, while C/S and D/A decrease V_{max} without affecting K_m, R/M demonstrated a marked increase in K_m, suggesting that removing this charge stabilization in fact greatly reduces the

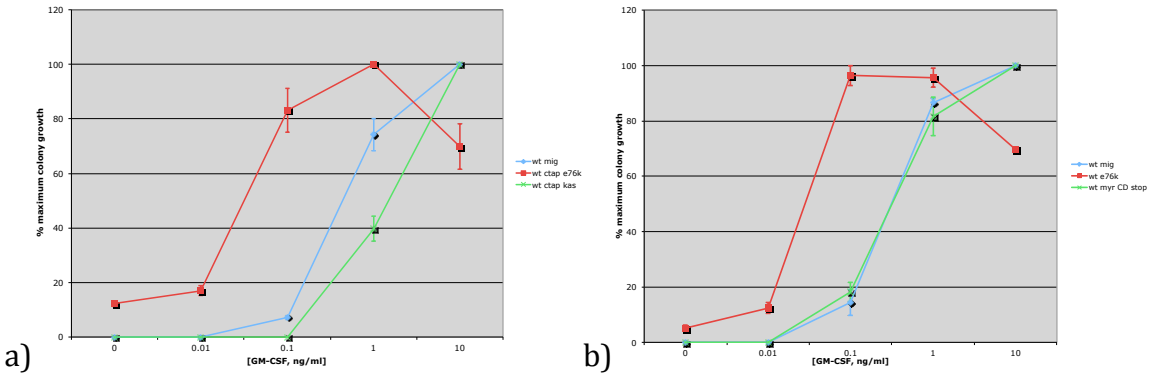


Figure 4. Effect of different SHP-2 variants on CFU-GM activity. a) Addition of double substrate trapping mutations results in dominant negative effect. b) Myristylated isolated catalytic domain is not constitutively active.

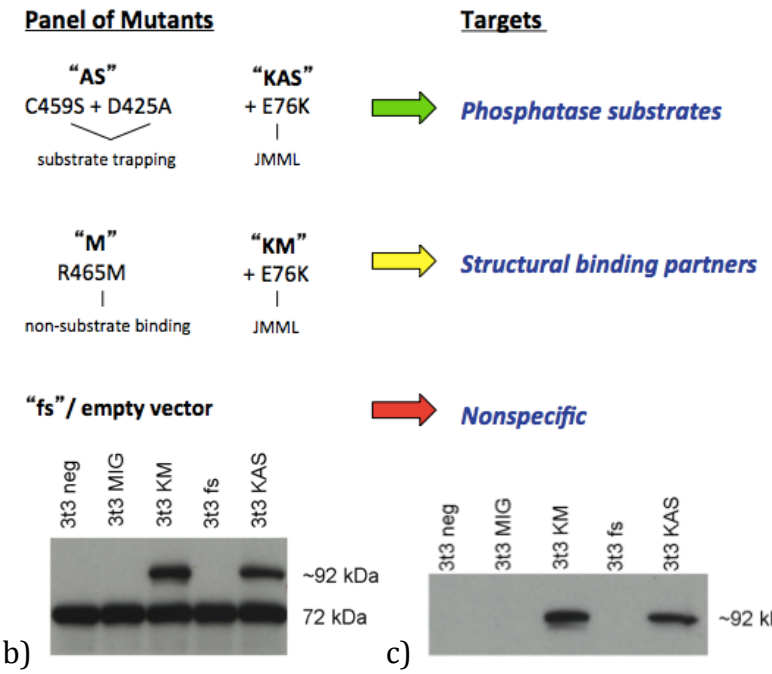
affinity of the enzyme for substrate. This was, of note, the same allele used by Mohi et al. to assess the catalytic dependence of E76K while avoiding potential confounding effects of substrate-trapping. Thus, while both of these mutants are catalytically inactive and encode a full-length SHP-2 molecule, the latter mutant, hereafter referred to as 'KM,' should theoretically be non-substrate binding, unlike the 'KAS' mutant, which should be substrate trapping. In Mohi et al. adoptive transfer experiment, the R/M, much like the C/S in Schubbert's experiments, abolished the CFU-GM phenotype of SHP-2 E76K, suggesting that the intact functioning of the SH2 domains and phosphotyrosine residues in the R/M mutant are not sufficient to preserve transforming ability (Mohi et al., 2005). Corresponding 'AS' and 'M' mutants were also generated in order to assess whether the E76K substitution might also affect substrate specificity.

The SH2 domains of SHP-2 are inherently troublesome for substrate identification purposes, as they bind to phosphotyrosine just as does the catalytic domain. In fact, the few instances in which csw binding sites on substrates like torso and Dos have been mapped suggest that it is entirely possible that a substrate

could present one or two phosphotyrosine residues to engage the SH2 domains and thereby recruit SHP-2 in order to be dephosphorylated on a third. This suggests that a genuine substrate might bind both an 'AS' and 'M' mutants equally well, meaning that while a putative binding partner binding 'M' but not 'AS' could effectively be ruled out, the converse might not necessarily be true. In an attempt to develop a more efficient 'bait' strategy that would avoid the confounding effect of the SH2 domains, we added a myristylation sequence to the catalytic domain of SHP-2 to see if constitutive membrane localization could replace the need for SH2-mediated targeting, assuming that the relevant substrates would be located at the membrane where the receptor complexes were formed. If this model were correct, a myristylated, isolated catalytic domain that was uninhibited, and therefore active, and properly localized to the membrane, should be transforming in its own right. However, to our surprise, when introduced into fetal liver cells, this 'myr-CD' mutant gave a CFU-GM phenotype indistinguishable from that of wild-type cells, suggesting that the SH2 domains were necessary for more than simply membrane localization (Fig. 4b). This is consistent with early experiments demonstrating that while in *Drosophila* the addition of a myristyl sequence to full-length csw resulted in constitutive activation (Allard et al., 1996), deletion of the N-SH2 domain in mice resulted in a nonfunctional protein (Saxton et al., 1997). The final panel of SHP-2 mutants thus consisted of full-length protein sequences with the E76K, C459S, D425A, and R465M substitutions to generate substrate-trapping and non-substrate binding versions of oncogenic and wild-type SHP-2 (Fig. 5a).

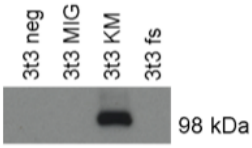
Affinity tag-based purification has been demonstrated to be preferable to

a)



b) IB: total Shp2

c) IP: IgG beads, IB: total Shp2



d) IP: IgG beads, IB: pGab2

Figure 5. NTAP-Shp2 constructs validated in 3T3 cells. a) Panel of SHP-2 mutants. b) Expression of NTAP constructs in transduced 3T3 cells. c) NTAP-Shp2 constructs pulled down using Protein A component of TAP tag. d) NTAP-Shp2 constructs pull down known binding partner, phospho-Gab2.

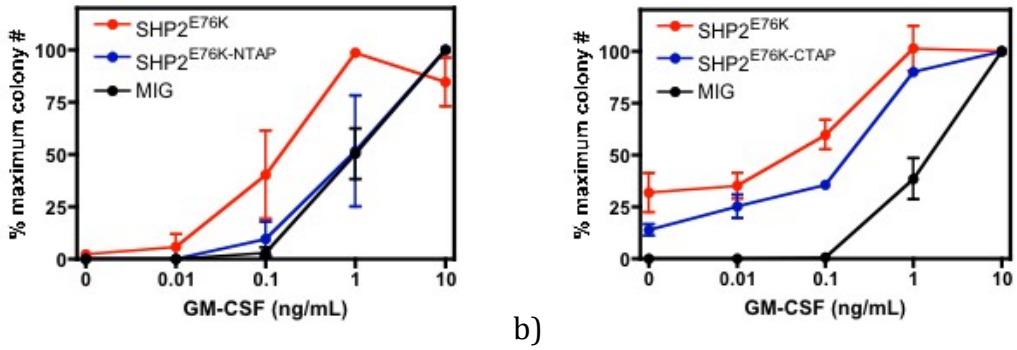
direct antibody-mediated isolation for substrate identification purposes, as immunoprecipitation with a SHP-2 antibody would result in recovery of endogenous wild-type protein in addition to our mutants of interest, as well as its associated binding partners, thus increasing the background noise and interfering with the identification of genuine substrates. The tandem affinity purification method, as described, was designed specifically to be used in cases where overexpression of a protein of interest might be problematic for identifying binding partners, leading either to mislocalization or unbalanced stoichiometry or both. As both of these were valid concerns for the isolation of a likely labile enzyme-

substrate complex whose interaction would be phosphorylation-dependent and involve an appreciable on/off rate, that while greatly diminished, would still be non-zero. For this reason, we decided to take advantage of the original 'TAP' tag developed by Rigaut et al which consists of two Protein A subunits of IgG and a calmodulin tag separated by a cleavage site for the tobacco etch virus. It has been noted in the literature that use of an affinity tag requires three preliminary validation steps: that the tagged protein is expressed, that the tag is accessible, and that the tagged protein is functional. We began with an N-terminal fusion of the TAP cassette, with the affinity tags being inserted immediately after the start codon, and introduced this NTAP-SHP2 construct into an MSCV-based retroviral expression vector for stable expression in a variety of cell types, first for validation and later for purification under appropriate experimental conditions.

The NTAP-SHP2 mutants were first introduced into 3T3 cells for validation of expression and found to be present at a level similar to that of the endogenous protein, as desired (Fig. 5b). Next, an IgG-mediated pulldown was performed in order to determine whether the Protein A components of the tag were accessible within a cellular context. Immunoblot detection using a SHP-2 antibody confirmed recovery of the NTAP-SHP2 'bait' by this method, suggesting that the native protein conformation did not render the tag inaccessible by burying it in the interior of the folded protein (Fig. 5c). This method also proved capable of pulling out known mammalian SHP-2 binding partner Gab2 (Fig. 5d). Though it is unclear whether Gab2 is a substrate or merely an adaptor-type binding partner of SHP-2, this result confirms the ability of this affinity tag to pull out SHP-2 in complex with other

proteins of interest.

The final validation step required demonstrating that the NTAP-SHP-2 protein was functional, that is, that the addition of the additional amino acid sequence bulk did not interfere with the tagged-protein's ability to function as an effective signal transduction molecule. Although SHP-2 function could have been assessed in any number of ways, we concluded that the most stringent test, and most relevant for the ultimate purpose we had in mind, would be to test the ability of an NTAP SHP-2 protein harboring the E76K substitution to transform primary murine hematopoietic progenitors in a CFU-GM assay. While the MIG NTAP SHP2 E76K-expressing cells did retain some traces of GM-CSF hypersensitivity, the factor-independent colony formation characteristic of this allele was no longer observed (Fig. 6a). As in vitro GM-CSF hypersensitivity is known to correlate with expression level of the oncogenic protein, this was likely at least in part due to the very low expression of the NTAP construct achieved in murine fetal liver cells. Since it is a



a) b)

Figure 6. Validation of TAP-Shp2 constructs in primary cells. a) MIG-NTAP-E76K does not confer GM-CSF hypersensitivity as efficiently as MIG-E76K in a CFU-GM assay. b) MIG-CTAP E76K demonstrates key features of GM-CSF hypersensitivity in a CFU-GM assay including factor independent growth and early saturation.

general rule-of-thumb in affinity tag experiments to generate both N- and C-terminal fusions to determine the optimal 'bait,' we had also cloned a CTAP SHP2 construct, with the affinity tags being inserted immediately before the stop codon. We found that MIG CTAP SHP-2 E76K was both expressed at a higher level and retained the transforming capability of a non-tagged SHP-2 E76K, and thus we proceeded with this as our bait (Fig. 6b).

The TAP protocol as originally described involves sequential rounds of affinity purification using first IgG agarose to capture the tandemly tagged bait, then incubating the bound beads with the tobacco etch virus protease in order to freeing the bait by cleaving off that portion of the tag, then using calmodulin binding peptide resin for another round of bait capture after which EGTA is used to elute SHP-2 and, in theory, only its very tightly bound (and now thoroughly washed) binding partners to be subjected to identification by mass spectrometry. Because of the large initial cell numbers required to perform sequential affinity purification and still end up with enough sufficient protein for analysis, this type of technology has primarily been employed in cell lines, although as technology has improved these types of studies have also begun to be performed in primary cells. Given the highly cell-and receptor-type specific nature of SHP-2 functions in signaling and our interest primarily in the role of SHP-2 in JMML, we first screened several human hematopoietic cell lines and found that the U937 human monocytic cell line, in addition to representing the cell lineage most obviously dysregulated in JMML, demonstrated a hypersensitive phosphorylation of STAT5 in response to GM-CSF similar to that described in JMML patient samples—a phenomenon we later

discovered was likely due to the fact that it harbors a weakly activating mutation in its endogenous *PTPN11* gene (Fig. 7a).

Because the presence of the endogenous enzyme, particularly in this case an activated endogenous enzyme, is not only a source of potential noise for antibody-mediated pulldown but also a source of competition for relevant substrates, it has been suggested that in light of recent advances in RNA interference-mediated

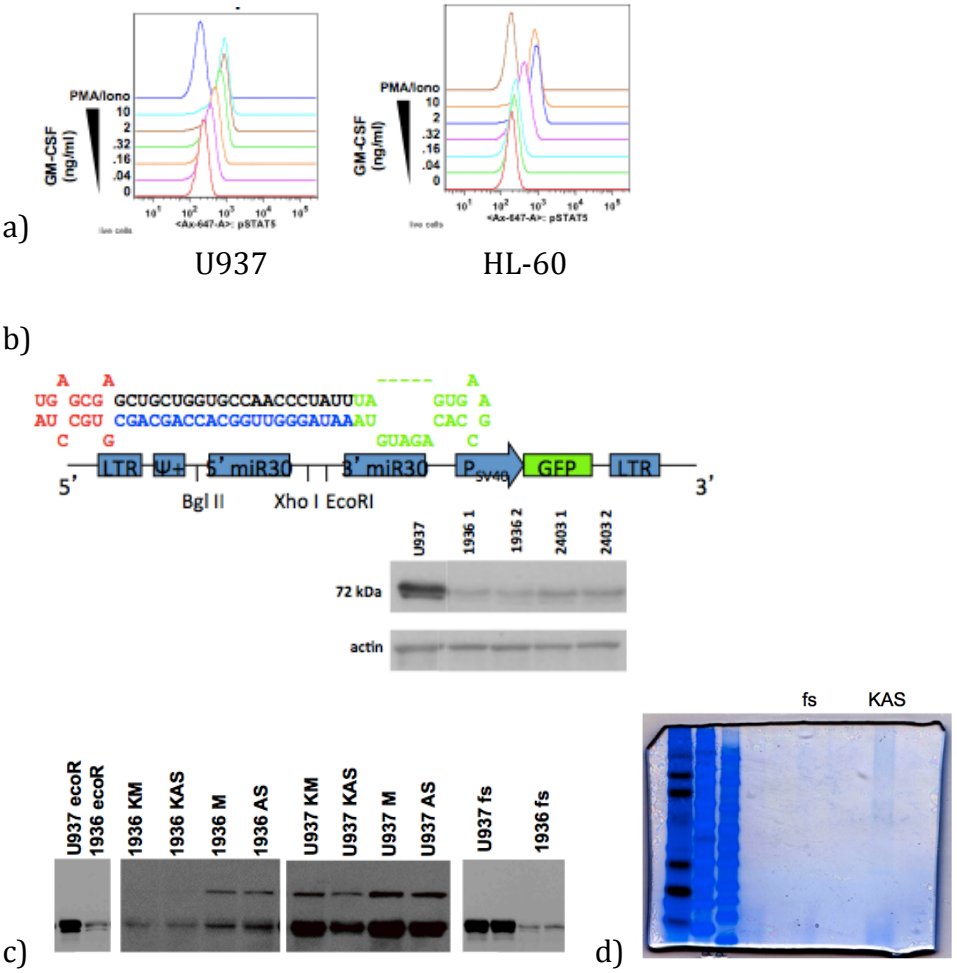


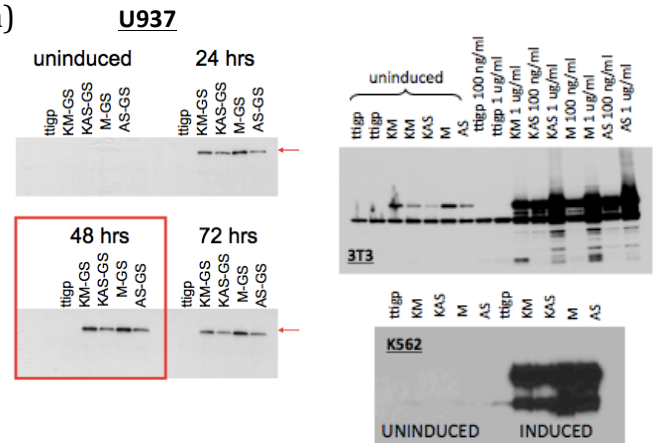
Figure 7. shRNA knockdown in U937 cells decreases expression of endogenous and exogenous SHP-2. a) GM-CSF hypersensitivity in STAT5 phosphorylation in U937 cells (left) as compared with another human leukemia cell line HL60 (right). b) Knockdown of SHP-2 expression using two sequences, 1936 and 2403. c) MIG-CTAP-SHP2 expression in shSHP2 knockdown and parental U937 cells. d) Coomassie stained gel of MIG-NTAP-SHP2 KAS and 'fs' control.

knockdown approaches, elimination or attenuation of the endogenous protein could markedly improve one's chances of isolating a bona fide substrate with an engineered substrate-trapping mutant. Thus using a mir-30 based backbone, we engineered and tested two different shRNAs against SHP-2 that specifically targeted the human (endogenous) nucleotide sequence, both of which proved to be effective in decreasing SHP-2 expression, with one sequence (1936) proving slightly better than the other (2403) (Fig. 7b). These shRNAs were GFP-tagged, thus requiring the CTAP SHP-2 constructs to be switched to a puromycin marker, and these MSCV-puro CTAP SHP-2 constructs were stably expressed in both parental and shSHP2 U937 cells. Although, as in the 3T3s, all tagged mutant constructs were expressed, in this case their expression levels were markedly diminished compared with the endogenous protein (Fig. 7c). Furthermore, in the shSHP2 cell lines, despite the species-specificity of the shRNA sequence, immunoblotting of the resulting cell lysates demonstrated apparent knockdown of both endogenous and exogenous SHP-2 protein levels, rending the latter nearly undetectable. Also of note, the 'AS'-containing mutants were consistently expressed at lower levels than their 'M' counterparts, presumably consistent with their biological activities, with this negative selection appearing to worsen over time (data not shown). Not surprisingly, subsequent TAP purification of approximately 500e6 of the CTAP SHP-2 mutant-expressing parental U937 cells subjected to gel analysis revealed a bait band barely visible by Coomassie stain and insufficient for MS analysis, suggesting that the low levels of expression determined by immunoblot translated into very low bait recovery by the TAP method (Fig. 7d).

This combination of very large cell input with sometimes very minimal protein recovery led to attempts by several groups to further optimize the TAP protocol. One such group, specifically seeking to optimize the tag coding sequence and composition for use in mammalian cells, developed a modified 'GS' tag consisting of Protein G subunits and a streptavidin moiety that was expressed at higher levels and resulted in an increase in recovery by an order of magnitude (Burckstummer et al., 2006). Given our issues with low expression and apparent negative selection against the substrate-trapping mutants of greatest interest to us, GS-tagged versions of the SHP-2 mutants were generated and cloned into a tet-inducible retroviral vector.

These tet-inducible mutants were then re-expressed in U937 cells and subjected to a slightly modified version of the TAP protocol, involving now a streptavidin binding peptide resin and biotin-based elution. Time course and dose response analysis of doxycycline exposure revealed maximum expression at 1 ug/ml and 48 hours, although expression of these fusion proteins was markedly higher at all time points than had been witnessed previously. As another unrelated line of investigation led us to suspect potentially high protease content in these monocytic cells, we sought to maximize bait expression by moving into two additional cell lines, the 3T3 line originally used for validation, and the human CML cell line K562, which we anticipated would have a high level of tyrosine phosphorylation from BCR-ABL expression. As expected, these lines demonstrated much higher levels of expression of the GS-tagged SHP-2 mutants, even showing low levels of expression in the uninduced state likely due to a leaky tet-responsive

a)



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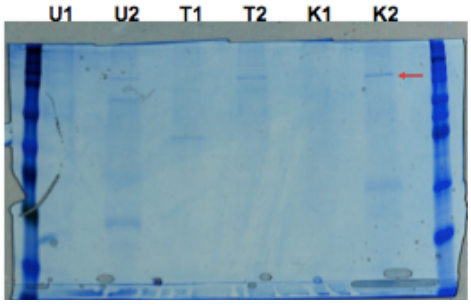

c)

Acc. #: Q06124 **Gene:** PTN11_HUMAN **Species:** HUMAN **Name:** Tyrosine-protein phosphatase non-receptor type 11

Protein MW: 68436.9 **Protein pI:** 6.9 **Protein Length:** 597

```
1   MTSRRWFHPN ITGVEAENLL LTRGVDGSFL ARPSKSNPGD FTLSVRRNGA VTHIKIQNTG DYYDLYGGEK FATLAELVQY
81  YMEHHGQLKE KNGDVIELKY PLNCADPTSE RWFHGHLSGK EAEKLLTEKG KHGSFLVRES QSHPGDFVLS VRTGDDKGES
161 NDGKSKVTHV MIRCQELKYD VGGGERFDSL TDLVEHYKKN PMVETLGTVL QLKQPLNTTR INAAEIESRV RELSKLAETT
241 DKVKQGFWEE FETLQQQECK LLYSRKEGQR QENKNKNRYK NILPFDHTRV VLHDGDPNEP VSDYINANII MPEFETKCNN
321 SKPKKSYIAT QGCLQNTVND FWRMVFQENS RVIVMTTKEV ERGKSKCVKY WPDEYALKEY GVMRVRNVKE SAAHDYTLRE
401 LKLSKVGQAL LQGNTERTVW QYHFRTWPDH GVPSDPGGVL DFLEEVHHKQ ESIMDAGPVV VHCSAGIGRT GTFIVIDILI
481 DIIREKGVDC DIDVPKTIQM VRSQRSGMVQ TEAQYRFIYM AVQHYIETLQ RRIEEEQKSK RKGHEYTNIK YSLADQTSGD
561 QSPLPPCTPT PPCAEMREDS ARVYENVGLM QQQKSFR
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Num Unique	% Cov	Best Disc Score	Best Expect Val
31	52.9	4.52	5.4e-7

Figure 8. tet-inducible GS-tagged SHP-2 constructs are expressed at higher levels. a) Time course of doxycycline induction reveals maximum expression at 48 hours in U937 cells. Dose response reveals maximum expression at 1ug/ml in 3T3 and K562 cells. 'ttigp' indicates empty vector. b) Coomassie staining of U937 (U), 3T3 (T), and K562 (K) cell lines. 1 and 2 refer to empty vector and 'KAS' respectively. c) Peptide coverage of SHP-2 by mass spectrometry.

element (Fig. 8a). TAP purification of empty vector- and 'KAS'-expressing cells from these lines demonstrate visible bait bands by Coomassie, efficient SHP-2 recovery, but once again, only contaminant recovery otherwise (Fig. 8b and 8c).

These data suggested that expression was not the only issue, since even when expressed at very high levels and recovered with reasonable efficiency, the SHP-2 bait appeared to be incapable of retaining its interactions with other proteins. Unlike the proteins identified in the original TAP publications which were components of relatively abundant and stable protein complexes, we sought to capture a signaling molecule in complex with a catalytic substrate that was likely far less abundant than the enzyme itself and whose initial binding and catalytic relationship was entirely phosphorylation-dependent. Upon closer consideration, it became clear that the unique nature of this protein interaction imposed some very real and unfavorable constraints, namely that the enzyme-substrate complexes we sought to isolate were likely inherently more labile and temporally-sensitive than those previously described. As such, the lengthy processing time required for the sequential rounds of purification and intervening TEV protease incubation became a significant concern.

Standard immunoprecipitation performed on a small scale with these same lysates using just the streptavidin portion of the tag revealed efficient recovery of the known binding partner, tyrosine phosphorylated Gab2 by immunoblot analysis, which further suggested that a quicker, simpler purification would facilitate the recovery of intact SHP-2-containing complexes. To this end, a large scale experiment was attempted using, again, only the streptavidin moiety for purification purposes and shortening the duration of the immunoprecipitation step, which had previously ranged from 3 hours to overnight for the Prot A/G and CBP/SBP incubations, to 30 and eventually 15 minutes. Furthermore, since the biotin elution is known to be

only about 50% efficient and additional losses are incurred during the TCA precipitation required prior to gel analysis, the specificity gained from this step was foregone in favor of the speed and maximal bait recovery afforded by boiling the SBP beads directly in SDS. Together these modifications not only greatly shortened the amount of time required for processing, but they also reduced the amount of input required by an order of magnitude (to 50e6 cells).

In order to maximize the availability of relevant phosphorylated substrates, the GS-tagged SHP-2 mutants were introduced into the GM-CSF dependent TF-1 human erythroleukemia cell line, which were starved and factor-stimulated immediately prior to purification. Initial experiments utilizing only the SHP-2 'KM' and 'KAS' mutants resulted in the successful identification of SHP-2 and a number of other potential proteins of interest by mass spectrometry (Fig. 9a). Subsequently, gel bands were excised immediately following staining and frozen prior to MS analysis to prevent diffusion into the destain solution (Fig. 9b). Finally, magnetic SBP beads were used in to eliminate the time and additional losses from centrifugation-based washes, and rather than being boiled in SDS and run out on a gel for band excision and digestion, the proteins were digested directly off the beads and prepared for MS analysis in solution. As expected, this greatly increased the yield of proteins identified, although at the expense of increased background from nonspecific binding. This strategy resulted in very high yield of recovered bait and an apparent increase in signal-to-noise as compared with either of the two previous methods.

The summary of the results from these 3 experiments is presented in Table 1. It should be noted that the background signal in these experiments was in large part

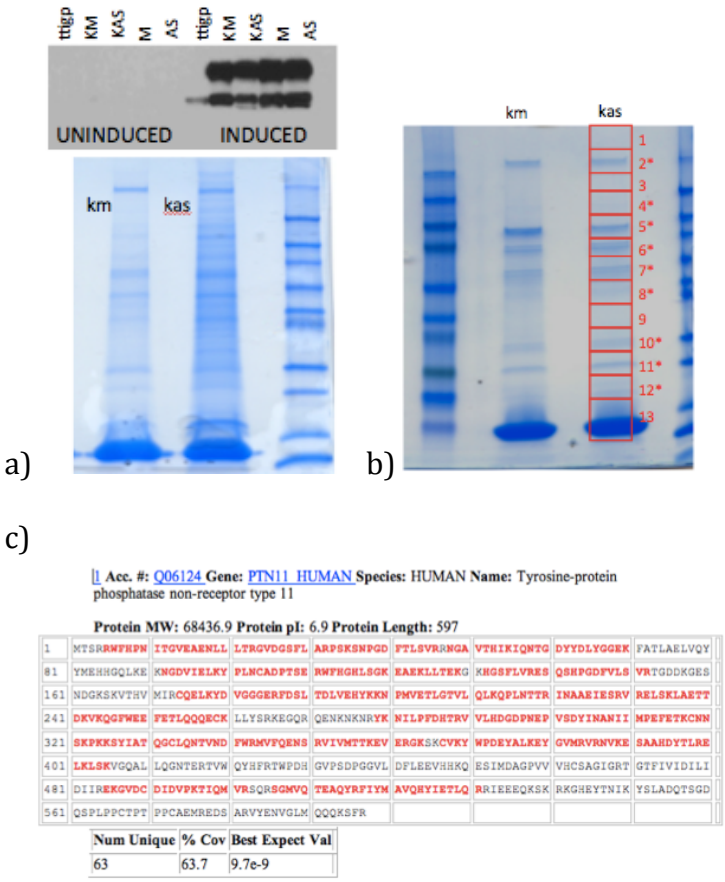


Figure 9. Shortened processing time and direct SDS elution improve recovery of putative substrates. a) Single-step SBP pulldown with 30 minute incubation and boiling of beads into SDS sample buffer. b) Same processing with gel bands excised and frozen before MS. c) Example of efficient SHP-2 peptide recovery with significant percent coverage.

related to the streptavidin moiety used for affinity purification. The tandem purification protocol and/or biotin elution would have contributed significantly to specificity in eliminating the signal from endogenous biotinylated proteins, but as yield was our priority, these proteins were manually disregarded, along with likely contaminants and high abundance proteins such as keratin and actin. However, it formally remains possible that some of these high abundance proteins, such as biotinylated glycolytic enzymes or members of the actin and tubulin family of

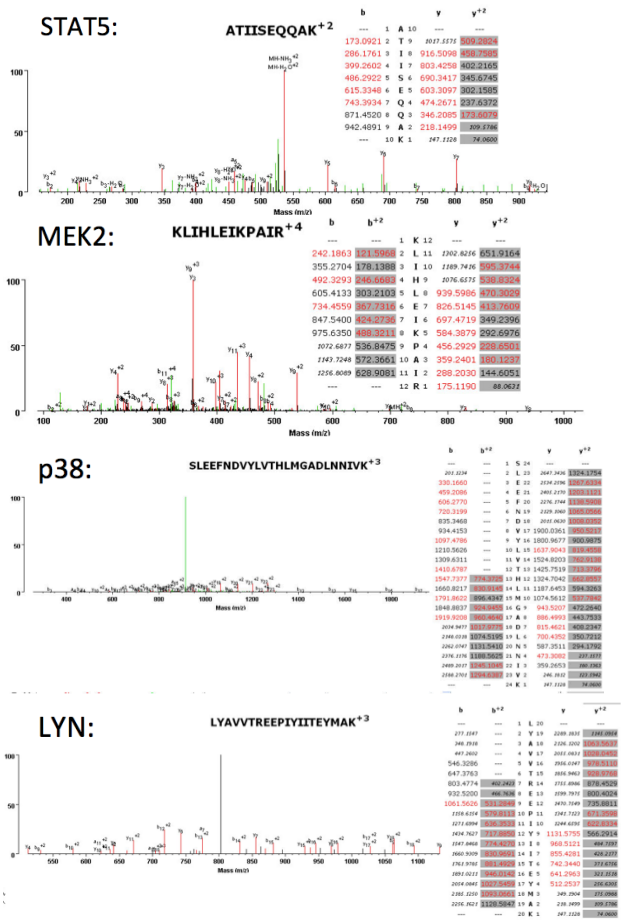
		KM		KAS	
Protein Name	Acc #	Num Unique	Peptide Count	Num Unique	Peptide Count
Tyrosine-protein phosphatase non-receptor type 11	Q06124	102	346	77	140
GRB2-associated-binding protein 1	Q13480	3	5	0	0
GRB2-associated-binding protein 2	Q9UQC2	5	5	2	0
GRB2-associated-binding protein 3	Q8WWW8	8	8	4	4
Signal transducer and activator of transcription 5A	P42229	1	1	5	4
Signal transducer and activator of transcription 3	P40763	0	0	6	7
Signal transducer and activator of transcription 1-alpha/beta	P42224	0	0	1	1
Serine/threonine-protein kinase PAK 2	Q13177	3	6	6	8
Mitogen-activated protein kinase 14	Q16539	1	2	5	8
Dual specificity mitogen-activated protein kinase kinase 2	P36507	1	2	3	3
Tyrosine-protein kinase Lyn	P07948	3	4	4	4
Cyclin-dependent kinase 1	P06493	8	12	10	10
Cyclin-dependent kinase inhibitor 2A, isoforms 1/2/3	P42771	2	3	3	3

Table 1. Summary of top candidates from MS analysis. 'Peptide Count' indicates total number of peptides recovered, while 'Num Unique' indicate number unique peptides.

proteins, could be genuine substrates. It was reassuring to pull out known SHP-2 binding partners such as Gab family of proteins, however, whether or not this interaction is catalytic or structural could not be determined by differential binding to the mutants. Other proteins of note were the STAT family of signal transduction molecules, MEK2, PAK2, p38, LYN, and CDK1.

Examination of peptide sequence traces identified from the putative substrates strongly suggested that these were highly unlikely to be non-specific peptide fragments (Fig. 10a). Attempted verification of these MS-identified binding partners was complicated by the fact that the IgG-based component of the GS tag cross-reacted with various antibody and detection reagents, thus making the GS-tagged SHP-2 mutants unsuitable for immunoblot validation. In order to preserve the specificity achieved by use of an affinity tag for the substrate-trapping (as opposed to endogenous) SHP-2 proteins, a FLAG epitope was added to the C-terminus of the various SHP-2 coding mutants and cloned into the same tet-inducible vector used previously. These FLAG SHP-2 mutants were introduced into

a)



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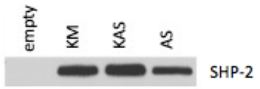


c)

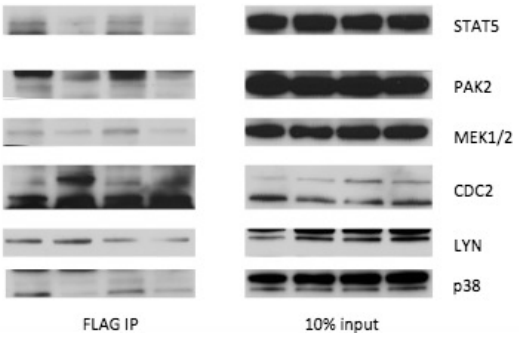


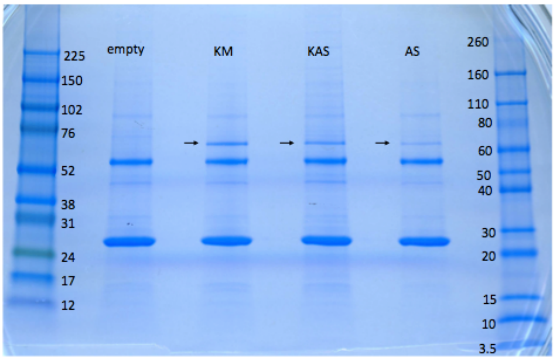
Figure 10. FLAG validation of putative interacting proteins. A) Example of peptide traces from proteins listed in Table 1 demonstrating specificity. B) Expression of FLAG-SHP2 mutants in TF-1 cells. c) FLAG immunoprecipitated and endogenous levels of putative substrate proteins.

the same TF-1 cell lines used for initial IP-MS analysis and demonstrated strong expression that was similar across mutants (Fig. 10b). Initial immunoblot analyses using antibodies directed against a number of MS 'hits' of interest demonstrated their expression in total lysate preparations across all cell lines, making them physiologically relevant proteins of interest, however anti-FLAG immunoprecipitation failed to demonstrate enrichment in pulldown by the SHP-2 mutant lines as compared with empty vector (Fig. 10c). However, low-level expression of each of these proteins (presumably background) was detectable across all lanes, making it difficult to rule these out as potential substrates. Other groups have found exogenous expression of both 'bait' and interacting protein(s) to be necessary to reproduce interactions identified via mass spectrometry, and given the likely scarcity of these phosphorylated substrates, these results indicate that further validation steps will require co-transfection with cDNAs encoding these proteins of interest.

Interestingly, large-scale purification of cells expressing the FLAG-tagged SHP-2 mutants and subsequent MS analysis resulted in very clear identification of bait on a Coomassie stained gel and correspondingly high peptide recovery for SHP-2 by mass spectrometry (Fig. 11a). These results were free of the biotinylated proteins, but still a large number of proteins were identified, a significant proportion of which were likely background. However one particular hit, parafibromin, demonstrated both significant peptide recovery and a recovery pattern consistent with that expected for a specific SHP-2 substrate in that it was absent from the empty vector preparation and enriched in the 'KAS' and 'AS' lanes

as compared with the hypothetically non-substrate binding 'KM' and 'M' preparations (Fig. 11b). Furthermore, unlike the putative substrates identified by the streptavidin-based affinity purification, interaction of the substrate trapping SHP-2 mutants with endogenous parafibromin was verified by immunoblotting and demonstrated a similar pattern to that seen for peptide recovery by MS (Fig. 11c). This interaction is of particular interest because it was recently identified by another group using a similar IP-MS-based approach looking for substrates of a different activating mutant of SHP-2 (T507K) identified from a hepatocellular carcinoma (Takahashi et al., 2011). They found parafibromin as well as another component of the RNA polymerase II-associated factor (PAF) complex in their myc-based affinity purification in 3T3 cells, but interestingly found that only parafibromin was specifically dephosphorylated by SHP-2. They proposed a mechanism by which SHP-2-mediated dephosphorylation of parafibromin resulted in the ability of parafibromin to stably bind beta catenin, resulting in accumulation of beta catenin in the nucleus and activation of downstream Wnt genes including cyclin D1 and c-myc. While diverging from the central role of Ras activation in SHP-2 mediated pathogenesis, this model provides a mechanism by which SHP-2's catalytic activity could contribute to oncogenesis. Experiments are underway to look at activation and nuclear accumulation of beta catenin in order to determine whether this mechanism is also operative in the transforming ability of the JMML mutant SHP2 E76K in hematopoietic cells.

a)



b)

	mock				Shp2_KM				Shp2_KAS				Shp2_AS			
Protein Name	Num Unique	Peptide Count	% Cov	Best Expect Value	Num Unique	Peptide Count	% Cov	Best Expect Value	Num Unique	Peptide Count	% Cov	Best Expect Value	Num Unique	Peptide Count	% Cov	Best Expect Value
Tyrosine-protein phosphatase non-receptor type 11	2	4	3.4	3.40E-04	69	818	64	1.50E-09	63	680	63.1	1.70E-09	50	350	59	3.60E-08
Parafibromin	1	1	1.9	0.0042	0	0	0	0	23	45	38.6	5.30E-05	11	19	21.5	1.70E-04

c)

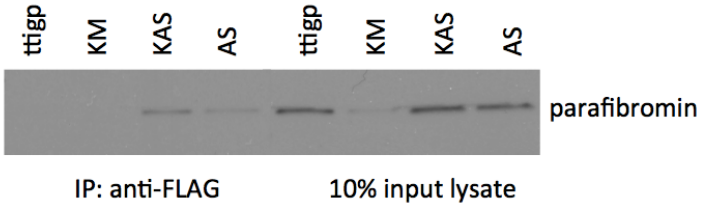


Figure 11. Parafibromin interacts specifically with substrate-trapping mutants of SHP-2. a) Coomassie stained gel of FLAG IP-MS of SHP-2 mutants. b) Peptide recovery of parafibromin is enriched in substrate-trapping samples. c) Western blot of FLAG immunoprecipitated lysate confirms interaction with endogenous parafibromin.

Discussion

Despite our best intentions in attempting to apply the most rigorous and advanced methodologies to aid in the nearly decade-old question of how SHP-2 contributes to leukemogenesis, ultimately the biological constraints proved to trump the technological, confirming the elusive nature of these critical effectors. Due to the unique role of SHP-2 as a PTP with a positive role in signaling, the dominant negative effect of the substrate trapping 'bait' was difficult for cells to tolerate and resulted in reduced expression over time. While problematic in its own

right, this proved particularly troublesome for substrate identification, as SHP-2 is known to be present well in excess of its tyrosine-phosphorylated binding partners, thus requiring that the tagged bait also be expressed well in excess of what might be required for isolation of stoichiometric binding partners in order to 'trap' sufficient quantities of its putative substrates to be detectable by mass spectrometry. Furthermore, the added stringency provided by the tandem affinity purification, though advantageous for a detection method as sensitive to noise as mass spectrometry, came at the expense of lengthy processing time and loss in recovery at every step, all the while the inherent lability of the enzyme-substrate complex and degradation-sensitivity of the phosphorylation modifications necessary for all binding interactions, catalytic and otherwise, made speed a priority for maximal recovery of low-abundance substrates. In hindsight the initial attempts in U937 cells, which were discovered later to have a weakly activating mutation in *PTPN11*, may have been hampered not only by high protease content but perhaps also by increased competition from a hyperactive endogenous enzyme.

Burckstummer et al., in their optimization of the GS tag for mammalian cell expression, actually calculated the degree of loss incurred at every step (Burckstummer et al., 2006). They speculated that depending on the bait, the starting cell number ranged from 5x10^7 to 1x10^9, with the lower end of that range being typical for HEK293 cells. This corresponds to a total cell lysate yield of approximately 35 mg of total protein, with stable expression of a TAP-tagged protein by retroviral transduction yielding roughly 700pmol of bait. They calculated that even with their optimized GS tag, only about 40% of this was bound

up in the first round of affinity purification, and 30% thereof after TEV cleavage, with the second round being the most efficient and resulting in the recovery of 75% of the remaining material. They found the biotin elution step was the most variable, with an average of 50% recovery, resulting in an overall bait recovery of 5% from the original lysate, corresponding to an absolute protein yield in the picomolar range. This degree of inefficiency is clearly suboptimal even under the best of circumstances, but even more so when coupled with the relative scarcity and transience of SHP-2-containing enzyme-substrate complexes that are formed in response to growth factor-mediated phosphorylation events and subject to appreciable turnover given half-lives that, despite being extended by the addition of substrate-trapping mutations, are non-negligible relative to the lengthy processing times required.

Furthermore, the 5% recovery, which amounts to roughly 35 pmol in the original example of HEK293 cells, refers to the amount of purified bait recovered, to say nothing of the relative recovery of its interacting proteins. In their calculations the authors consider a stoichiometry of interacting proteins ranging from 2:1 to 1:20, with SHP-2 likely falling on the latter side of that spectrum, and estimate potential co-purification yields between 70 and 1.5pmol, with the latter figure representing the lower limit of detectability by silver stain and LC MS-MS from a relatively complex sample. For all its theoretical advantages, the technical limitations of this approach proved to be incompatible with the biological parameters of our question, thus forcing us to make concessions on several levels to increase yield at the expense of specificity. The replacement of a constitutive MSCV-

based vector with a tet-inducible promoter helped overcome some of the negative selection brought on by expression of the dominant-negative substrate-trapping mutant and increase bait expression, as did switching from the original yeast AC tag to the mammalian-optimized GS tag. These modifications resulted in high enough expression levels across several different cell lines to yield consistent and reproducible detection of SHP-2 by MS, with reasonable peptide coverage, but still without any other interactors. As such, further sacrifices in specificity were made with an eye toward speed and yield.

Single-step purification was opted for over the tandem approach, as well as quick resuspension/centrifugation-based washes and rather than flow through column chromatography, with magnetic beads ultimately removing the need even for centrifugation. Given the documented variability and inefficiency of the final biotin elution, elution was instead performed by boiling in SDS and gel analysis, with eventually even the latter aspect being forgone in favor of direct in-solution digestion and MS analysis, thus leaving the mixture more complex in order to prevent the losses to gel electrophoresis and storage. The IgG reactivity of the GS tag necessitated generation of FLAG-tagged versions of all of our 'bait' protein, which were then subjected to similarly abbreviated processing, producing increased noise but also an increase in apparent signal. The protein 'hits' resulting from the peptides recovered by MS analysis of these, quite literally, 'quick and dirty' sample preparations revealed several making repeat appearances that were potentially interesting and biologically relevant.

McDaniel et al. demonstrated that the p21 activated kinase (PAK) family of proteins might play a role in Ras activation of MAPK, as Pak1-/- mice crossed with Nf1+/- mice resulted in an attenuation of the increased proliferation and elevated pERK seen in Nf1 heterozygous mast cells (McDaniel et al., 2008). Interestingly, they also implicated p38 and MEK, both recovered by our peptide analysis, in this pathway. MEK was implicated as a target of PAK1 kinase activity, with loss of Pak1 resulting in decreased phosphorylation of MEK at serine 298, which is thought to be important for 'priming' the interaction between Mek1 and Raf-1 and resulting in increased Raf-1 directed phosphorylation of MEk1 at Ser 217/221. p38 inhibitor SB203580 was found to attenuate SCF-induced migration of wild-type and Nf1 heterozygous mast cells, but did not further reduce migration of *Pak1*$^{-/-}$ or *Nf1*$^{+/-}$;*Pak1*$^{-/-}$ mast cells, suggesting that Pak1 and p38 were acting via the same pathway. Lyn and Cdk1 were also intriguing given the implication of Src family kinases downstream of SHP-2 in various contexts, with Lyn being suggested as a potential substrate in the hematopoietic compartment (Futami et al., 2011). Cdk1 is a known target of SFKs and crucial for proper cell cycle regulation, which is of obvious importance for the growth-factor independent cell survival and proliferation observed in leukemia. Cdk1 has also been demonstrated to play a role in the endomitosis (DNA replication without cell division) undergone in polyploidy megakaryocytes. The role of Lyn and megakaryopoiesis downstream of SHP-2 will be discussed further in a subsequent chapter.

STAT5 was also of obvious biological relevance for leukemia and pSTAT5 was unexpectedly found to be elevated in U937 cells upon SHP-2 knockdown (data

not shown), consistent with it being a potential substrate. However this is contrary to work by Kotecha et al. suggesting that hyperphosphorylation and activation of STAT5 was a hallmark of JMML (Kotecha et al., 2008). The complexity of this relationship is highlighted by the fact that another group had previously reported STAT5 to be a substrate of SHP-2 downstream of IL-3 receptor signaling in murine Ba/f3 cells, but under most perplexing circumstances. Although SHP-2 was found to play an essential role in IL-3 signaling in Ba/f3 cells (Yu et al., 2003), overexpression of SHP-2 was found to result in increased Jak2 activation but decreased phosphorylation of STAT5 and the expression of its downstream targets Bcl-xl and pim-1, suggesting SHP-2 could also play a negative role in hematopoietic signaling by dephosphorylating STAT5 (Chen et al., 2004). Consistent with this, overexpression of SHP-2 in primary bone marrow was found to compromise progenitor differentiation and proliferation and enhance cell death induced by growth factor. However, since this stood in direct contrast with the supposed gain-of-function effects of SHP-2 mutations identified in leukemia, this same group introduced both SHP-2 E76K and wild-type SHP-2 into mice and found that while the former caused MPD, as had been previously published, overexpression of wild-type SHP2 decreased hematopoietic potential of the transduced cells in recipient animals. Furthermore, despite increased catalytic activity, STAT5 dephosphorylation was decreased in the context of SHP-2 E76K (Yu et al., 2006), which the authors suggested reflected that the oncogenic effect of SHP-2 E76K was related to more than its catalytic ability.

Once identified, our first goal was to validate putative substrates by conventional targeted approaches, namely immunoprecipitation and/or immunoblotting using specific antibodies to demonstrate direct binding to the substrate-trapping Shp2 mutant. Although we were unable to detect phosphorylated forms of the endogenous proteins binding specifically to the substrate trapping mutant for most of our 'hits,' often times co-expression of PTP and substrate are required as this interaction can be limited by the abundance of the latter as well as stoichiometric constraints, so an enzyme-substrate relationship cannot be ruled out until those experiments have been performed. However, for parafibromin, the endogenous interaction was found to bind to the substrate trapping mutant, confirming the work of Takahashi et al. in validating this as a substrate in 3T3 cells. This would be the first demonstration of this interaction in hematopoietic cells in the context of the JMML mutant SHP-2 E76K.

Returning to the Tiganis and Bennett criteria for PTP substrate identification, once direct binding to a substrate trapping mutant has been confirmed, functional validation in the form of increased/decreased phosphorylation of the target protein in response to knockdown/overexpression of Shp2 would be required to demonstrate that binding were not just an artifact of overexpression or representative of an adaptor-type interaction. As we have already generated the necessary reagents for both experiments, that is, SHP-2 knockdown and expression of a SHP-2 hypermorph (E76K), the determination of parafibromin or any of the other putative substrates would require demonstration of increased phosphorylation in the former case and decreased phosphorylation in the latter as

compared with wild-type. Demonstration of direct dephosphorylation in an in vitro assay would be the final criteria, with purified SHP-2 being mixed with the phosphorylated substrate and phosphorylation monitored using anti phosphotyrosine antibodies or release of radiolabelled phosphate, as this would eliminate the potential for an indirect relationship in which both were part of a complex but not an enzyme-substrate pair. However, as discussed previously, this method is thought to be most prone to overinterpretation, and particularly in the case of SHP-2 could prove problematic as it would require that the substrate also be responsible for providing the phosphotyrosine-containing ligand to engage the autoinhibitory SH2 domains, or else the assay would need to be performed with an isolated catalytic domain, which has been demonstrated to be a much more promiscuous assay given the relaxed constraints accompanying loss of the regulatory domains.

However, in the case of SHP-2, we have a much more stringent criteria which is that the identified substrate account for the biological function in question, namely, the ability of SHP-2 E76K to transform hematopoietic cells. The CFU-GM assay is the ideal readout for this purpose, as factor-independent growth and GM-CSF hypersensitivity are considered hallmarks of JMML, and the substrate-trapping, catalytically inactive C459S and 'AS' (C459S+D425A) mutations have already been demonstrated to abolish this JMML-like in vitro phenotype. Constitutive dephosphorylation of the genuine SHP-2 substrate in this disease, then, should phenocopy the effect of expressing the hyperactive SHP-2 E76K mutant. If SHP-2 E76K were to be co-expressed with an shRNA directed against the substrate and

then plated in a CFU-GM assay to look for modulation of the GM-CSF hypersensitivity, formally speaking, there would be two potential outcomes: if the substrate is a positive regulator of growth signaling that is activated by dephosphorylation, then knocking it down should abrogate the GM-CSF hypersensitivity phenotype. If the substrate is a negative regulator of growth signaling that is inactivated by dephosphorylation, then knocking it down should confer GM-CSF hypersensitivity even in the presence of wild-type Shp2. The substrate could also be overexpressed, but the expected outcomes would depend on the stoichiometry of the substrate with the Shp2 phosphatase, as well as that of the substrate with its relevant kinase. One would also have to consider in that case any potential confounding effects of expressing non-physiologic levels of the substrate protein on its own, if that were alone sufficient somehow to affect proliferation or survival. Furthermore, modulating the expression level of the substrate may not accurately reflect the effect of modulating its phosphorylation status.

The most specific assay, to that effect, would be to mutate the phosphorylated tyrosine residue(s) on the putative substrate to a phenylalanine to mimic its dephosphorylated state, or conversely to a negatively charged residue like glutamate to mimic its phosphorylated state, though this latter substitution would not of course be capable of reproducing any phosphotyrosine-dependent binding interactions. However, biological context is very useful for these purposes, as, for instance, the proposed role of parafibromin in regulating beta-catenin would suggest that expression of a constitutively activated beta catenin should also phenocopy the CFU-GM behavior of SHP-2 E76K. Furthermore, identification of a

novel pathway rather than a novel substrate could prove to be of greater clinical value as an opportunity for therapeutic intervention.

Materials and Methods

SHP2 constructs. Murine *PTPN11* cDNAs encoding the E76K and C459S substitutions were cloned into the pENTR2B entry vector as described by Schubbert et al. (ref??). *PTPN11* constructs encoding the additional D/A and R/M substitutions were similarly generated by site-directed mutagenesis and introduced into pENTR2B, with restriction enzyme digestion and ligation used to generate the various combinations indicated. These constructs were then transferred from the entry clones into Gateway-modified MSCV-IRES-GFP (MIG-GW) or MSCV-IRES-puromycin (MSCV-puro) retroviral expression vectors using Gateway LR Klonase II (Invitrogen, Grand Island, NY). shRNA sequences targeting human *PTPN11* were obtained from the laboratory of Dr. Scott Lowe (CSHL) and cloned into their mir30-based LMS expression vector, which contains a GFP marker for selection. Sequences were as follows (target sequence in bold):

1936:

TCGAGAAGGTATATTGCTGTTGACAGTGAGCGAC**AGCATTATATTGAAACACT**ATAGT

GAAGCCACAGATGTATAGTGTTTCAATATAATGCTGGTGCCTACTGCCTCGG

2403:

TCGAGAAGGTATATTGCTGTTGACAGTGAGCGAT**ACAATGTAGACAACATTAT**ATAGT

GAAGCCACAGATGTATATAATGTTGTCTACATTGTAGTGCCTACTGCCTCGG

The myr-CD construct was generated by direct primer-mediated amplification of the catalytic domain of *PTPN11* with addition of the 14 amino acid myristyl sequence MGSSKSKPKDPSQR derived from v-src to the N-terminus of the PTP domain and addition of a stop codon to the C-terminus. Primer sequence as follows:

5' myrCD fwd:

GATCGTCGACATGGGCAGCAGCAAGAGCAAGCCCAAGGACCCCAGCCAGAGAATCAATGC

TGCTGAA

FLAG constructs and purification: FLAG-SHP-2 constructs were generated by primer-mediated amplification and addition using the following reverse primer: 5'-GACAGGAATTCCTACTTATCGTCGTCATCCTTGTAATCTCTGAAACTCCTCTG, with the underlined portion encoding the FLAG peptide sequence: DYKDDDDK. FLAG immunoprecipitation was performed using Anti-FLAG M2 affinity gel (Sigma-Aldrich, St. Louis, MO) according to manufacturer's instructions for 15 minutes in the cold room, followed by SDS gel electrophoresis and Western blot detection using a murine FLAG M2 monoclonal antibody (Sigma-Aldrich). Later, Anti-FLAG M2 magnetic beads (Sigma-Aldrich) were used, also according to manufacturer's instructions.

shSHP2. shRNA sequences targeting human *PTPN11* were obtained from

and cloned into their mir30-based LMS expression vector, which contains a GFP marker for selection. Sequences were as follows (target sequence in bold):

1936:

TCGAGAAGGTATATTGCTGTTGACAGTGAGCGAC**AGCATTATATTGAAACACT**ATAGT

GAAGCCACAGATGTATAGTGTTTCAATATAATGCTGGTGCCTACTGCCTCGG

2403:

TCGAGAAGGTATATTGCTGTTGACAGTGAGCGAT**ACAATGTAGACAACATTAT**ATAGT

GAAGCCACAGATGTATATAATGTTGTCTACATTGTAGTGCCTACTGCCTCGG

TAP tag constructs. Both N- and C-terminal versions of the yeast 'AC' TAP cassette were generously provided by the laboratory of Dr. Frank McCormick. The NTAP and CTAP tag sequences were cloned from these cassettes and fused to the appropriate termini of the pENTR2B SHP-2 mutant (KM, KAS, M, AS) constructs before being recombined into the MIG-GW retroviral expression vector. The NTAP tag was also fused to a frameshifted sequence containing a four base pair insertion at the 5' end of the Shp2 coding sequence (fs) in order to control for non-specific binding. The C-terminal 'GS' TAP cassette was obtained with permission from the Center for Molecular Medicine (pCEMM-CTAP-GS) and the tag sequence cloned into a tet-inducible retroviral expression vector generously provided by the laboratory of Dr. Scott Lowe (TTIGP). *PTPN11* mutant cDNA sequences (KM, KAS, M, AS) were then cloned into the resulting TTIGP-GS vector, which contains a tet-responsive element (TRE) upstream of the cDNA cloning site in addition to both GFP and puromycin selectable markers. Use of these tet-inducible vectors required prior stable

transduction of a retroviral vector encoding the reverse tet transactivator and ecotropic receptor under a neomycin selection marker (RIEN). Expression was induced by the addition of sterile-filtered doxycycline at a final concentration of 1 ug/ml for 24-72 hours, as indicated. Puromycin selection was performed at final concentration of 2ng/ml and neomycin with a final concentration of 1 mg/ml Geneticin solution (Invitrogen).

Cell lines: U937 and TF-1 cell lines were obtained from ATCC. K562 cells were generously provided by the laboratory of Dr. Neil Shah, and 3T3 and 293T from Dr. Kevin Shannon. U937 and K562 cells maintained in RPMI 1640 (UCSF CCF formulation) with 10% filtered, heat inactivated fetal bovine serum (Hyclone) and penicillin/streptomycin/glutamine. TF-1 cells were maintained in this same base media with the addition of fresh human GM-CSF at a final concentration of 2 ng/ml (Peprotech, Rocky Hill, NJ). 293T and 3T3 cells were maintained in DME-H21 (UCSF CCF formulation) with 10% filtered, heat inactivated FBS and penicillin/streptomycin/glutamine. Primary murine cells were collected in IMDM with 20% FBS with penicillin/streptomycin/glutamine. All human cell lines were modified to express the ecotropic receptor either with a vector containing the ecotropic receptor alone and selected by puromycin (ecoR-puro) or neomycin (RIEN).

Retroviral transduction: Briefly, 293T cells were plated at ~80% confluence in 10 cm tissue-culture treated plates one day prior to transfection. Transfection was

performed using Lipofectamine 2000 reagent using 10ug each of Ecopac and DNA construct(s). The transfection mixture was prepared according to instructions and incubated for 30 minutes before being added to the 293T cells. The Lipofectamine-containing 293T media was replaced with fresh media after 24 hours, at which time recipient cells were plated at a density of 0.5-1e6 cells per ml in 24-well Falcon tissue-culture plates. The viral supernatant was collected 48 hours post-transfection using a 10 ml syringe and passed through a 0.45 micron filter. Polybrene was added to the viral supernatant at a final concentration of 5 ug/ml for primary murine cells and 10 ug/ml for cell lines. Recipient cells were centrifuged in a tabletop Beckmann centrifuge at 2100 rpm for 5 minutes and their media replaced with 2 ml/well of viral supernatant. Spinfection was then carried out at 2100 rpm for 1.5 hours followed by 2 hours of incubation at 37deg, after which the virus was removed and replaced with fresh media. GFP analysis and/or sorting occurred 48 hours post-spinfection on BD LSRII or FACS Aria machines, respectively.

Primary cells: For experiments involving murine fetal liver cells, E14.5 embryos were harvested from pregnant wild-type C57Bl/6 mice and fetal livers isolated and homogenized as described (Birnbaum et al. 2000). Fetal liver cells were cultured in stimulation media containing StemSpan SFEM (StemCell Technologies, Vancouver, BC), 15% FBS, 100 ng/ml stem cell factor (SCF) (Peprotech), 50 ng/ml FLT-3 ligand (Peprotech), 100 ng/ml IL-11 (R&D Technologies, Minneapolis, MN), 50 ng/ml IL-6 (Peprotech), penicillin, streptomycin, and glutamine for 48 hours prior to spinfection.

Colony assays: After transduction, murine fetal liver cells were sorted directly into methylcellulose medium (M3231, Stem Cell Technologies) supplemented with IMDM, betamercaptoethanol, penicillin, streptomycin, and glutamine. Recombinant murine GM-CSF (Peprotech) was then added at increasing doses up to a maximum final concentration of 10 ng/ml. For each GM-CSF dose and condition, 10,000 GFP cells were sorted into 1.2 ml of media and plated in 35mm untreated tissue culture plates. After 7 days of incubation, the colonies (CFU-GM) were counted by microscopy.

Western blot analysis: Cells were pelleted and lysed in solution containing 1% NP-40; 50 mM Tris-HCl, pH 7.4; 2 mM EDTA; 30 mM NaF; 30 mM β-glycerophosphate; 20 mM $Na_4P_2O_7$; 1 mM Na_3VO_4; and Complete protease inhibitor cocktail (Roche, Basel, Switzerland). The protein concentration was determined using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). 50 ug lysate was separated on a 10% polyacrylamide gel (Bio-Rad, Hercules, CA) and transferred to an Immobilon-P PVDF membrane (Millipore, Billerica, MA). Membranes were blocked overnight at 4°C in TBS-T (Tris-buffered saline, 0.05% Tween-20) with 5% BSA or milk, then incubated with primary antibodies in fresh blocking solution for 180 minutes at room temperature and washed in TBS-T with 0.15% Tween-20. Secondary detection was achieved by incubating with horseradish peroxidase–conjugated donkey anti–rabbit (DAKO USA, Carpinteria, CA) or anti-mouse (GE Healthcare Life Sciences, Pittsburgh, PA) antibodies for 50 minutes at room

temperature followed by washing and enhanced chemiluminescence (ECL) detection (GE Healthcare Life Sciences).

Antibodies: Western blotting experiments were performed using antibodies to: pErk T202/Y204 (Cell Signaling Technology, cat. #9101), pGab2 Y452 (Cell Signaling Technology, cat. #3882), Lyn (Cell Signaling Technology, cat. #2796), Cdc2 (Cell Signaling Technology, cat. #9116), CDC73/parafibromin (Cell Signaling Technology, cat. #8126), Stat5 (Cell Signaling Technology, cat. #9358), Mek1/2 (Cell Signaling Technology, cat. #8727), Pak2 (Cell Signaling Technology, cat. #2615), p38 (Cell Signaling Technology, cat. #9228), Shp2 (PTP1D) (BD Biosciences, cat. #610621) Flow-cytometry detection was performed using a directly conjugated pSTAT5-Alx647 Y694 antibody (BD Biosciences, cat. # 612599).

Flow cytometric analysis of phospho-proteins: Following stimulation, cells were fixed with paraformaldehyde at a final concentration of 2% (16% ampules; Electron Microscopy Sciences, Hatfield, PA) at room temperature for 10 minutes. The cells were then washed twice with PBS, permeabilized with ice-cold 95% methanol (Electron Microscopy Sciences) while vortexing, placed on ice for 20 minutes, and then stored at −20°C at minimum overnight and up to 2 weeks. Upon removal from storage, the samples were washed 3 times with PBS and incubated at 4°C for 1 hour in FACS buffer (Hanks balanced salt solution containing 4% FBS; Hyclone, Logan, UT). The cells were pelleted and then incubated with anti-CD16/CD32 (murine Fc receptor) antibody (2.4G2; BD Biosciences, San Jose, CA) on ice for 15 minutes.

Unconjugated primary antibodies were added at optimized concentrations and incubated at room temperature for 30 minutes. Samples were washed once with FACS buffer. Secondary and directly conjugated antibodies were added at optimized concentrations and incubated in the dark at room temperature for 30 minutes. Samples were washed once with PBS and analyzed on an LSRII flow cytometer (BD Biosciences) equipped with 488-nm and 633-nm lasers. Data were collected using DIVA software (BD Biosciences) and analyzed using FlowJo (Tree Star, Ashland, OR).

TAP purification: Cells were lysed in 1% Triton buffer (1% Triton X-100, 25mM Tris pH 7.5, 150mM NaCl, 1mM EDTA,1mM EGTA, 20mM NaF, 1mM NaVO4, 1mM DTT, and complete mini protease inhibitor tablet-Roche). Pooled lysates were purified as described by Gavin et al. (Gavin, Bosche et al. 2002), concentrated by TCA precipitation, separated on a Criterion TGX 4-12% gel (Bio-Rad) , and stained with SimplyBlue SafeStain (Invitrogen). Excised gel bands were digested with trypsin and analyzed by tandem mass spectrometry. In-gel digestion and mass spectrometry were performed by Juan Oses of the laboratory of Al Burlingame (UCSF) as described below.

In-gel digestion. Gels were cut along the lanes in several pieces, and proteins were digested in-gel with trypsin as described previously (J. Rosenfeld, J. Capdeveille, J.C. Guillemot and P. Ferrara. Anal. Biochem. 203 (1992), pp. 173–179) (donatello.ucsf.edu/ingel.html). Briefly, gel slices were diced into small pieces (1 mm^2) and washed three times in 50% MeCN 20mM NH_4HCO_3. Gel pieces were

allowed to rehydrate in a solution containing 10 mM DTT 20 mM NH_4HCO_3 in water, and then incubated at 56C for 60 min. The supernatant was removed, and gel slices were covered with 55 Mm Iodoacetamide in 20 mM NH_4HCO_3 and incubated for 45 min at RT. After this, samples were washed again 3 times in 50% MeCN 20mM NH_4HCO_3, and then gel pieces were evaporated to dryness under vacuum. 10 ul of a 20 mM NH_4HCO_3 solution containing 100 ng sequencing grade modified trypsin (Promega, Madison, WI) was added per gel slice. 20mM NH_4HCO_3 was then added as needed to cover the gel pieces. Digestion was allowed to proceed overnight at 37C. Product peptides were extracted from the gel pieces by incubation with 50 % MeCN 5% formic acid in water. The extracted digests were vacuum-evaporated and resuspended in 20 µl 0.1% formic acid in water. Peptides were extracted using C18 ZipTips (Millipore) according to the manufacturer's protocol. Eluates of the ZipTips were vacuum-evaporated and peptides resuspended in 10 µl 0.1% formic acid in water.

Reverse-phase LC-MS/MS Analysis. The digests were separated by nano-flow liquid chromatography using a 75-µm x 150-mm reverse phase 1.7 µm BEH 130 C18 column (Waters) at a flow rate of 600 nL/min in a NanoAcquityTM Ultra performance UPLC system (Waters). Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. Following equilibration of the column in 5% solvent B, an aliquot of each digest (5 µl) was injected, then the organic content of the mobile phase was increased linearly to 40% over 60 min, and then to 50% in 1 min. The liquid chromatography eluate was coupled to a hybrid linear ion trap-Orbitrap mass spectrometer (LTQ-Orbitrap Velos, Thermo Scientific,

San Jose, CA) equipped with a nanoelectrospray ion source. Spraying was from an uncoated 15-μm-inner diameter spraying needle (New Objective, Woburn, MA). Peptides were analyzed in positive ion mode and in information-dependent acquisition mode to automatically switch between MS and MS/MS acquisition. MS spectra were acquired in profile mode using the Orbitrap analyzer in the m/z range between 300 and 1800. For each MS spectrum, the 6 most intense multiple charged ions over a threshold of 1000 counts were selected to perform CID experiments. Product ions were analyzed on the linear ion trap in centroid mode. The CID collision energy was automatically set to 25%. A dynamic exclusion window of 0.5 Da was applied that prevented the same m/z from being selected for 180 s after its acquisition.

Peaklists were generated using in-house software based on the Raw_Extract script from Xcalibur v2.4 (Thermo Fisher Scientific, San Jose, CA). The peak lists were searched against the human subset of the SwissProt database as of July 6, 2011 containing 20238 entries using in-house ProteinProspector version 5.8.0 (a public version is available on line). A randomized version of all entries was concatenated to the database for estimation of false discovery rates in the searches. Peptide tolerance in searches was 20 ppm for precursor and 0.8 Da for product ions, respectively. Peptides containing two miscleavages were allowed. Carbamidomethylation of cysteine was allowed as constant modification; acetylation of the N terminus of the protein, pyroglutamate formation from N terminal glutamine and oxidation of methionine, were allowed as variable modifications. The number of modification was limited to two per peptide. Protein

hits were considered significant when two or more peptide sequences matched a protein entry and the Prospector score was above the significancy level. A minimal ProteinProspector protein score of 20, a peptide score of 15, a maximum expectation value of 0.05 and a minimal discriminant score threshold of 0.0 were used for initial identification criteria. For identifications based on one peptide sequence with high scores, the MS/MS spectrum was reinterpreted manually by matching all the observed fragment ions to a theoretical fragmentation obtained using MS Product (Protein Prospector) (Clauser et al., 1999).

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Chapter 3:

Role of Src Family kinases in SHP-2 mediated transformation and signaling

Introduction

The catalytic activity of the JMML-associated mutant SHP-2 E76K is known to be required for transformation of hematopoietic progenitors, but the key substrates that are dephosphorylated to promote leukemogenesis are unknown. In addition to the substrate-trapping approach already described, Tiganis and Bennett also described in their review on protein tyrosine phosphatase (PTP) substrate identification the importance of demonstrating the modulation of phosphotyrosine levels of putative substrates by overexpression or knockdown of the PTP in question (Tiganis and Bennett, 2007). They note that this approach could be complicated by regulatory mechanisms governing PTP activity, as overexpression of a PTP like SHP-2, for instance, may not result in the expected increase in substrate dephosphorylation since catalytic activity depends also upon SH2 domain engagement. As such, they recommend that overexpression studies be complemented by loss-of-function analysis, which should conversely result in increased substrate tyrosine phosphorylation. The activating effect of the SHP-2 E76K mutation is ideal for these purposes, as it circumvents the potentially convoluted effect of PTP overexpression and provides a clean gain-off-function system with which to evaluate downstream effects on phosphotyrosine signaling. This combined with the availability of phosphotyrosine-specific antibodies like 4G10 for immunoprecipitation provides yet another unbiased approach by which SHP-2 activity could be assessed.

The Src family kinases (SFKs) are protein tyrosine kinases with known transforming potential and like SHP-2, a tightly regulated and highly

phosphorylation-dependent mechanism of activation (Xu et al., 1997). In addition to their membrane-localizing myristylation sequence and catalytic kinase domain, the SFKs contain SH2 and SH3 domains and a C-terminal phosphotyrosine residue. All of these components are known to be critical for signaling, but the latter three in particular which participate in an elegant autoinhibitory configuration by which the SH2 domain engages the C-terminal phosphotyrosine while the SH3 domain interacts with a conserved polyproline region within the SH2-kinase linker sequence, thus disrupting catalytic activity despite leaving the active site unoccluded. Thus, like SHP-2, the SFKs are regulated by the formation of intramolecular contacts that result in basal auto-inhibition. Despite their similar structure, however, the SFK model is distinct in that tyrosine phosphorylation is inhibitory (rather than activating, as has been proposed for SHP-2) and the inhibition of SHP-2 is achieved by direct interaction of the N-SH2 domain, which retains its phosphotyrosine-binding ability, with the PTP domain, not with the C-terminal phosphotyrosine. Both, however, play important roles in signaling and hematopoiesis, and the critical function of the inhibitory phosphorylation at Y527 makes the SFKs an intriguing target for phosphatase-mediated regulation.

SFKs are known to play an important role in hematopoiesis, as all of the known mammalian SFKs are expressed in the various hematopoietic lineages, with many cell types expressing multiple SFKs and several of the SFKs being expressed exclusively in the hematopoietic compartment. Accordingly, murine models of SFK loss show a marked propensity toward hematologic dysregulation. Hck, Fgr and Lyn are expressed in terminally differentiated myelomonocytic cells including

neutrophils, monocytes and macrophages. Mice lacking Hck or Fgr or both displayed normal hematopoietic development, although phagocytosis was noted to be impaired in *Hck*$^{-/-}$ macrophages and doubly mutant mice demonstrated an increased susceptibility to infection with *Listeria monocytogenes*, indicating a role for these SFKs in the immune response to intracellular pathogens (Lowell et al., 1994). Of note, no significant decrease in tyrosine phosphorylation was observed in single or doubly mutant mice suggesting the possibility of compensatory SFK signaling via alternate isoforms, and indeed the kinase activity of Lyn was found to be increased in *Hck*$^{-/-}$ macrophages. Interestingly, later studies demonstrated that neutrophils and dendritic cells from these doubly mutant animals demonstrated increased MAPK activation, F-actin polymerization, calcium flux, and chemotactic responses to chemokine stimulation, which was attributed to the role of these SFKs in mediating the inhibitory activity of the PIR-B receptor (Zhang et al., 2005). Thus these SFKs appear to play both positive and negative roles in regulating immune cell function depending on their context.

Lyn is also highly expressed in hematopoietic cells and has been shown to be particularly important in B cells and BCR signaling, with Lyn null mice suffering from a deficit of mature B cells despite normal numbers of pre-B cell precursors in the bone marrow initially attributed to attenuated BCR signaling (Nishizumi et al., 1995). However subsequent studies demonstrated that despite delays in early BCR signaling events, BCR crosslinking in *Lyn*$^{-/-}$ in fact led to enhanced MAPK activation and enhanced B cell proliferation in response to anti-IgM treatment (Chan et al., 1997). This was attributed to defects in BCR inhibition caused by the loss of Lyn-

mediated phosphorylation of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) on inhibitory receptors like FcγRIIb1. Despite suffering a reduction in peripheral B cells, aged *Lyn*$^{-/-}$ mice were found to develop a lupus-like autoimmune disease due to accumulation of IgM-secreting plasma cells, eventually leading to the development of immune-mediated glomerulonephritis. Thus Lyn participates in both initiation and inhibition of BCR signaling. In addition to this paradoxical B cell phenotype, aged *Lyn*$^{-/-}$ mice were also found to display a myeloid phenotype with splenomegaly and macrophage tumors, again attributed to the role of Lyn in ITIM-dependent inhibitory signaling (Harder et al., 2001).

This complicated relationship is highlighted by the comparison of *Lyn*$^{-/-}$ mice with mice expressing an activated allele of Lyn (Y508F) in which the inhibitory C-terminal phosphotyrosine has been mutated to phenylalanine. Similar activated alleles of Lck and Src have been shown to develop thymic lymphomas (Abraham et al., 1991) and mammary neoplasia (Webster et al., 1995), respectively, but the Lyn Y508F, or Lyn$^{up/up}$, animals, despite demonstrating the expected increases in phosphotyrosine and kinase activity confirming expression of the gain-of-function allele in the hematopoietic compartment, failed to develop any hematologic malignancies, and in fact had decreased spleen weights compared to wild-type. Of note, however, they also had nearly twice as many neutrophils in spleen and peripheral blood compared with wild-type. Aged *Lyn*$^{-/-}$ mice, by contrast, in addition to the previously noted splenomegaly, demonstrated an accumulation of myelomonocytic cells in liver and lymph nodes, as well as in lungs, ovaries, pancreas, bowel and kidneys, with some older mice even developing large

aggregates on their tails, ears, and legs. Closer examination of younger *Lyn*$^{-/-}$ mice revealed an increased proportion of neutrophils and macrophages in the spleen and monocytes in peripheral blood, as well as an increase in splenic and peripheral blood CFU-M and CFU-GM, although the colony forming ability of bone marrow progenitors was similar to wild-type. Bone marrow derived macrophages from *Lyn*$^{-/-}$ mice also showed modest hypersensitivity to CSF-1 and GM-CSF. Lyn$^{up/up}$ animals demonstrated myeloid progenitor numbers and dose-dependent colony formation similar to that observed in wild-type animals.

Interestingly, more recent studies have shown that Lyn deficient animals also demonstrate an increase in megakaryopoiesis, with an increased number of megakaryocytic progenitors as well as mature megakaryocytes in the spleen and bone marrow (Lannutti et al., 2006). Megakaryocytes are derived from the megakaryocyte-erythroid progenitor cell, or MEP, population of pluripotent hematopoietic stem cells. Their differentiation and maturation is primarily regulated by the cytokine thrombopoietin (TPO), which directs the cellular enlargement and endomitosis, or DNA replication without cell division, characteristic of this lineage, resulting in the polyploid giant cells responsible for platelet production. Genetic deletion of the genes for TPO or its cognate receptor, Mpl, result in mice with a 70 to 90% reduction in the number of megakaryocytes and platelets (de Sauvage et al., 1996; Gurney et al., 1994). TPO binding to the cytokine receptor Mpl results in activation of the JAK/STAT signaling axis (Drachman and Kaushansky, 1997), which is necessary for survival and proliferation, as well as the Ras and PI3K pathways (Geddis et al., 2001; Rojnuckarin

et al., 1999). However, Lannutti et al. demonstrated that SFKs were also phosphorylated in response to TPO stimulation in primary murine megakaryocytes, specifically Lyn and Fyn (Lannutti et al., 2003). Inhibition of these kinases in Mpl-expressing Ba/f3 cells was found to enhance TPO-dependent proliferation and induce megakaryocytic differentiation and nuclear polyploidy, which the authors attribute increased and sustained MAPK activation (Lannutti and Drachman, 2004). *Lyn-/-* megakaryocytes were similarly shown to exhibit enhanced MAPK and AKT phosphorylation in response to TPO stimulation, suggesting that Lyn might negatively regulate megakaryopoiesis by activation of MAPK.

Crossing of the complex $Lyn^{-/-}$ and $Hck^{-/-}/Fgr^{-/-}$ models of positive and negative regulation to generate triple null $Hck^{-/-}/Fgr^{-/-}/Lyn^{-/-}$ null mutant mice resulted in fertile animals without any evident phenotype beyond that observed in the $Lyn^{-/-}$ single null animals (Meng and Lowell, 1997). Elicited peritoneal bone marrow-derived macrophages from these animals were studied to determine the role of these SFKs in lipopolysaccharide (LPS)-mediated signal transduction and in spite of a dramatic reduction in overall tyrosine phosphorylation and absence of any apparent compensatory expression of alternate SFK isoforms, these cells were found to demonstrate normal LPS-induced cytokine secretion, nitrite production, and tumor cell cytotoxicity activity. Phosphorylation of MAPK and JNK kinases occurred normally after LPS stimulation, suggesting that HFL are not required for LPS-mediated signal transduction. Although loss of these three myeloid SFKs left macrophage signaling seemingly intact, a later study found that they were required for BCR-ABL induced B-ALL. Hu et al. found that BCR-ABL activated these same

three SFKs in B-lymphoid cells, and retroviral transduction of triple-null bone marrow with BCR-ABL efficiently induced CML but not B-ALL in transplantation recipients. Treatment with a dual Src/Abl inhibitor, CGP76030, was found to impair proliferation of B-cells expressing BCR ABL and prolong survival of mice with B-ALL but not CML mice, and combination of imatinib with this inhibitor was found to be superior to imatinib alone. Together these data suggested that it was SFK inhibition that was responsible for the B-ALL-specific therapeutic effect and added benefit combined with imatinib, thus implicating SFKs are potential therapeutic targets in Ph+ B-ALL (Hu et al., 2004).

Given the overwhelming evidence demonstrating that SHP-2 is a positive effector of Ras/MAPK signaling, it is important to note that as two of the oldest known oncogenes, Src and Ras have a long and storied history. In 1986 it was demonstrated that microinjection of neutralizing antibodies against Ras blocked v-Src mediated transformation of NIH-3T3 cells (Smith et al., 1986). Later, another group found that Src was able to transform certain cell types in the absence of Ras activation, suggesting that other pathways might be involved as well (Aftab et al., 1997). Penuel et al. then demonstrated that Ras and PI3K initiated parallel pathways that both contributed to transformation, with inhibition of both being required block v-Src mediated transformation in chicken embryo fibroblasts (Penuel and Martin, 1999). More recent studies have suggested that Src-mediated activation of Ras may occur not at the plasma membrane, but rather on the intracellular membranes of the Golgi apparatus and the endoplasmic reticulum.

Bivona et al. demonstrated that signaling at these 'endomembranes' was dependent on SFK-mediated phosphorylation of Plcgamma1 to facilitate translocation of RasGRP1 and activation of Ras (Bivona et al., 2003).

Based on this role for SFKs in Ras activation, Neel et al. proposed a model by which SHP-2 regulates Ras by indirectly modulating inhibitory SFK phosphorylation. By examining the response of immortalized fibroblasts from SHP-2 null mice, the authors observed a decrease in the activating SFK phosphorylation (Y416) and increased basal inhibitory phosphorylation (Y527) which failed to dephosphorylate in response to growth factor stimulation (Zhang et al., 2004). EGF-stimulated phosphorylation of the SFK targets p62Dok, Vav2, and Plcgamma1 were also found to be decreased in SHP-2 null cells, while tyrosine phosphorylation of EGFR, gab1, and Shc were unaffected, similar to fibroblasts lacking Src, Fyn, and Yes ($SYF^{-/-}$), suggesting an SFK-specific effect. Direct dephosphorylation of SFKs by SHP-2 was considered unlikely for 'quantitative' reasons, as basal Src activity was found to be decreased by at least 50% in SHP-2 deficient fibroblasts, suggesting regulation of a parallel pathway. They instead proposed a model by which SHP-2 prevents proper localization of the inhibitory c-Src kinase (Csk) by dephosphorylating the Csk-targeting protein PAG, thereby resulting in sustained activation of Src and downstream activation of Ras on endomembranes. Subsequent generation of a novel conditional SHP-2 knockout mouse model resulted in peri-implantation lethality that was attributed to a requirement for SHP-2 in trophoblast stem cells. SHP2 deletion was found to impair FGF4-evoked activation of Src, Ras, and Erk, suggesting a similar mechanism to that described in fibroblasts. SHP-2 deletion was

found to result in increased stability of the pro-apoptotic protein Bim, as did inhibitors of MEK and Src, suggesting that FGF4 promotes trophoblast stem cell survival by phosphorylating Bim through a SHP-2/Src/Ras pathway (Yang et al., 2006).

In this study we set out to identify phosphotyrosine-containing proteins whose phosphorylation was modulated by SHP2 knockdown or overexpression of the hyperactive JMML-associated E76K mutant. Surprisingly, we observed that global phosphotyrosine levels were decreased by SHP2 knockdown and increased by SHP2 E76K, a paradoxical result for a tyrosine phosphatase. This suggested a potential action of SHP-2 on a protein tyrosine kinase, and upon further investigation, expression of the SHP2 E76K was found to result in SFK activation. To determine whether a SFK might prove to be the elusive SHP2 substrate, we looked at activated alleles of Src as well as the SFK Lyn, which is thought to be one of the major SFK isoforms in the hematopoietic lineage and an important downstream effector in hematopoietic growth factor signaling. Despite the evidence of SFK activation in response to E76K, activated alleles of Src (Y527F) and Lyn (Y508F) were unable to phenocopy the effects of E76K expression in hematopoietic cells. Conversely, the absence of Lyn did not abolish the factor-independent colony formation or growth factor hypersensitivity mediated by SHP2 E76K in a CFU-GM assay, although it did blunt overall colony formation. This suggests that while SFKs are likely important for GM-CSF signaling, it is unlikely to be the critical substrate for SHP2 E76K-mediated transformation.

Results

Given the catalytic function of SHP-2 as a protein tyrosine phosphatase, we sought to identify proteins containing phosphotyrosine moieties modulated by expression of SHP2. We began by generating retroviral shRNA vectors targeting human *PTPN11* and introducing them into the human monocytic U937 cell line to achieve stable knockdown of SHP-2 expression (Fig. 1a). Immunoblotting with the phosphotyrosine-specific 4G10 antibody we hoped to identify bands of decreased

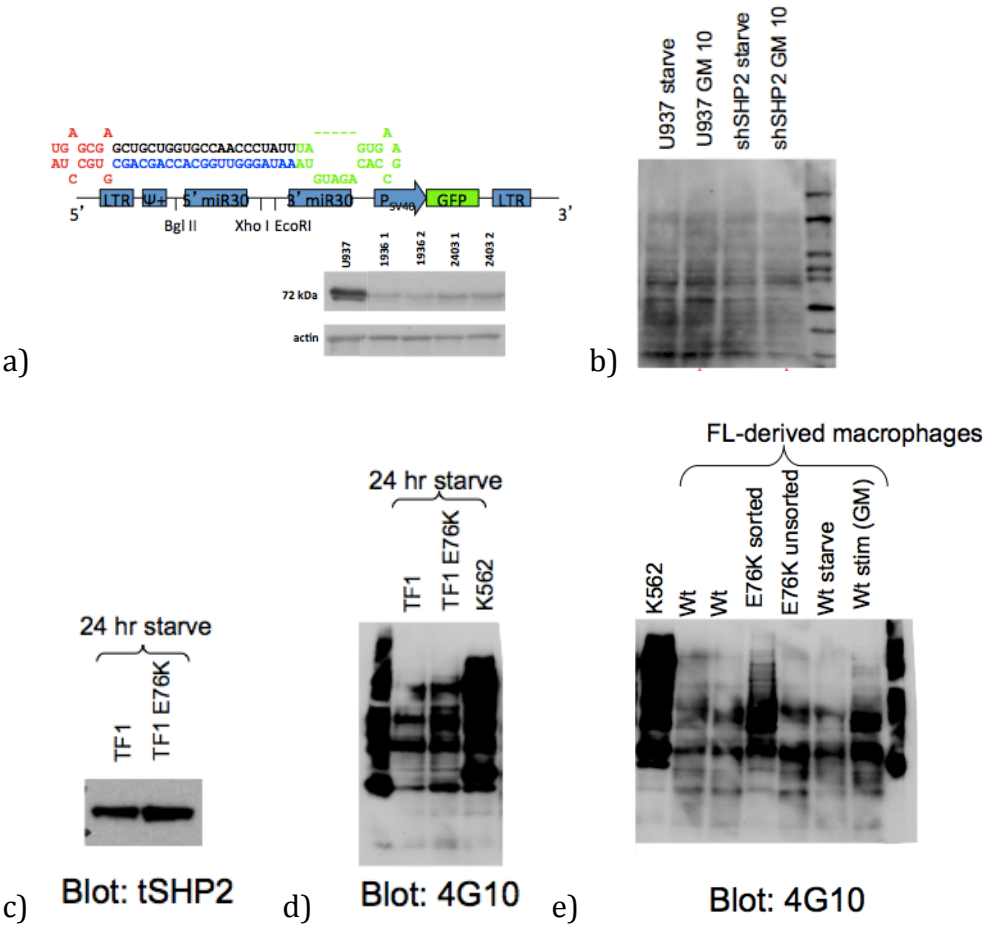


Figure 1. SHP-2 knockdown or E76K expression affects global phosphotyrosine. a) SHP-2 knockdown in U937 cells using two different targeting sequences, 1936 and 2403. b) 4G10 immunoblot of lysates from shSHP2 U937 cells expressing the 1936 shRNA. c) Expression of SHP-2 E76K in TF-1 cells. d) Expression of SHP-2 E76K increases total phosphotyrosine in TF-1 and e) primary fetal liver derived macrophages.

phosphotyrosine levels were in fact decreased (Fig. 1b). Although surprising given intensity in response to SHP-2 knockdown, but instead we found that global the function of SHP-2 as a phosphatase, this was also consistent with its role as a positive effector of growth factor signaling. In light of this unexpected result, we did the converse experiment using overexpression of the JMML-associated SHP2 E76K mutant, which is known to be catalytically hyperactive. Just as knockdown decreased overall phosphotyrosine, we found that overexpression of SHP2 E76K increased overall phosphotyrosine both in a GM-CSF dependent human hematopoietic TF-1 cell line and also in fetal liver-derived macrophages (Fig. 1c).

We chose the study the effects of SHP2 E76K in TF-1 cells because it had been published previously that SHP2 E76K can transform these cells to growth factor independence (Ren et al., 2007). We also found this to be the case (Fig. 2a) and interrogated signaling in the factor-free state as a means of understanding how SHP-2 might be promoting growth factor independence/hypersensitivity in the disease state. As expected given the role of SHP-2 in Ras signaling, we observed an increase in ERK phosphorylation in the absence of growth factor (Fig. 2b). However, the magnitude of the effect of SHP-2 E76K overexpression on global phosphotyrosine suggested that the SHP-2-mediated dephosphorylation might actually be directed at modulating the activity of a tyrosine kinase. In TF-1 cells starved of growth factor for 24 hours, we observed decreased phosphorylation of the inhibitory residue Y527 and increased phosphorylation of the activating residue Y416, suggesting that hyperactivation of SHP-2 catalytic activity promotes SFK activation (Fig. 2c).

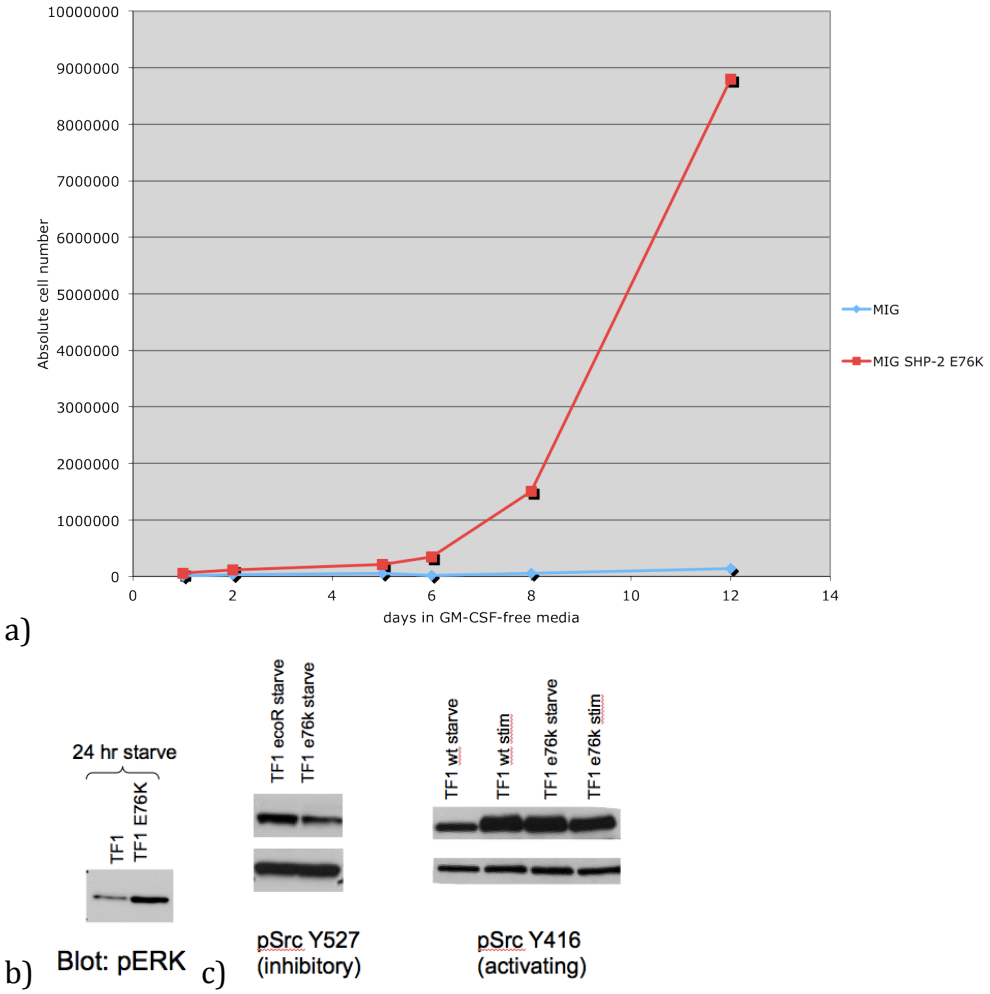
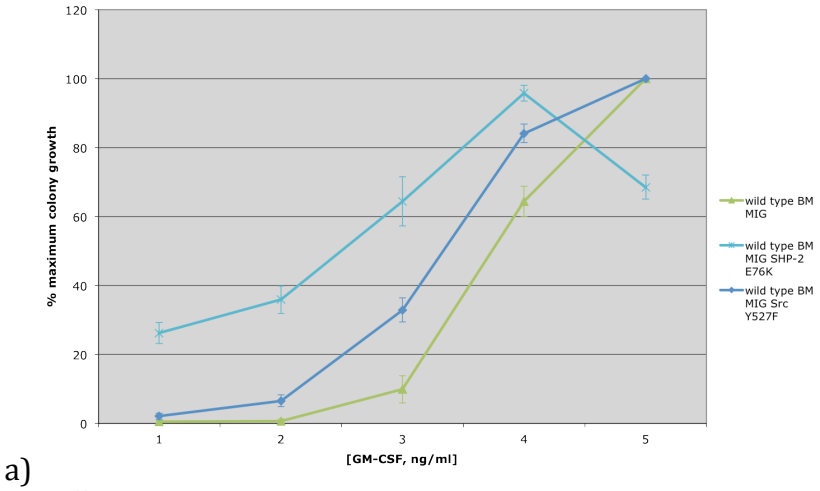


Figure 2. Expression of SHP-2 E76K transforms TF-1 cells. a) SHP-2 E76K renders the GM-CSF dependent TF-1 cell line factor independent, b) increases basal phospho-ERK, and c) increases basal SFK activation as evidenced by decreased inhibitory pY527 and increased activating pY416.

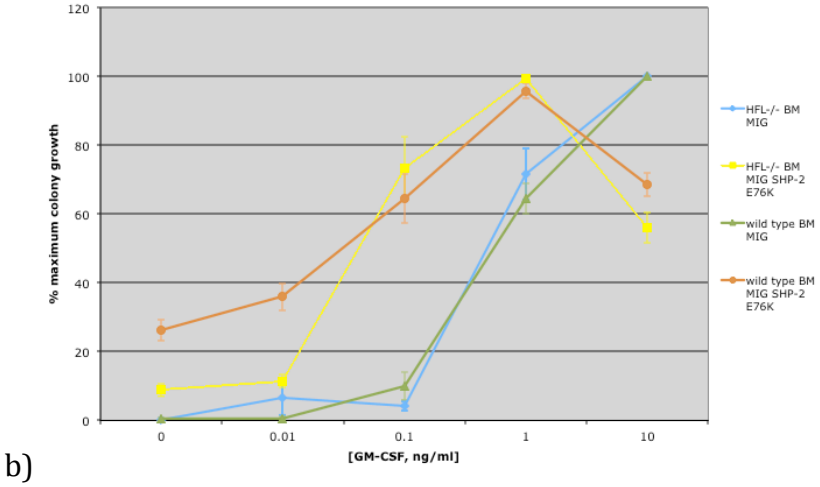
In order to examine whether SFKs might be the critical substrate dephosphorylated by SHP2, we used activated alleles of c-Src and Lyn in which the C-terminal site of inhibitory phosphorylation by Csk, Y527 in c-Src and Y508 in Lyn, were mutated to phenylalanine. In TF-1 cells, overexpression of these mutants was insufficient to render the cells factor-independent, whereas an activated allele of K-Ras (G12D) succeeded in doing so, more efficiently, in fact, than SHP-2 (data not

shown). The fact that 'constitutively dephosphorylated' Src or Lyn mutants could not phenocopy a hyperactive SHP2 mutant in this in vitro assay suggested that though activated, SFKs might not be the critical target of SHP2-mediated transformation. To further this analysis, we then introduced these same mutants into primary murine bone marrow and plated the cells in a CFU-GM assay. Consistent with their inability to transform TF-1 cells to factor independence, the activated Src Y527F allele failed to reproduce the factor-independent growth or GM-CSF hypersensitivity characteristic of cells expressing SHP-2 E76K, although some mild hypersensitivity was observed (Fig. 3a). Furthermore, expression of SHP2 E76K in bone marrow cells from mice lacking the three major hematopoietic SFK isoforms Hck, Fgr, and Lyn, still displayed factor-independent colony formation and hypersensitivity (Fig. 3b). As expected, loss of SFK activity also failed to prevent K-Ras G12D-mediated transformation (Fig. 3c). Together these data suggest that SFKs are unlikely to be the critical effectors of SHP2-mediated transformation in hematopoietic cells.

Because Lyn in particular has been implicated in SHP-2 signaling, the same experiments were repeated specifically with activated alleles of Lyn (Y508F) and *Lyn-/-* bone marrow. Interestingly, unlike the Src Y527F allele, expression of the activated Lyn actually seemed to suppress colony formation below that of wild-type, perhaps indicating that its documented negative regulatory role in hematopoietic signaling could play a role in the monocyte/macrophage lineage (Fig. 4a). Loss of Lyn also failed to prevent SHP-2 E76K (Fig. 4b) or K-Ras G12D (Fig. 4c) from transforming *Lyn-/-* bone marrow progenitors.



a)



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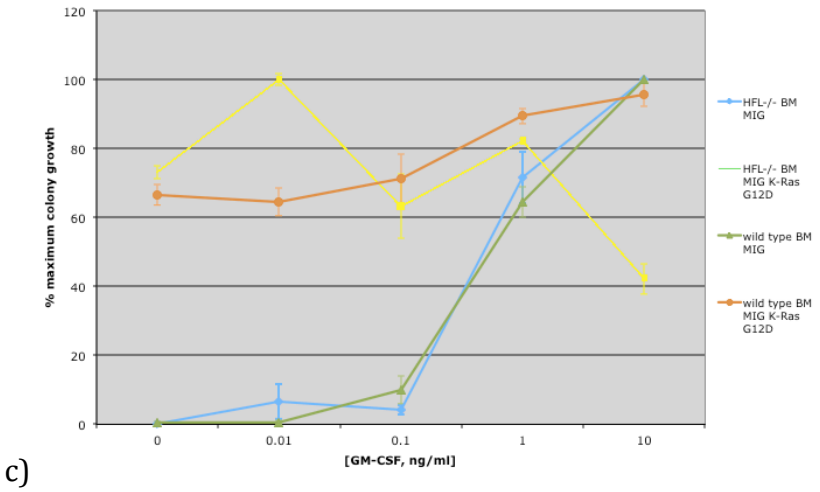

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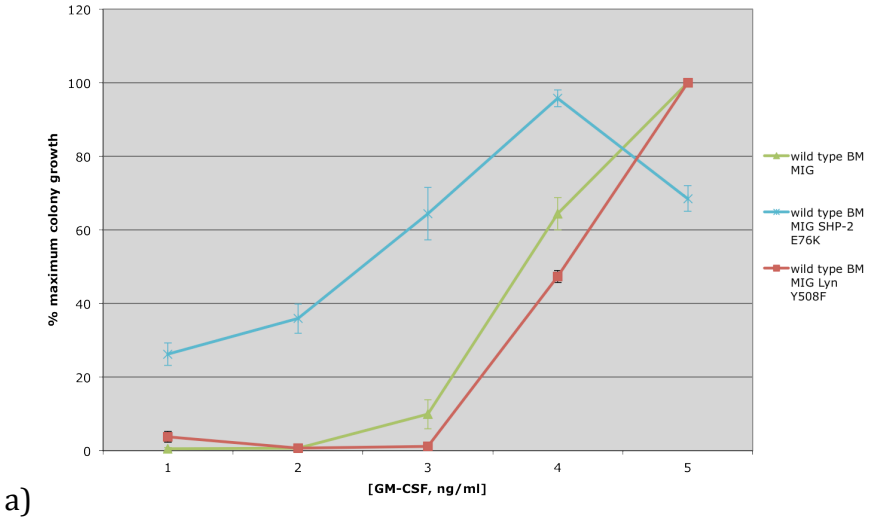
Figure 3. Effects of gain and loss of SFK activity in CFU-GM assay. a) Retroviral transduction of wild type murine bone marrow with the activated Src Y527F does not phenocopy SHP-2 E76K expression, although it does confer slight hypersensitivity. b) Loss of all myeloid SFKs, Hck, Fgr, and Lyn ($HFL^{-/-}$), does not prevent SHP-2 E76K-mediated transformation. c) Oncogenic K-Ras G12D does not require SFK activity for transforming activity.



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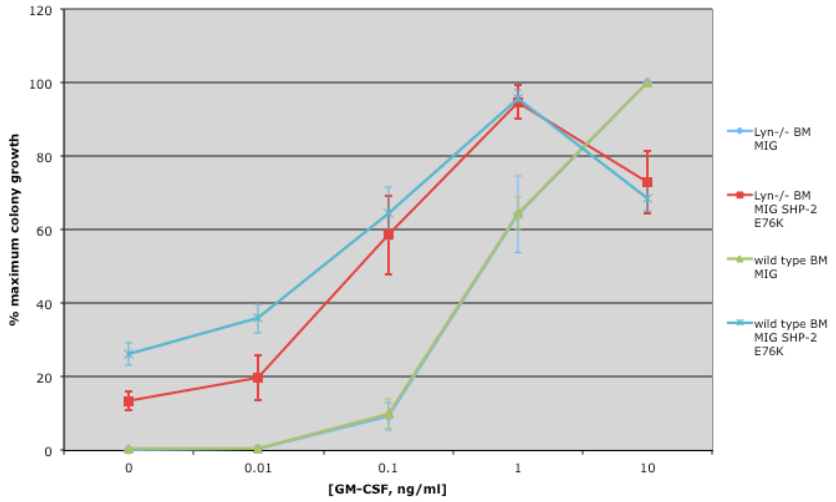

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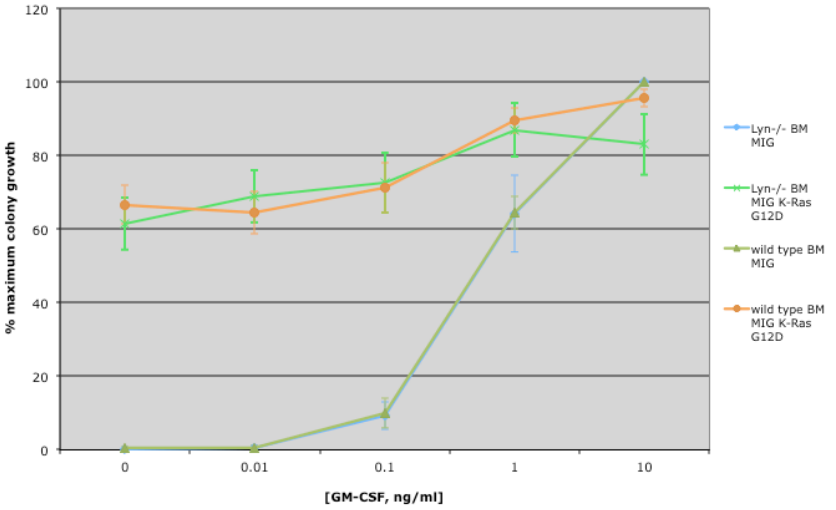

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Figure 4. Effect of gain- and loss-of-function of Lyn on SHP-2 mediated transformation. A) Expression of activated Lyn Y508F does not phenocopy SHP-2 E76K and in fact has a slightly growth suppressive effect. b) Loss of Lyn does not prevent transformation by SHP-2 E76K or c) K-Ras G12D.

However, despite the inability of the activated SFK mutants to induce factor-independent colony formation or GM-CSF hypersensitivity, expression of these mutants did result in a striking increase in overall colony number (Fig. 5a). Unlike Src, however, Lyn Y508F produced fewer absolute numbers of colonies (Fig. 5b). This paradoxical result is reminiscent of the mouse models of Lyn (*Lyn-/-* and $Lyn^{up/up}$) where gain and loss of Lyn function was found to result in strangely similar phenotypes, suggesting positive and negative roles even within the same tissue types. Surprisingly loss of Lyn had a growth-suppressive effect on absolute colony formation in G12D-expressing bone marrow cells as well (Fig. 5d). A comparison of absolute colony number in the absence or presence of GM-CSF suggests that while SFKs may not be the critical dephosphorylated substrate effectors of SHP-2, they may be important overall for GM-CSF mediated signaling (Fig. 5e). In fact, a reexamination of the initial evidence of SFK activation in the context of SHP-2 E76K supports this view, as the noted basal activation stands in contrast to the response to GM-CSF stimulation. Parental TF-1 cells demonstrate SFK activation in response to GM-CSF stimulation, as previously noted by other groups. However, despite basal elevation of SFK phosphorylation (pY416) in the absence of growth factor, TF-1 cells expressing SHP-2 E76K demonstrate a decreased phosphorylation in response to GM-CSF stimulation.

Although initially dismissed as simply an incidental observation, another incidental observation, namely re-engineering of the TF-1 SHP-2 E76K cell line with the retrovirally introduced-ecotropic receptor under a different selectable marker (neomycin instead of puromycin) resulted in massive overexpression of the

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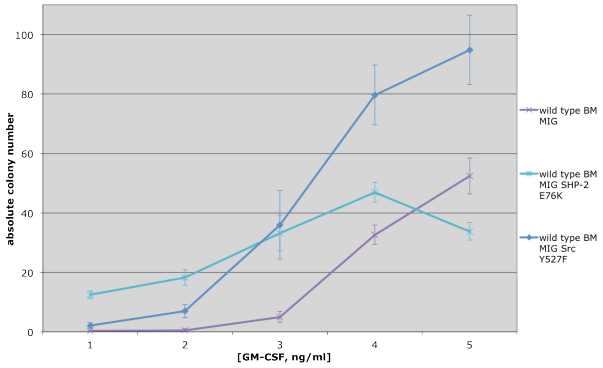

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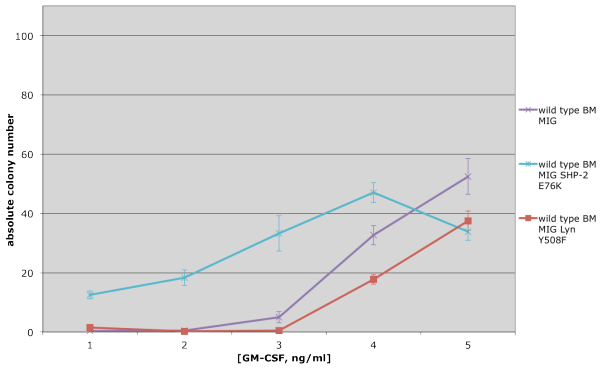

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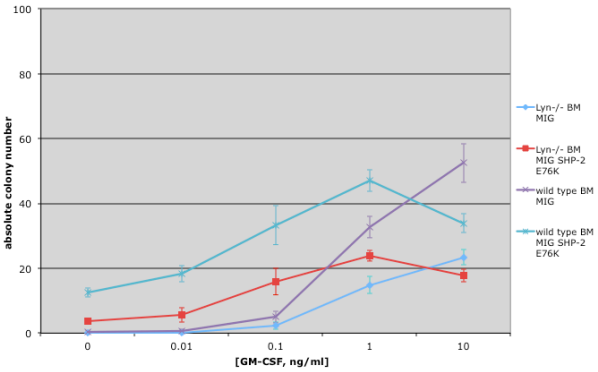

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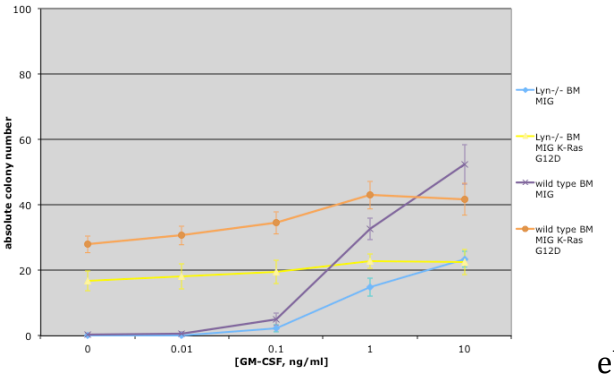

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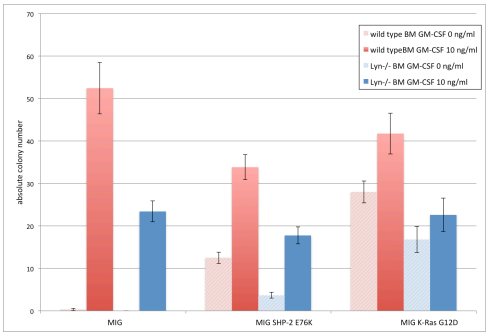

Figure 5. Paradoxical effects of SFK gain- and loss-of function on absolute colony formation. a) Expression of activated Src Y527F results in an increase in absolute colony number. b) Unlike Src Y527F, activated Lyn also results in decreased absolute colony formation. c) Loss of Lyn results in decreased absolute colony formation by SHP-2 E76K as compared with wild-type. d) Loss of Hck/Fgr/Lyn results in normal if not slightly increased absolute colony formation by SHP-2 E76K. c) Loss of Lyn results in decreased absolute colony formation by K-Ras G12D. d) Effect of Lyn loss on absolute colony count is more pronounced at maximum dose of GM-CSF (10 ng/ml) than at basal.

oncogene (Fig. 6a) and a marked change in phenotype. While previously E76K expression resulted in factor-independent growth but no morphologic evidence of transformation, these high-overexpressing cells grew noticeably larger after transduction. Of note, expression of K-Ras G12D under this new selectable marker similarly resulted in dramatic morphologic shift, this time toward an adherent macrophage-like appearance (Fig. 6b and c), suggesting that in both cases the high levels of expression provided a stronger push towards differentiation as opposed to proliferation. The dramatic increase in cell size observed in the setting of SHP-2 E76K suggested a potentially multinucleate phenotype, as TF-1 cells are known to have megakaryocytic potential.

Cytospin preparations of these cells confirmed a multinucleate phenotype that was consistent with megakaryocytic differentiation, although the basophilic cytoplasmic staining observed would not be consistent with normally differentiated megakaryocytes (Fig. 6d). However, it was also noted that the parental TF-1 cells display unusually large nuclei with potentially 2N+ DNA content, suggesting that differentiation of these cells would likely result in an abnormal megakaryocytic appearance as well (personal communication, Scott Kogan). More specific immunophenotyping of these cells with the human megakaryocytic markers CD41 and vWF will be required for proper lineage validation, as will experiments investigating this potential in primary cells. Differentiation down this particular lineage was particularly intriguing because of the known role of the hematopoietic SFK Lyn in megakaryopoiesis, with Lyn being a known negative regulator of TPO signaling and megakaryocytic differentiation. This would be consistent with the

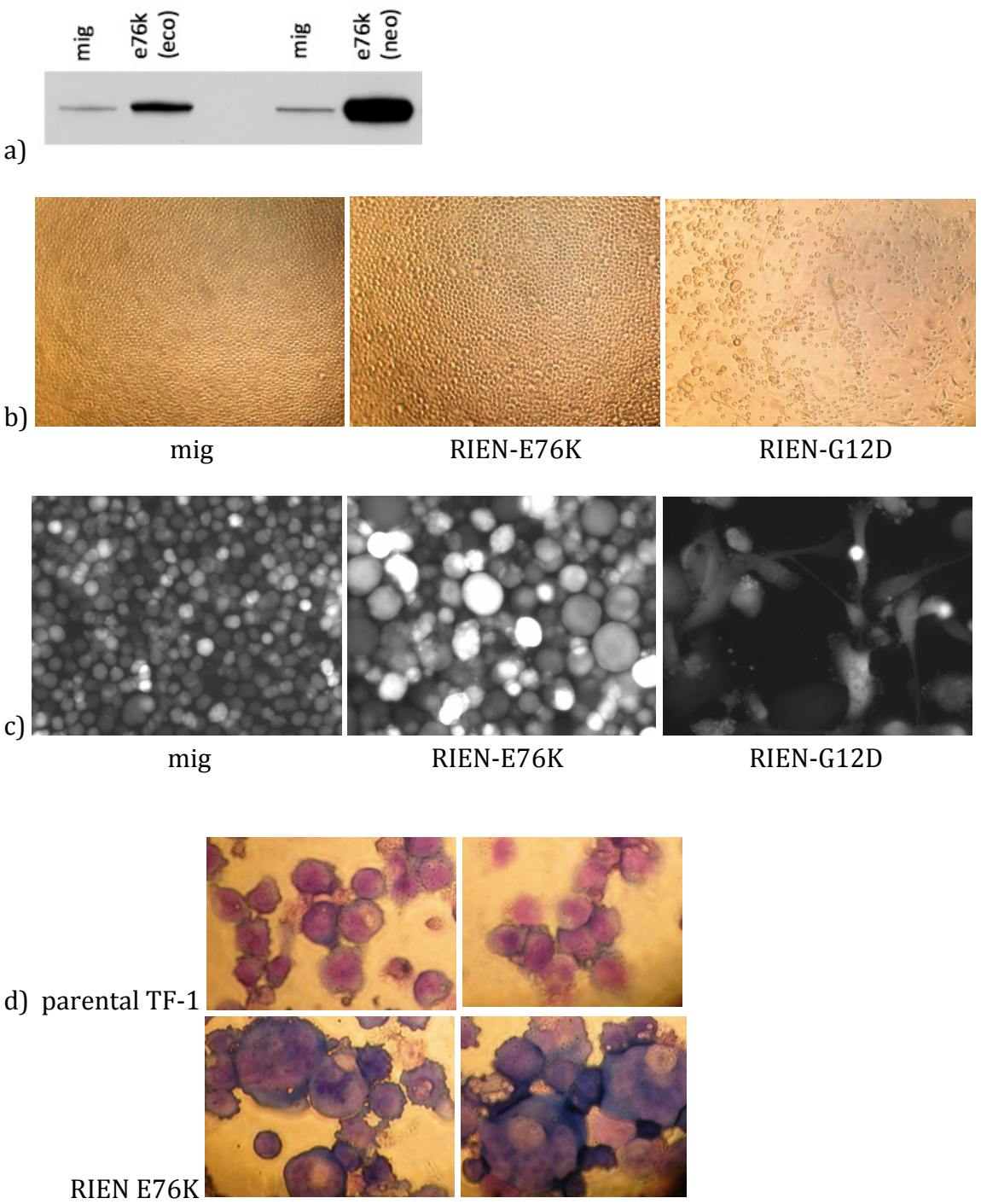

Figure 6. High overexpression of SHP-2 E76K or K-Ras G12D in TF-1 cells results in morphological change. a) Level of SHP-2 E76K expression under the RIEN-modified cells. Images taken by b) light microscopy and c) fluorescence microscopy (detecting GFP from retroviral MIG vector) demonstrating differences in size and shape. d) Cytospin preparation and Giemsa staining of parental and E76K-expressing TF-1 cells demonstrate marked changes in nuclear size and cytoplasmic color.

observed decrease in SFK phosphorylation observed in E76K-expressing cells in response to GM-CSF, and suggests a complex mechanism by which basal SFK activation in the absence of growth factor might promote proliferation, while inhibition of Lyn activity in the presence of growth factor might promote differentiation specifically toward a megakaryocytic fate. Further studies with isoform-specific SFK antibodies will help clarify this question, as differential activation of the hematopoietic SFK members is likely, with inhibition of Lyn in particular being of functional significance with regards to megakaryocytic differentiation. Of note, treatment of TF-1 cells with the SFK inhibitor SU6656 resulted in similar enlargement of cell size (data not shown). SU6656 has also been published to promote endomitosis, a form of endoreplication in which DNA replication occurs in the absence of cytokinesis, a characteristic feature of the multinucleate megakaryocyte (Lannutti et al., 2005).

Together these data suggest that SFKs, though not the critical substrate for SHP-2 in JMML, may be the critical effector of a novel SHP-2 pathway promoting megakaryocytic differentiation downstream of the GM-CSF receptor.

Discussion

Here we have presented data demonstrating that modulation of SHP-2 activity surprisingly affects global phosphotyrosine levels in hematopoietic cells, with knockdown resulting in decreased overall pY content and expression of the JMML-associated E76K mutant resulting in a global increase. This latter effect was found to result from SFK activation, particularly in the absence of growth factor, in a

GM-CSF dependent human leukemia cell line that is transformed to factor-independent growth by E76K expression. Several recent lines of evidence have implicated SFKs as being important for the activity of SHP-2 in fibroblasts (Zhang et al., 2004), trophoblasts (Yang et al., 2006), skeletal muscle (Fornaro et al., 2006), and most recently in granulocytes (Zhu et al., 2004). In examining immortalized fibroblasts derived from SHP-2 null mice, Zhang et al. similarly observed that genetic deletion of *Ptpn11* resulted in a global decrease in phosphotyrosine in bone-marrow derived mast cells in a manner that phenocopied deletion of Src (Zhang et al., 2004).

Corey et al. recently demonstrated phosphorylation of the SFK Lyn downstream of the G-CSF receptor and proposed that Gab2-mediated recruitment of SHP-2 to the receptor was responsible for dephosphorylation of the inhibitory phosphotyrosine at Y507 (Futami et al., 2011). Like them, we also observed phosphorylation of SFK in response to GM-CSF stimulation and saw an increase in basal phosphorylation in the presence of a hyperactive SHP-2 mutant. However, the inability of the 'constitutively dephosphorylated' Lyn Y507F mutant to confer factor independence on the GM-CSF dependent TF-1 cell line in the same manner as SHP-2 E76K or K-Ras G12D suggests that a direct catalytic relationship between SHP2 and LYN is unlikely in this context. It is important to note that despite the similarity in nomenclature, the GM-CSF and G-CSF receptors are in fact quite distinct structurally and belong to different classes of cytokine receptor, with GM-CSFR bearing closer resemblance to the IL-3 receptor and G-CSFR to the erythropoietin receptor. Furthermore, despite the decreased spleen weight and lack of apparent hematologic

malignancy in the $Lyn^{up/up}$ mice, the neutrophil lineage was specifically and quite markedly increased, suggesting that Lyn may play a lineage-restricted role in promoting proliferation of granulocytes (Harder et al., 2001). The development instead in Lyn-deficient animals of an MPD-like phenotype with significant macrophage infiltration of non-hematopoietic tissues further suggests that SHP-2 mediated activation of Lyn by dephosphorylation of its inhibitory C-terminal phosphotyrosine is unlikely to account for the role of SHP-2 in JMML.

As described previously, five SFK members are primarily expressed in leukocytes: Blk, Fgr, Hck, Lyn and Lck, and of these, myeloid cells preferentially express Hck, Fgr, and Lyn. *$Hck^{-/-}/Fgr^{-/-}/Lyn^{-/-}$* triple null mice are viable and fertile, and no other SFKs were detected in macrophages derived from the bone marrow of these mice (Meng and Lowell, 1997). As these three SFKs are thought to be the only ones expressed in early hematopoietic progenitor and myeloid cells, we sought to determine whether they were required for the factor-independent growth and hypersensitivity produced by SHP-2 E76K in a CFU-GM assay. Surprisingly these cells were not resistant to transformation, suggesting that these SFKs are not the primary effectors of oncogenic SHP-2. The same experiment was repeated with $Lyn^{-/-}$ bone marrow with the same result. Consistent with this result, expression of activated alleles of Src or Lyn did not confer growth factor independence or hypersensitivity to wild-type bone marrow cells. Again, in the original studies performed with the *$Hck^{-/-}/Fgr^{-/-}/Lyn^{-/-}$* mice, the authors found that despite significant reduction of total protein phosphotyrosine, functional analyses indicate that bone marrow–derived macrophages had no major defects in LPS-induced

activation and activated the ERK1/2 and JNK kinases, as well as the transcription factor NF-κB, as well as wild-type macrophages. Given the importance of Ras/MAPK signaling in the biology of JMML, these findings support the idea that SFKs are likely not responsible for the role of SHP-2 as a positive regulator of this signaling axis.

Curiously, however, expression of the constitutively active Src Y527F allele in wild-type bone marrow produced an increase in overall colony number in a dose-dependent fashion. While not able to mimic the growth factor independence or hypersensitivity of JMML-associated mutations in SHP-2 or K-Ras, this suggests that activation of Src kinase activity can augment response to GM-CSF signaling. Similarly, genetic deletion of Lyn resulted in a marked decrease in colony number, even in the control empty vector-expressing cells. Together these data indicate that SFKs may be important for GM-CSF signaling, but not for SHP2-mediated transformation. This idea is supported by recent studies done by Perugini et al., in which two different activated mutants of the beta-common chain of the GM-CSF receptor were investigated (Perugini et al., 2010). The GM-CSF receptor has a highly complex dodecameric structure consisting of two hexamers composed each of alpha and beta chains. The authors used an 'intracellular' V449E transmembrane mutant consisting exclusively of beta-common as well as an FIΔ 'extracellular' mutant that required GMRα for its activation. They found that the transmembrane V449E mutant selectively activated JAK2/STAT5 and ERK pathways, whereas the GMRα-dependent FIΔ mutant selectively activated PI3-K/AKT and NFκB pathways and signaled primarily through the Src family kinases. They also demonstrated a novel interaction between the SH3 domains of Lyn and Src with a polyproline region

in GMRα, suggesting a potential mechanism for the SFK-dependence of the FIΔ mutant. These data suggest that the unique heteromeric structure of the GM-CSFR might allow for alternative modes of receptor activation that could act independently in the unliganded state but synergistically in the presence of growth factor. Such a model in which GM-CSF signaling can occur through two parallel pathways would support the notion that Src might be important downstream of GM-CSFR activation but independent from the activation of the SHP2/RAS/MAPK pathway.

This model of parallel pathway activation by the GM-CSF receptor could also provide a basis for the paradoxical SFK inhibition observed in the presence of ligand, with SFK activation by an unliganded GMRα being distinct from potentially inhibitory effects of synergistic ligand-bound GM-CSFR signaling, which might involve more feedback or downstream regulation. Although TPO is the primary cytokine responsible for megakaryocyte differentiation and maturation, GM-CSF has also been shown to stimulate in vitro megakaryopoiesis (Kaushansky et al., 1986). The morphologic change observed in TF-1 cells expressing SHP-2 E76K and grown in the presence of GM-CSF toward a markedly megakaryocytic phenotype is consistent with the apparent inhibition of SFK activity in response to GM-CSF stimulation after overnight factor-starvation. In a wholly unrelated attempt to assess the effect of SFK inhibition on cell growth, we similarly observed marked enlargement of cells in response to treatment with SU6656, which we later discovered was consistent with published findings of Lannutti et al., demonstrating polyploidization in response to this agent (Lannutti et al., 2005). Thrombocytopenia

is a common finding in JMML patients, and although megakaryopoiesis has not been particularly well-characterized in mouse models of JMML-associated mutations, these data suggest that the noted involvement of SFK inhibition and MAPK activation in megakaryopoiesis could be of clinical importance. The recent observation of centrosomal amplification and aneuploidy in knock-in mice expressing SHP-2 E76K suggest that the same mechanisms governing physiologic processes in megakaryocytes may also serve, when dysregulated, to generate aneuploidy and genomic instability and thereby promote oncogenesis (Xu et al., 2010).

These data have important therapeutic implications as inhibition of SFKs in JMML may effectively stunt GM-CSF-stimulated growth of both mutant and wild-type cells but would not directly interfere with the underlying mechanism of leukemogenesis. Pending confirmation by further immunophenotypic analysis, this would be the first evidence of a role for SHP-2 in megakaryocytic differentiation. Whether this is a physiologic role or could be specific to oncogenic SHP-2 due to altered substrate specificity remains to be determined. However, the role of SFKs and Ras in SHP-2-mediated signaling and the implication of both these pathways in megakaryopoiesis warrant further investigation into the role of SHP-2 in normal megakaryopoiesis and in the thrombocytopenia observed in JMML.

Materials and Methods

Cell lines: U937 and TF-1 cell lines were obtained from ATCC (Manassus, VA). 293T

cells were a generous gift from the laboratory of Dr. Kevin Shannon. U937 cells were maintained in RPMI 1640 (UCSF CCF formulation) with 10% filtered, heat inactivated, characterized fetal bovine serum (Hyclone Laboratories, Logan, UT) and penicillin, streptomycin, and glutamine. TF-1 cells were maintained in this same base media with the addition of fresh human GM-CSF at a final concentration of 2 ng/ml (Peprotech, Rocky Hill, NJ). 293T cells were maintained in DME-H21 (UCSF CCF formulation) with 10% filtered, heat inactivated FBS and penicillin, streptomycin, and glutamine. Primary murine cells were collected in IMDM with 20% FBS with penicillin, streptomycin, and glutamine.

All human cell lines were modified to express the ecotropic receptor either under a puromycin (ecoR-puro) or neomycin (RIEN) selectable marker, with the vectors generously provided by the laboratory of Dr. Scott Lowe (CSHL).

Primary cells: For experiments involving murine bone marrow cells, adult C57Bl/6 mice were sacrificed and all bones were removed and flushed with IMDM 20% fetal bovine serum (Hyclone). The resulting cell suspension was filtered through a 70um filter and red blood cells lysed with buffer containing ammonium chloride, potassium hydrogen carbonate and EDTA. The bone marrow cells were then cultured in stimulation media containing Myelocult (Stemcell Technologies, Vancouver, BC, Canada), 15% fetal bovine serum (Hyclone), 100 ng/ml IL-3, 100 ng/ml IL-6, 10 ng/ml SCF (all cytokines from Peprotech), penicillin, streptomycin, and glutamine for 3 hours prior to spinfection. For macrophage experiments, bone marrow or fetal liver cells were plated on 6cm untreated plates in Iscove's Modified

Dulbecco's Medium with 20% fetal bovine serum (Hyclone) , penicillin, streptomycin, glutamine, and 50 ng/ml of macrophage colony stimulating factor (M-CSF) (Peprotech). After 6-8 days, macrophages were starved in IMDM for 24 hours and then stimulated with 10 ng/ml murine GM-CSF (Peprotech) for 15 minutes. Cells were scraped off the plate using enzyme-free dissociation buffer (UCSF CCF formulation) and then harvested for Western blot analysis. Bones from *Lyn*$^{-/-}$ and *Hck*$^{-/-}$/*Fgr*$^{-/-}$/*Lyn*$^{-/-}$ mice were generously provided by the laboratory of Dr. Cliff Lowell (UCSF).

Proliferation assays: For proliferation assays, cells were counted by trypan blue exclusion on a hemacytometer, washed in calcium- and magnesium-free phosphate-buffered saline (UCSF CCF) and resuspended in GM-CSF-free media at a final concentration of 50,000 cells/ml in 6- or 24-well tissue culture treated plates. Cells were plated in triplicate and counted every other day by trypan blue exclusion using a hemacytometer for 14 days.

Colony assays: After transduction, murine fetal liver or bone marrow cells were sorted directly into methylcellulose medium (M3231, Stem Cell Technologies) supplemented with IMDM, betamercaptoethanol, penicillin, streptomycin, and glutamine. Recombinant murine GM-CSF (Peprotech) was then added at increasing doses up to a maximum final concentration of 10 ng/ml. For each GM-CSF dose and condition, 10,000 GFP+ cells were sorted into 1.2 ml of media and plated in 35mm

untreated tissue culture plates. After 7 days of incubation, the colonies (CFU-GM) were counted by microscopy.

Constructs. Murine *PTPN11* cDNA containing the 226G>A mutation encoding the E76K substitutions were cloned into the pENTR2B entry vector as described by Schubbert et al. (Schubbert et al., 2005) and then transferred into Gateway-modified MSCV-IRES-GFP (MIG-GW) retroviral expression vectors using Gateway LR Klonase II (Invitrogen, Grand Island, NY). shRNA sequences targeting human *PTPN11* were selected with generous help from the laboratory of Scott Lowe (CSHL) and cloned into their mir30-based LMS expression vector, which contains a GFP marker for selection. Sequence for shSHP2 1936 is as follows (target sequence in bold):
TCGAGAAGGTATATTGCTGTTGACAGTGAGCGAC**AGCATTATATTGAAACACT**ATAGT
GAAGCCACAGATGTATAGTGTTTCAATATAATGCTGGTGCCTACTGCCTCGG
pCA Src Y527F and Lyn Y508F constructs were generously provided by the laboratory of Dr. Cliff Lowell (UCSF). The cDNA sequences from these vectors were cloned into pENTR2B and recombined into the MIG-GW vector for retroviral expression.

Retroviral transduction: Briefly, 293T cells were plated at ~80% confluence in 10 cm tissue-culture treated plates one day prior to transfection. Transfection was performed using Lipofectamine 2000 (Invitrogen) reagent using 10ug each of Ecopac and DNA construct(s). The transfection mixture was prepared according to instructions and incubated for 30 minutes before being added to the 293T cells. The

Lipofectamine-containing 293T media was replaced with fresh media after 24 hours, at which time recipient cells were plated at a density of 0.5-1e6 cells per ml in 24-well Falcon tissue-culture plates. The viral supernatant was collected 48 hours post-transfection using a 10 ml syringe and passed through a 0.45 micron filter. Polybrene was added to the viral supernatant at a final concentration of 5 ug/ml for primary murine cells and 10 ug/ml for cell lines. Recipient cells were centrifuged in a tabletop Beckmann centrifuge at 2100 rpm for 5 minutes and their media replaced with 2 ml/well of viral supernatant. Spinfection was then carried out at 2100 rpm for 1.5 hours followed by 2 hours of incubation at 37deg, after which the virus was removed and replaced with fresh media. GFP analysis and/or sorting occurred 48 hours post-spinfection on BD LSRII or FACS Aria (BD Biosciences, San Jose, CA) machines, respectively.

Colony assays: After transduction, murine fetal liver cells were sorted directly into methylcellulose medium (M3231, Stem Cell Technologies) supplemented with IMDM, betamercaptoethanol, penicillin, streptomycin, and glutamine. Recombinant murine GM-CSF (Peprotech) was then added at increasing doses up to a maximum final concentration of 10 ng/ml. For each GM-CSF dose and condition, 10,000 GFP cells were sorted into 1.2 ml of media and plated in 35mm untreated tissue culture plates. After 7 days of incubation, the colonies (CFU-GM) were counted by microscopy. These experiments were performed in duplicate or triplicate in at least two independent biological replicates.

Western blot analysis: Cells were pelleted and lysed in solution containing 1% NP-40; 50 mM Tris-HCl, pH 7.4; 2 mM EDTA; 30 mM NaF; 30 mM β-glycerophosphate; 20 mM $Na_4P_2O_7$; 1 mM Na_3VO_4; and Complete protease inhibitor cocktail (Roche, Basel, Switzerland). The protein concentration was determined using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). 50 ug lysate was separated on a 10% polyacrylamide gel (Bio-Rad, Hercules, CA) and transferred to an Immobilon-P PVDF membrane (Millipore, Billerica, MA). Membranes were blocked overnight at 4°C in TBS-T (Tris-buffered saline, 0.05% Tween-20) with 5% BSA or milk, then incubated with primary antibodies in fresh blocking solution for 180 minutes at room temperature and washed in TBS-T with 0.15% Tween-20. Secondary detection was achieved by incubating with horseradish peroxidase–conjugated donkey anti–rabbit (DAKO USA, Carpinteria, CA) or anti-mouse (**???)** antibodies for 50 minutes at room temperature followed by washing and enhanced chemiluminescence (ECL) detection (GE Healthcare, Piscataway, NJ).

Antibodies: Western blotting experiments were performed using antibodies to pERK T202/Y204 (Cell Signaling Technology, cat. #9101), pSrc Y416 (Cell Signaling Technology, cat. #6943), pSrc Y527 (Cell Signaling Technology, cat. #2105), anti-phosphotyrosine, clone 4G10 (Millipore, cat. #05-321), and SHP2 (PTP1D) (BD Biosciences, cat. # 610621).

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Chapter 4:

Identification and characterization of *CBL* mutations in JMML

Introduction

Myeloproliferative neoplasms (MPNs) are clonal malignancies characterized by overproduction of immature and mature myeloid lineage cells. In particular, juvenile myelomonocytic leukemia (JMML) is an aggressive MPN of childhood characterized by malignant transformation in the stem cell compartment with clonal proliferation of progeny that variably retain the capacity to differentiate (reviewed in (Niemeyer and Kratz, 2008)). Hematopoietic stem cell transplantation (HSCT) is the only curative therapy for JMML; however, relapse rates approach 30% with this modality (Locatelli et al., 2005). While spontaneous remissions occur in some infants (Flotho et al., 2008; Matsuda et al., 2007), the underlying mechanism for this is unknown.

Extensive molecular data implicate germline and somatic mutations that deregulate Ras signaling as key initiating events in JMML, with studies showing that 60% of patients harbor an oncogenic mutation in *PTPN11*, *NRAS*, or *KRAS* while another 15% have neurofibromatosis type 1 (NF1) and demonstrate loss of the normal *NF1* allele in leukemic cells (Kalra et al., 1994; Loh et al., 2003; Niemeyer et al., 1997; Shannon et al., 1994; Tartaglia et al., 2003). Patients with the myeloproliferative subtype of chronic myelomonocytic leukemia (CMML), a similar MPN of adulthood, frequently acquire *NRAS*, *KRAS*, and *JAK2* mutations (Levine et al., 2005; Onida et al., 2002). Genetically accurate mouse models recapitulate these diseases, supporting the hypothesis that hyperactive Ras is necessary and sufficient to cause MPN (Braun et al., 2004; Chan et al., 2004; Le et al., 2004; Mohi et al., 2005). A hallmark feature of JMML and CMML is the formation of abnormally high numbers

of granulocyte-macrophage colony-forming units (CFU-GM) in methylcellulose cultures containing low concentrations of GM-CSF (Emanuel et al., 1991),(Ramshaw et al., 2002). Phosphorylation of the β^c chain of the GM-CSF receptor by the JAK2 kinase creates docking sites for adapters and signal relay molecules, resulting in activation of Ras and of downstream Ras effectors including ERK and S6.

Recently, we and others used high density single nucleotide polymorphism arrays to analyze blood and bone marrow specimens from patients with MPN (Dunbar et al., 2008; Grand et al., 2009; Loh et al., 2009; Sanada et al., 2009). These studies revealed copy-neutral loss of heterozygosity (acquired isodisomy) of a region on chromosome 11q in a subset of cases, and subsequent studies demonstrated homozygous mutations in *CBL*. Approximately 10-15% of children with de novo JMML are estimated to harbor homozygous *CBL* mutations(Loh et al., 2009; Makishima et al., 2009). *CBL* mutations are acquired somatically in adults with MPN (Dunbar et al., 2008; Grand et al., 2009; Sanada et al., 2009).

Children with NF1 and Noonan syndrome (NS) are predisposed to JMML (Bader-Meunier et al., 1997; Rauen et al.; Shannon et al., 1994; Tartaglia et al., 2002; Tartaglia et al., 2003), and we therefore considered the possibility that germline *CBL* mutations occur in some affected infants and young children. A review of the medical records of the 21 children with JMML found to have *CBL* mutations enrolled in the EWOG-MDS studies or treated at USCF (16 of 21 were previously included in a screen of a larger international cohort(Loh et al., 2009)) uncovered an unexpectedly high percentage with developmental delay and other congenital anomalies, which included cryptorchidism, and impaired growth (Tables 1 and 2). All children met

Table 1. Hematological Features at Diagnosis, Hematopoietic Stem Cell Transplantation and Current Status in 21 Children with homozygous *CBL* Mutations in JMML Cells

ID	Sex	Age and Hematological Features at Diagnosis						HSCT		Last follow-up	
		Age (years)	WBC ($x10^9$/L)	% blasts PB/BM	Platelets ($x10^9$/L)	Spleen Size[a]	Karyotype	Age (years)	MC / CC	Alive/ Dead	Age (years)
A053	M	0.1	112	1 / 2	20	9	normal	-	-	A	18.2
A054	F	1.1	36	1 / 4	95	7	normal	-	-	A	17.7
D256	M	3.6	13	1 / 6	18	10	normal	-	-	A	13.0
D048	F	1.6	23	0 / 2	46	9	normal	-	-	D	9.9
D389	M	0.6	14	0 / 6	67	8	normal	-	-	A	7.5
D088	M	0.8	46	3 / nd	32	12	normal	-	-	D	7.3
NL075	F	1.5	55	5 / 2	55	12	normal	1.8	MC	A	7.3
CZ039	M	1.1	29	3 / 4	284	6	normal	1.2	MC/CC[c]	A	7.0
D451	M	0.7	27	2 / 3	86	6	normal	1.2	MC/CC[d]	A	6.2
D647	F	2.5	67	1 / 0	117	6	normal	3.9	CC	A	6.1
I066	F	5.0	21	2 / 10	33	5	normal	5.7	MC	D	6.0
SC084	M	1.4	33	0 / 0	33	13	normal	2.0	NA	A	4.8
D104	M	2.2	27	0 / 2	232	5	normal	-	-	D	3.9
D347	M	0.9	72	4 / 6	48	10	45,XY,-16	1.2	MC	D	3.4
D703	F	1.4	27	2 / 1	15	4	46,XX+der (8)	1.7	MC	A	3.0
UPN1333	M	0.6	36	3 / 4	46	4	normal	1.1	MC	D	2.6
D251	F	0.6	31	0 / 20	37	4	normal	1.1	CC	D	2.4
UPN1778	M	1.3	22	0 / 1.5	61	5	normal	-	-	A	2.0
UPN1125	F	1.3	196	4 / 5	47	4	normal	1.7	CC	D	2.1
D774	F	1.5	22	0 / 4	266	3	normal	-	-	A	1.5
UPN1241	F	0.6	43	5 / 3	62	4	normal	1.1	MC	D	1.5

ID, patient identification. M, male. F, female. WBC, white blood count. PB, peripheral blood. BM, bone marrow. HSCT, -hematopoietic stem cell transplantation. A, alive. D, dead. MC, mixed chimerism. CC, complete chimerism. NA, not analyzed.
[a]in cm below costal margin in left midclavicular line, [b]All patients had received a myeloablative preparative regimen prior to HSCT. One patient with MC died of progressive disease (UPN 1333), the other deaths were due to transplant related toxicities. [c]patient converted back to CC after donor lymphocyte infusion, [d]patient converted back to CC without therapy

Table 2. *CBL* Mutation and Non-Hematological features in 21 Children with JMML

ID	*CBL* Mutation				Café au lait spots[m]	JXG	Crypt-orchism	Growth < 3rd percentile	Dev. delay	Hearing loss	Optic atrophy	Hyper-tension[n]	Cardio-myopathy
	Mutation	Germ	Mo	Fa									
A053[a]	p.C396R	+	-	NA	-	-	-	-	+	+	+	+	+
A054	p.Y371H	+	+	NA	-	+	female	-	-	-	-	-	-
D256[b]	p.W408R	+	-	-	+	+	-	+	+	-	+	+	-
D048[c]	p.C384R	NA	NA	NA	-	-	female	+	+	-	+	+	+
D389[d]	p.Y371C	+	-	+	+	-	+	-	+	+	+	+	+
D088	p.Y371D	NA	NA	NA	-	-	+	+	-	-	-	-	-
NL075	p.Y371H	+	-	-	-	+	female	-	-	-	-	-	-
CZ039	p.C404R	+	-	-	-	-	-	-	-	-	-	-	-
D451	p.Y371H	+	-	-	-	-	+	-	+	-	-	-	-
D647	c.1228-2A>G splice site	+	-	-	-	-	female	+	+	-	-	-	-
I066	c.1228-2A>G splice site	NA	NA	NA	-	-	female	-	-	-	-	-	-
SC084	p.Y371H	+	+	-	-	-	-	-	+	-	-	-	-
D104[e]	p.C384R	NA	NA	NA	-	+	-	-	-	-	-	-	-
D347[f]	c.1096-1G>C splice site	+	-	NA	+	+	+	+	+	-	-	-	-
D703	p.C384R	+	-	+	-	-	female	-	-	-	-	-	-
UPN1333	p.Y371H	+	+	-	-	-	-	-	+	+	-	-	-
D251[g]	p.L380P	+	NA	NA	-	+	female	+	+	-	-	-	-
UPN1778[h]	p.Y371N	+	-	NA	-	-	-	-	-	-	-	-	-
UPN1125	p.Y371H	+	+	-	-	-	female	-	-	-	-	+	-
D774[k]	p.H398R	+	+	-	-	-	female	-	-	-	-	-	-
UPN1241	p.Y371H	+	-	-	+	-	female	-	-	-	-	-	--

ID, patient identification, Germ, germline. Mo, mother. Fa, Father. NA, not analyzed. JXG, juvenile xanthogranuloma. Dev., developmental.

[a]autoimmune thyroiditis with anti-thyroglobulin antibodies; echocardiography with mitral insufficiency grade II, myocardial hypertrophy, hypoelastic and hypoplastic ascending aorta and arch of aorta

[b]Diagnosis of intracranial germinoma at age 9 years with homozygous CBL mutation in the brain tumor

[c]Diagnosed with Takayasu arteritis, type III, died 9 months after diagnosis of vasculitis and stenting of major arteries

[d]Father with history of Hodgkin lymphoma

[e]Sudden death at home

[f]Died of cerebral hypoxia, also had a history of supraventricular tachycardia

[g]Prior to HSCT, was diagnosed with erythroderma, post-HSCT: toxic epidermal necrolysis, acute liver failure, liver Tx, subtotal brain infarction, apallic syndrome

[h]Pectus excavatum

[k]Older sister with heterozygous mutation

[m]Patients indicated by **+** have 1-2 café au lait spots, there was no patient with 6 or more café au lait spots

[n]Measurements of blood pressure following HSCT were excluded from analysis

diagnostic criteria for JMML(Chan et al., 2008; Hasle et al., 2003) but six patients with a follow-up of more than seven years did not undergo transplantation for various reasons. Of these, one died of progressive JMML (D088), but the MPN improved spontaneously in five others. All of these patients continued to display variable degrees of splenomegaly in the presence of normal blood counts and exhibited the persistence of a homozygous *CBL* mutation in CD4, CD8, CD14, and CD19 sorted cells from their peripheral blood at last follow-up. In addition, four of these patients have developed clinical signs consistent with vascular pathology, including optic atrophy, hypertension, and an acquired cardiomyopathy; one was diagnosed with Takayasu arteritis, type III by angiography (Figure 1e). Another patient (D256) developed an intracranial germinoma that was shown to harbor the same homozygous *CBL* mutation as in his bone marrow. Of note, among the patients treated with HSCT, there was a high rate of conversion to stable mixed chimerism (8/11 patients with available data) (Table 1).

We analyzed normal tissues from 17 of these children and detected a heterozygous *CBL* mutation in each of them. Mutational analysis of parental DNA was informative in 13 families and confirmed autosomal inheritance of a *CBL* mutation in seven (Figure 1a, 1b, and Table 2). Patients UPN1333 and UPN1125 were from large pedigrees in which several individuals had died of JMML (Figure 1a and b).

The proband in family 1 (UPN1333, V:1) was referred after transplantation for JMML. Initially diagnosed at 7 months of age, he received his first HSCT at age 13 months and then developed mixed chimerism 6 months later. A blood sample

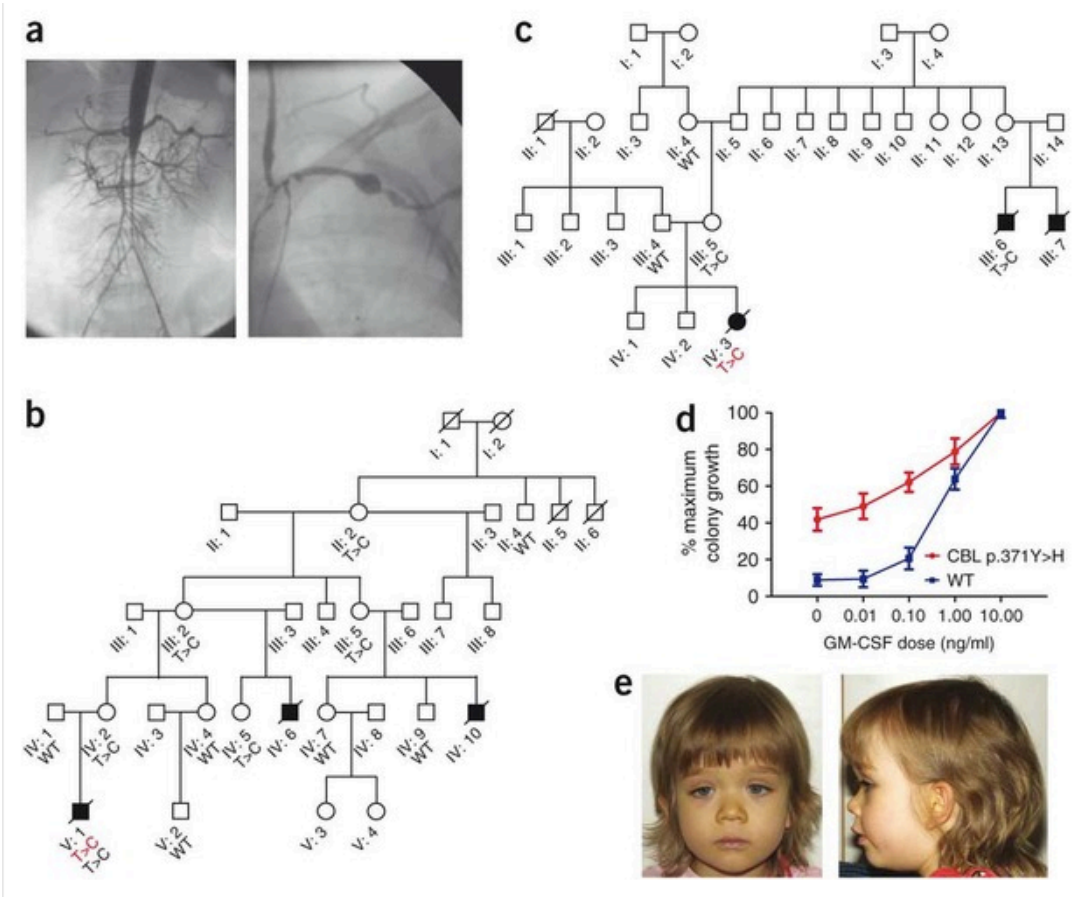

Figure 1. Germline mutations in *CBL* can be inherited in an autosomal dominant fashion and are associated with a phenotype, GM-CSF hypersensitivity and vasculitis. Panel (a) The family tree of UPN1333 is shown in panel a, where the diseased bone marrow of UPN1333 displayed a homozygous *CBL* c.1111T>C (red) mutation as well as a heterozygous lesion from his buccal swab (black). Only women appear to be heterozygote carriers, and only boys appear to be affected by JMML in this family. Panel (b) The bone marrow of UPN1125 demonstrated a homozygous *CBL* mutation—her mother (III:5) is a known carrier, and she had two male cousins dying from JMML (III:6, III:7). Her father and aunt refused participation in this study. Panel (c) demonstrates a classic GM-CSF hypersensitivity response on a colony assay for patients with CBL mutations (n=3) versus normal (n=13). Error bars represent standard error of the mean (s.e.m.) Panel (d) shows one toddler (D703) diagnosed with JMML and a homozygous mutation at p.C384R. She displays frontal bossing, downslanting palpebral fissures, hypertelorism, and a low nasal bridge. Of note, both father and daughter also display bilateral ptosis. Panel (e) demonstrates the angiograms from the aorta and left subclavian artery from patient D048 nine months after the diagnosis of Takayasu arteritis type III.

displayed a homozygous *CBL* mutation at c.1111T>C (Y371H). Importantly, analysis of buccal swab DNA revealed both the mutant c.1111T>C allele and a wild-type 1111T allele. The family pedigree is shown in Figure 1a. Both maternal relatives died from progressive JMML. Peripheral blood or buccal swabs from extended family members revealed multiple heterozygous individuals (Figure 1a). Interestingly, detailed medical history from the affected maternal great-grandmother revealed a history of infant leukemia characterized by a high white blood cell count and splenomegaly that resolved spontaneously. Sorted B (CD19) and T (CD3) cells, and granulocytes from her peripheral blood currently display a heterozygous c.1111T> C. Leukemia cells from UPN 1333 displayed a classic pattern of GM-CSF hypersensitivity in methylcellulose cultures (Figure 1c) and increased phosphorylation of STAT5 in response to low doses of GM-CSF (Kotecha et al., 2008). Peripheral blood mononuclear cells from his heterozygous mother did not display either of these features, suggesting that homozygosity for the mutant *CBL* allele is essential for these cellular behaviors (data not shown).

The proband in family 2 (UPN1125, IV:3) was a girl diagnosed at 15 months of age with JMML. She also harbored a homozygous c.1111T>C *CBL* mutation in her bone marrow. The family history revealed that her mother had two male first cousins who were diagnosed with JMML at 6 months of age and died before age 10. The first boy had archived frozen liver tissue available from autopsy and demonstrated a heterozygous c.1111T>C mutation. Interestingly, he also developed clinical signs and laboratory values consistent with small vessel vasculitis prior to his death. Buccal swabs were obtained from a limited number of family members

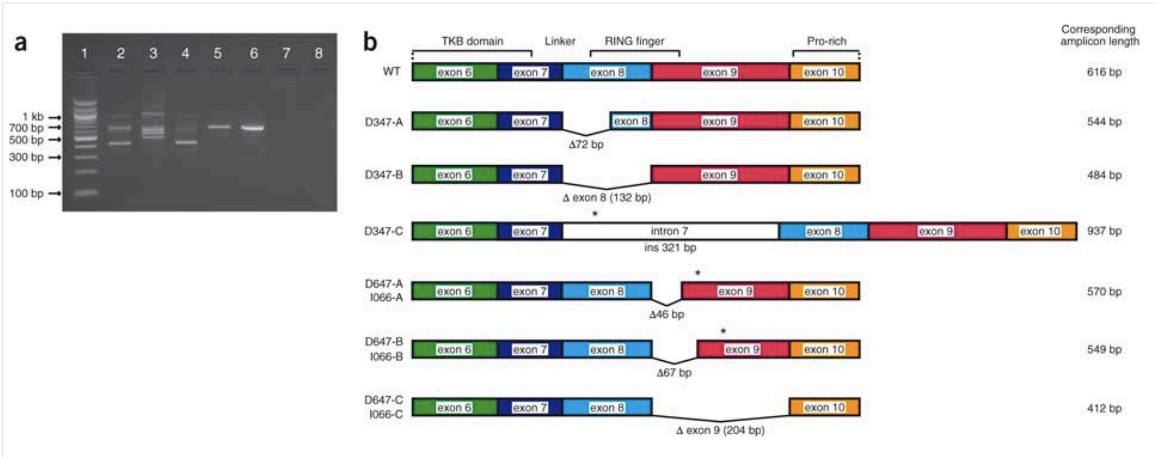

Figure 2. Consequences of splice site mutations in cDNA from D347, 647, and I066. Panel (a) RT-PCR using an exon 6 forward primer and an exon 10 reverse primer on cDNA generated from these patients. The wild-type amplicon is 616 base pairs long. Lane 1: MW ladder, Lane 2: I066, Lane 3: D347, Lane 4: D647, Lane 5: *CBL* point mutant, Lane 6: HM2833 *CBL* wild-type, Lane 7: Genomic DNA control, Lane 8: no template control. Panel (b) is a schematic representation of the splice site variants detected either recurrently (D347) or that were shared by I066 and D647. Deletions of base pairs are indicated by Δ# of base pairs and insertions by ins # base pairs. Premature stop codons are indicated by asterisks (*).

(Figure 1b) of UPN1125, and her mother was found to carry a heterozygous *CBL* mutation.

Three patients in our series displayed homozygous splice site mutations (Table 2, I066, D647, and D347). RT-PCR demonstrated several new splice products arising from these mutations (Figure 2); most notable are the 2 splice site variants that either delete the entirety of exon 8 (D347) or exon 9 (I066 and D647) or retain an interstitial intron (e.g. intron 7 for D347). Each of the deletion splice variants encodes a protein that is predicted to lack critical regions of the linker and RING finger domains, while the intron 7 retention introduces a premature stop codon to abort translation upstream of the RING finger domain.

To investigate the functional properties of the variant mutant Cbl proteins encoded by homozygous point mutations, we first studied the effect of the common p.Y371H substitution on the growth of primary hematopoietic cells from murine fetal liver. This system reproduces the hypersensitivity to GM-CSF that is characteristic of JMML (Emanuel et al., 1991; Schubbert et al., 2005). Fetal liver cells transduced with retroviral vectors expressing wild-type or mutant Cbl proteins demonstrated no increased sensitivity to GM-CSF (Figure 3a). Similarly, expression of p.Y371H Cbl in a BaF3-EpoR cell line did not confer cytokine independence (Figure 3b). Based on the observation that JMML cells invariably lose the normal *CBL* allele and on recently published data(Sanada et al., 2009), we reasoned that reduction of normal Cbl expression might be mandatory to deregulate hematopoietic growth. Therefore, we introduced a short hairpin RNA, which markedly reduced the expression of murine Cbl in BaF3-EpoR cell lines (Figure 3c). We next transduced these cells with a series of wild-type and mutant human constructs and demonstrated strong expression of exogenous Cbl (Figure 3c). In this context, we observed cytokine independent proliferation (Figure 3d) upon expression of p.Y371H Cbl and a known murine oncogenic Cbl protein (70Z)(Andoniou et al., 1994; Blake et al., 1991; Langdon et al., 1989). The 70Z oncogenic protein deletes out 17 amino acids located from position 366-382 in the linker domain. Furthermore, the p.Y371H and 70Z transduced cells demonstrated hypersensitivity to increasing concentrations of human EPO (Figure 3e), mimicking the growth factor hypersensitivity seen in JMML (Figure 1d).

We next examined the biochemical consequences of mutant Cbl expression.

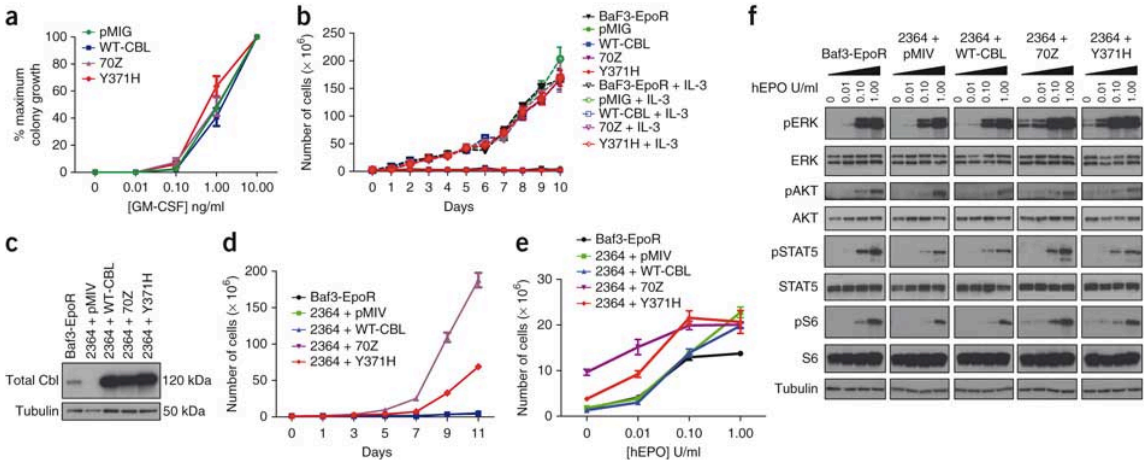

Figure 3. p.Y371H does not confer cytokine sensitivity or cytokine independent growth until silencing of murine Cbl. Panel (a) Transduction of p.Y371H or the known murine oncogenic mutant 70Z in wild-type hematopoietic cells from fetal liver, did not confer hypersensitivity to GM-CSF, nor did expression of these mutants in BaF3-EpoR cells result in cytokine independent growth (b). Panel (c) An shRNA to murine Cbl (cbl.2364) demonstrated near complete shutdown of expression in BaF3-EpoR cells by Western blot with re-expression upon introduction of the human WT, 70Z, or p.Y371H in these same cell lines. Panel (d) Both the p.Y371H and 70Z Cbl conferred cytokine independent growth in the presence of cbl.2364. Controls included the Venus vector pMIV and BaF3-EpoR. Error bars for triplicate replicates (s.e.m.) are shown and when not visible, indicate tight clustering. Using a paired t-test: day 7 comparing 2364+ WT-Cbl versus 2364+p.Y371H, p-value= 0.017, and at day 9: p-value <0.001. Panel (e) Serial transduction of the hairpin (2364) and p.Y371H or 70Z constructs also conferred hypersensitive growth after assessing cell proliferation on day 5 in increasing concentrations of Epo. Using a paired t-test at each concentration of Epo when comparing 2364+WT-Cbl versus 2364+p.Y371H: Epo 0 unit/ml: p = 0.036, Epo 0.01 units/ml: p= 0.0015, Epo 0.1 units/ml: p= 0.029, Epo 1 unit/ml (saturating dose) P= 0.697. Panel (f) Both the p.Y371H and 70Z containing cells demonstrated activation of pERK, pAKT, and pS6 in the absence of Epo or in low dose 0.01 unit/mL of Epo in comparison to negative controls. All cell proliferation work was done in triplicate.

In cells depleted of endogenous Cbl and expressing exogenous p.Y371H and 70Z Cbl proteins, we observed constitutive phosphorylation of ERK, AKT and S6 in cells deprived of cytokine. We also found heightened responses to low dose EPO (Figure

2f).

In order to determine if mutant Cbl proteins retain E3 ligase activity, we assessed their ability to promote ubiquitylation of a known Cbl substrate. Soon after activation, the epidermal growth factor receptor (EGFR) undergoes Cbl-dependent polyubiquitylation and proteosomal degradation. HA-Cbl(p.Y371H)-expressing HEK293 cells exhibited markedly elevated levels of phosphorylated EGFR upon EGF stimulation in comparison to HA-Cbl(WT)-expressing counterpart (Figure 4a), which indicates a possible defect in clearance of pEGFR by Cbl(p.Y371H). Cbl(p.C384R) mutant exhibited a similar defect in pEGFR clearance (data not shown). Upon polyubiquitylation of targets, Cbl promotes its own polyubiquitylation and subsequent auto-degradation. Consistent with this model, levels of Cbl(WT), but not Cbl(p.Y371H or p.C384R), rapidly diminished following EGF treatment in the absence of proteasome inhibitor MG132 (Figure 4a, b and data not shown,). However, Cbl(WT) levels were markedly stabilized in the presence of MG132 while Cbl(p.Y371H) levels remained relatively high irrespective of MG132 (Figure 4b), which suggest that Cbl mutants p.Y371H and p.C384R have an inherent defect in E3 function. Consistent with this notion, Cbl(WT) promoted robust polyubiquitylation of pEGFR while p.Y371H and p.C384R showed diminished capacity to polyubiquitylate pEGFR in an in vitro ubiquitylation assay (data not shown), similar to that of Cbl(70Z), which is known to be defective in E3 ligase activity, and in concordance with recently published data by Sanada et al (Sanada et al., 2009), who demonstrated that p.Y371S and p.Q367P mutants detected in human patients also have diminished ubiquitylation activity. The Y371 residue most

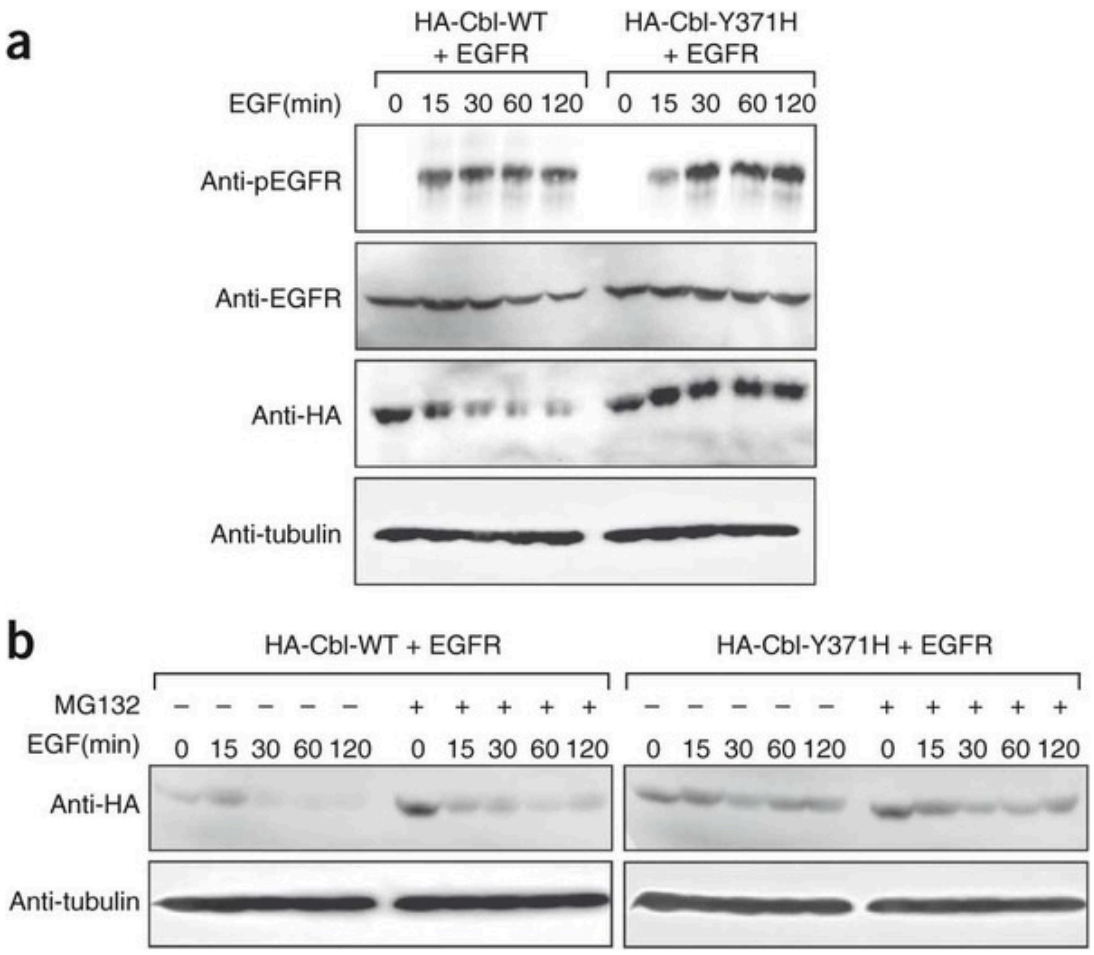

Figure 4. Cbl mutants exhibit prolonged protein turnover and are associated with increased phosphorylated EGFR levels upon EGF stimulation. Panel (a) HEK293 cells transfected with plasmids encoding EGFR in combination with HA-Cbl(WT) or HA-Cbl(p.Y371H) were serum starved for 18 h followed by 15 min EGF (50 ng/ml) stimulation. Cells were then washed and maintained in serum-free media for the indicated periods of time. Equal amounts of whole cell extracts were resolved on SDS-PAGE and immunoblotted with the indicated antibodies. Panel (b) HEK293 cells transfected with plasmids encoding EGFR in combination with HA-Cbl(WT) or HA-Cbl(p.Y371H) were serum starved for 18 h followed by 15 min EGF (50 ng/ml) stimulation. Cells were then washed and maintained in serum-free media with (+) or without (-) MG132 for the indicated periods of time. Equal amounts of whole cell extracts were resolved on SDS-PAGE and immunoblotted with the indicated antibodies.

commonly affected in germline *CBL* mutations has been the focus of extensive biochemical analysis (Sanada et al., 2009). This literature supports a key role for Y371 in maintaining the integrity of the alpha-helical structure of the linker region,

which plays a critical role in substrate specificity. Interestingly, Y371 has been found to be phosphorylated despite its predicted location away from the protein surface. Substitution of this residue with phenylalanine results in a non-oncogenic form of the protein, which lacks E3 ligase activity. Conversely substitution with a glutamate constitutively activates E3 ligase activity (Kassenbrock and Anderson, 2004; Thien and Langdon, 2001).

In most ways, *CBL* appears to function as a classic tumor suppressor gene in this cohort, with germline heterozygosity predisposing to neoplasia upon reduction to homozygosity in target tissues. However, the predominance of specific missense mutations makes *CBL* distinct from most tumor suppressor genes such as *RB* and *NF1*, which typically demonstrate more severe loss of function mutations such as deletion or protein truncation. This suggests that the mutant Cbl proteins retain an essential biochemical function (Loh et al., 2009; Sanada et al., 2009). It is further supported by our data showing that exclusive expression of a mutant *CBL* allele has positive effects on cytokine signaling and proliferation. The specific disruption of E3 ligase activity may leave intact adapter functions, resulting in a relative imbalance of CBL's positive and negative roles in signal transduction. Sanada et al hypothesized that this may result in inhibitory effects on Cbl-b, a related family member(Sanada et al., 2009). This is similar to what has been reported for p53, a classic tumor suppressor gene with specific gain of function mutations in the context of loss of heterozygosity (Dittmer et al., 1993; Lang et al., 2004). Beyond missense mutations, truncated Cbl proteins are also known to confer transforming effects by countering the negative action of full-length Cbl on RTK signaling. The *Drosophila* analogue of

the mammalian v-Cbl oncogene (i.e. Dv-Cbl), for example, has been demonstrated to function as oncogenic dominant-negative variant whose expression resulted in vivo in enhanced signaling of the EGFR cascade and cooperated with activating mutations in the Ras pathway to ultimately produce melanotic tumors in flies (Robertson et al., 2000)

In addition, clinical data reveals that some patients may experience spontaneous resolution of JMML but go on to develop clinical features consistent with vasculitis and other autoimmune phenomena later in life. Interestingly, autoimmunity has also characterized genetically engineered mouse strains with Cbl mutations. *Lck-Cre*$^{+}$ c-*Cbl*$^{flox/flox}$ *Cbl-b*$^{-/-}$ mice, which delete c-Cbl in T cells, develop severe vascular lesions with massive infiltration of T-cells and high concentrations of anti-double stranded DNA antibodies (Naramura et al., 2002). Furthermore, these T cells are hypersensitive to T cell receptor signaling and display prolonged ERK phosphorylation. Similarly, *Cd19-Cre*$^{+}$ c-*Cbl*$^{flox/flox}$ *Cbl-b*$^{-/-}$ mice that delete c-*Cbl* in B cells develop a lupus-like syndrome associated with perivascular infiltration and hyperactivation of B cell receptor signaling (Kitaura et al., 2007). In mice, loss of *Cbl-b* is required for these phenotypes. Oncogenic c-*Cbl* proteins may inhibit *Cbl-b in vivo*, resulting in a functional loss of both *Cbl* and *Cbl-b*, which in turn contributes to dysregulated lymphocyte signaling and subsequent vasculitis. Indeed, the redundant role of c-*Cbl* and *Cbl-b* was further explored by Sanada, et al, who demonstrated the inhibitory effects of c-*Cbl* p.Y371S on wild-type *Cbl-b* (Sanada et al., 2009). It is of clinical interest that patients with JMML and homozygous *CBL* mutations who undergo HSCT are not known to develop vasculitis later in life,

implying that a normal immune system is critical to preventing this late manifestation.

We describe a new syndrome in which affected children display several congenital anomalies that overlap with NF1, NS, and Legius, suggesting that the affected proteins converge on the Ras/MAPK pathway. Indeed, several Ras/MAPK pathway proteins regulate developmental programs in multiple species - for instance, the Drosophila homologs of each of these genes--*CBL* (D-cbl), *PTPN11* (csw), *NF1*, and *SPRED* perform critical functions for growth and patterning (Hashimoto et al., 2002; Oishi et al., 2006; Pai et al., 2000; The et al., 1997).

Patients with germline *CBL* mutations are at increased risk of developing JMML, which may follow an aggressive clinical course or resolve without treatment. Some affected individuals develop vasculitis later in life. The *CBL* mutations found in JMML can arise de novo or can be transmitted through the germline, and human leukemia samples invariably show loss of the normal *CBL* allele. Consistent with this tumor suppressor function, JMML-associated Cbl proteins confer cytokine hypersensitivity in transduced BaF3-EpoR cells in the absence of wild-type Cbl, have defective E3 ligase activity, and constitutively activate key Ras effector pathways. The role of aberrant Cbl signaling in vasculitis remains to be determined, and it will be particularly interesting to investigate if patients with germline *CBL* mutations who have been cured of JMML after HSCT remain at risk of developing vasculitis. It is also of great interest that some of these patients continue to display homozygous *CBL* mutations in their peripheral blood despite having improved their blood counts. Finally, our data provide strong evidence that Cbl is a key negative regulator of Ras

signaling networks in hematopoietic cells and it will be important to identify key targets of the Cbl ubiquitin ligase and to uncover other biochemical mechanisms involved in growth control.

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Materials and Methods

*Subjects***.** Patients were diagnosed and treated either in Europe under the auspices of the European Working Group of Myelodysplastic Syndromes in Childhood (EWOG-MDS) or enrolled as research subjects at the University of California, San Francisco. The Committees on Human Research at each of the institutions in EWOG-MDS, as well as UCSF approved these studies. Informed consent was obtained from parents or guardians, and in the case of pedigree analysis, all screened relatives. Family and clinical histories were reviewed, as were physical exams at diagnosis.

*Mutation Screening***.** Bone marrow or peripheral blood samples at diagnosis were obtained. Mononuclear cells were isolated using standard Histopaque 1111. Buccal swabs, fibroblasts, or tissues unaffected by tumor were also obtained when available. Genomic DNA was extracted using PureGene reagents (Qiagen, Foster City, CA). Patients were screened for mutations in *CBL*, *NRAS*, *KRAS*, and *PTPN11* as previously described (Kalra et al., 1994; Loh et al., 2009; Loh et al., 2003). RNA was prepared according to usual methods. cDNA was generated using the SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen). Splice variants were identified through PCR using an annealing temperature of 58 °C and the following primers: 5'-TTGAGGGAACACATACTCGCT-3' and 5'-TATGTTACTGCTGATGGGAACA-3'. Splice variants were gel extracted using the QIAquick Gel Extraction Kit (Qiagen). The resulting fragments were subcloned using the TOPO® TA Cloning Kit (Invitrogen) according to the manufacturer's instructions. Positive colonies were

picked, mini prepped using the QIAprep Spin Miniprep Kit (Qiagen), and sequenced with standard M13 Forward (-20) and M13 Reverse primers.

*Cbl and shRNA expression constructs***.** For the CFU-GM experiments, Gateway technology (Invitrogen) was used to clone WT and mutant *Cbl* cDNAs into the murine stem cell virus (MSCV) backbone containing a green fluorescent protein (GFP) cassette driven by an internal ribosome entry site (IRES) downstream of the *Cbl* sequence (pMIG). The human WT and 70Z Cbl plasmids were a kind gift from Hamid Band (Levkowitz et al., 1998). For the subsequent experiments using Ba/F3 cells, the same *Cbl* cDNAs were cloned into an MSCV-IRES-Venus (pMIV) backbone to allow for co-transduction with the GFP-tagged shRNA. miR30 based shRNA sequences targeting murine *Cbl* were graciously designed by Johannes Zuber and Scott Lowe. We selected one of these putative sequences (cbl.2364) and custom ordered a single 110-bp oligonucleotide (Bioneer) to serve as a template for PCR amplification. PCR products were digested with *XhoI* and *EcoRI* and ligated into the LTR-driven MiR30 SV40-GFP (LMS) MSCV-based vector (also graciously provided by the Lowe lab) to produce the LMS-2364 retrovirus encoding the *Cbl* shRNA. The sequence for cbl.2364 is:
TCGAGAAGGTATATTGCTGTTGACAGTGAGCGATACCTATGAAGCGATGTATAATAGTG
AAGCCACAGATGTATTATACATCGCTTCATAGGTACTGCCTACTGCCTCGG.

Hematopoietic progenitor assays. All experimental procedures involving mice were reviewed and approved by the UCSF Committee on Animal Research. These assays

were performed as described previously using murine fetal liver cells transduced with MSCV-*Cbl*-IRES-GFP retroviruses engineered to express WT or mutant Cbl proteins. For the human CFU-GM assays, mononuclear cells from peripheral blood or bone marrow were plated in MethoCult H4230 (StemCell Technologies), supplemented with recombinant human GM-CSF (Peprotech) and counted 14 days later as described previously [28]. For the CFU-GM assays, GFP-positive cells were sorted using a FACS Aria (BD Biosciences) and then seeded in methylcellulose medium (M3231; StemCell Technologies) (Schubbert et al., 2007), supplemented with recombinant murine GM-CSF (Peprotech). Colonies were counted by indirect microscopy after 8 days.

*Cell Viability and Proliferation assays and Western blots***.** Murine pro-B Ba/F3 cells were transduced with MSCV-EpoR-IRES-puro as described previously (Mullighan et al., 2009). These cells were maintained in RPMI-1640 with 10% FCS (HyClone), penicillin, streptomycin, L-glutamine, 10 ng/ml puromycin (Calbiochem), and 10 μg/ml murine IL-3 (Peprotech). The Ba/F3-EpoR cells were transduced with the LMS-2364 construct or the LMS vector alone. GFP-positive cells were sorted on a FACS Aria (BD Biosciences) and then transduced with the MSCV-*Cbl*-IRES-Venus retroviruses expressing WT or mutant *Cbl* proteins. Cells positive for both GFP and YFP expression were sorted on the FACS Aria and studied in proliferation and Western blot assays.

For the proliferation assays, cells were washed 3x and then cultured for 6 hours in

cytokine-free media before being plated in 6-well plates at a density of 500,000 cells/ml at increasing doses of hEPO (R&D Systems). Growth was monitored every other day using a ViCell cell counter (Beckman Coulter).

For Western blot analysis, cells were washed 3x and cultured for 6 hours in cytokine-free media before being stimulated for 15 minutes with increasing doses of hEPO (R&D Systems). Whole-cell lysates were blotted and probed with the following antibodies: anti-phospho-p44/42 mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 (ERK1/2) (Thr202/Tyr204, cat. 9101), anti-phospho-AKT (S473, cat. 4060), anti-phospho-S6 (Ser235/236, cat. 2211) (all from Cell Signaling Technology: catalog); anti-phospho-STAT5 (cat. 44-390G) (Invitrogen) and anti-alpha-tubulin (Abnova). ERK1/2 (cat. 9102), AKT (cat. 9272), S6 (2217), and STAT5 (9363) antibodies were from Cell Signaling Technology.

*HEKCells***.** HEK293 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Wisent, St. Bruno, QC, Canada) at 37°C in a humidified 5% CO_2 atmosphere.

*Antibodies***.** Mouse monoclonal antibodies against HA (12CA5) and α-tubulin were obtained from Boehringer Ingelheim (Laval, QC, Canada) and Sigma (Milwaukee,

WI), respectively. Rabbit polyclonal antibodies against pEGFR, EGFR and ubiquitin were obtained from Upstate (Temecula, CA), Santa Cruz Biotechnology (Santa Cruz, CA) and DAKO Canada (Mississauga, ON, Canada), respectively. EGF ligand was obtained from Sigma (Milwaukee, WI). MG132 proteasome inhibitor was obtained from Boston Biochem (Cambridge, MA).

*Plasmids***.** Plasmids encoding HA-CBL(WT, 70Z, Y371H, C384R) were subcloned into the pcDNA-DEST4.0 vector via Gateway Cloning technology (Invitrogen, Carlsbad, CA) and confirmed by DNA sequencing. Plasmid encoding EGFR was generated as previously described (Wang et al., 2005).

*Immunoblotting and immunoprecipitation***.** Immunoprecipitation and immunoblotting were performed as described previously (Ohh et al., 1998). Cells were lysed in EBC buffer (50mM Tris pH 8.0, 120mM NaCl and 0.5% NP-40) supplemented with protease and phosphatase inhibitors (Roche, Laval, QC, Canada). Cell lysates were immunoprecipitated with indicated antibodies in the presence of Protein-A agarose beads (Repligen, Waltham, MA). Bound proteins were washed five times with NETN buffer (20mM Tris pH 8.0, 120mM NaCl, 1mM EDTA, and 0.5% NP-40), eluted by boiling in sodium dodecyl sulfate (SDS)-containing sample buffer, and resolved by SDS-polyacrylamide gel electrophoresis (PAGE).

*In vitro ubiquitylation assay***.** In vitro ubiquitylation assay was performed as described previously (Ohh et al., 2000)

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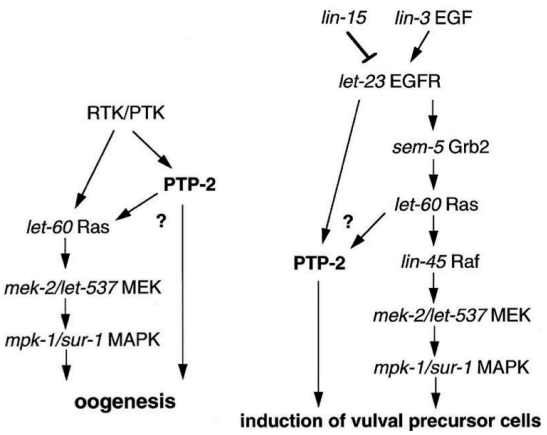
Chapter 5:

Conclusions and Future Directions

The vast amount of genetic and biochemical data, across tissue types and organisms, in physiologic and pathologic states, that has been generated since the cloning of SHP-2 and its homologs over two decades ago in an attempt to address this seemingly straightforward question as to how this SH2-domain containing protein tyrosine phosphatase functions as a signaling molecule is striking in the consistency of its inconsistencies. Early on it was recognized that this evolutionarily conserved PTP functions primarily as a positive effector of growth factor signaling and is intimately linked to the Ras/MAPK signaling pathway, but the consensus more or less ends there. A review of even the 'simplest' models derived from classic genetic experiments in *C. elegans* and *Drosophila* reveals a striking degree of complex epistasis, replete with multiple arrows and question marks that persist to this day (Allard et al., 1996; Cleghon et al., 1998; Gutch et al., 1998). Even within a single growth factor signaling pathway, the importance of both catalytic and non-catalytic functions of SHP-2, acting both upstream and downstream of Ras, have been demonstrated time and again (Fig. 1).

The relationship between SHP-2 and Ras/MAPK pathway effectors in development has been duly confirmed in human disease, with the identification of phenocopying, mutually exclusive, gain-of-function mutations in juvenile myelomonocytic leukemia and in the inherited 'RASopathies' like Noonan syndrome. However, even here the relationship is convoluted, as *PTPN11* mutations are responsible for 90% of cases of LEOPARD syndrome (Legius et al., 2002), as compared with 50% of cases of the phenotypically very similar Noonan syndrome (Tartaglia et al., 2001), but evidence suggests their pathogenesis is distinct.

a)



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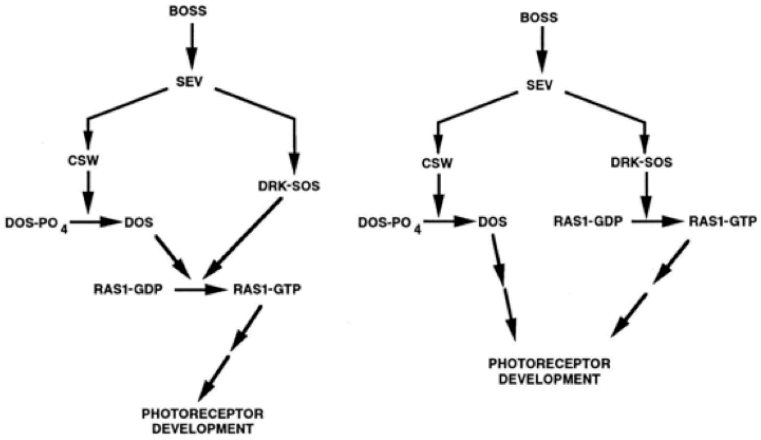

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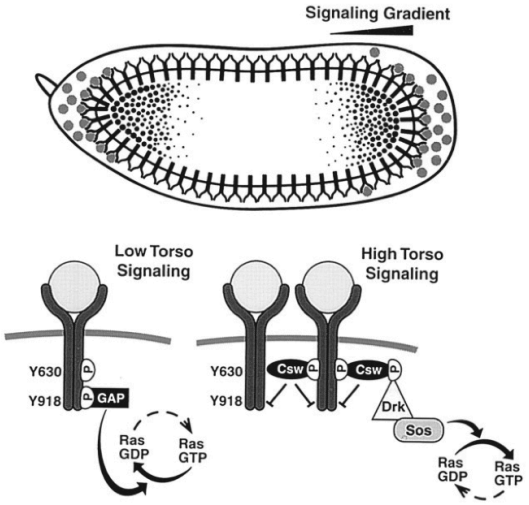

Figure 1. Models of SHP-2 homolog signaling downstream of growth factor receptors. a) Models for *C. elegans ptp-2* signaling during oogenesis and vulval development. Adapted from Gutch et al (Gutch et al., 1998). b) Models for *Drosophila* CSW and DOS during R7 differentiation. Adapted from Allard et al (Allard et al., 1996). c) Models for *Drosophila* Torso-dependent signal transduction. Adapted from Cleghon et al (Cleghon et al., 1998).

LEOPARD syndrome (LS) is characterized by lentigines (dark freckle-like melanocytic lesions) and hypertrophic cardiomyopathy, which are rarely observed in *PTPN11*-associated NS, as well as a predisposition to acute leukemia and neuroblastoma, as compared with the transient JMML-like MPD observed in NS patients. The mutations identified in these diseases are biochemically distinct as well, as nearly all *PTPN11* mutations identified in JMML and NS target the interface between the N-SH2 and PTP domains and result in increased catalytic activity, whereas LS-associated mutations target the catalytic domain and affect catalytic residues, resulting in mutants with decreased or absent catalytic activity that, in fact, exert dominant negative effects in vitro (Kontaridis et al., 2006) Modeling of these mutants in zebrafish suggested, again, a dual role for SHP-2 in pathogenesis, with catalytic activity being required for activation of MAPK but catalytic-independent function of the LS-associated SHP-2 mutants being sufficient to inhibit p53-mediated cell death in neural crest cells (Fig. 2).

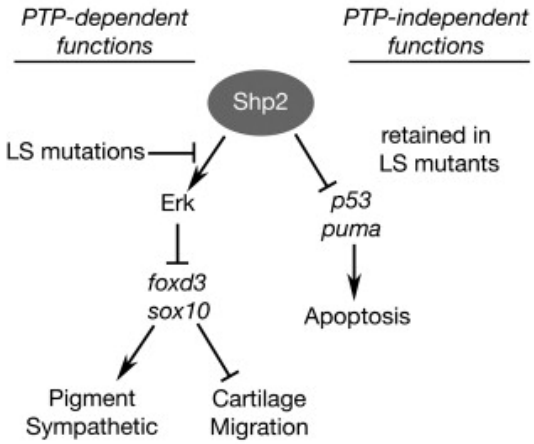

Figure 2. Model of catalytic-dependent and -independent functions of SHP-2 during neural crest development and subsequent effects of LS mutants. Adapted from Stewart et al (Stewart et al., 2010).

SHP-2 is also structurally convoluted in that each and every one of its protein interaction motifs is phosphotyrosine-dependent, whether it be its own phosphotyrosine residues at Y542 and Y580, or its N- and C-SH2 domains, or its PTP domain. Thus the identification of a phosphorylation-dependent protein interaction leaves open the question of whether this relationship is adaptor in nature, or catalytic, or both, as in the case of csw and torso, particularly given that engagement of the SH2 domains (by phosphotyrosine) is required for catalytic activity (tyrosine dephosphorylation). Evidence even suggests that this modular reciprocity could result in an additional intramolecular interaction, with not only the N-SH2 domain creating autoinhibitory contacts with the PTP domain to block substrate access, but also the N- and C-SH2 domains interacting with the C-terminal phosphotyrosine residues to relieve its own autoinhibition and promote PTP activation. The thorough mutational biochemical analysis done by Keilhack et al. (Keilhack et al., 2005) further highlights the implications of this parallel organization, as different mutations depending on their location were found to affect catalytic activity either directly by promoting the active state or indirectly by increasing the affinity of the SH2 domains for its phosphotyrosine-containing peptide ligands. Their work and that of Flint et al (Flint et al., 1997) demonstrating the varying effects of specific substitutions on catalytic activity and substrate affinity reinforce the interrelatedness of these domains and their complex regulation.

The overwhelming conclusion to be drawn from all this work is: 'it depends.' That is, that the specific action of SHP-2, whether catalytic-dependent or -independent, upstream or downstream of Ras/MAPK, or involving some other

pathway altogether, truly depends on the specific cell-type, growth-factor, and in the case of a pathologic function, the specific genotype and phenotype in question. To that end, our studies are unique in that they have focused quite specifically on the role of SHP-2 E76K in hematopoietic cells in response to GM-CSF signaling and began from as unbiased a perspective as we could manage, with no expectations derived from previous studies in heterologous systems with wild-type or different mutant versions of SHP-2. Our specific decision to compare a substrate trapping E76K containing the C459S+D425A combination optimized by Agazie et al. with a non-substrate binding E76K containing the R465M substitution described by Flint et al. to strongly decrease substrate affinity was also particularly suited to the specific identification of enzyme-substrate, rather than purely structural adaptor-type interactions.

The IP-MS approach proved to be efficient for the recovery of SHP-2 by either tandem- or single-affinity tag purification, but the recovery of binding partners proved to be much more challenging. This was likely due to two features: first, the purification of a temporally constrained, non-stoichiometric enzyme-substrate complex as compared with the components of the ribonucleoprotein spliceosome complex purified from yeast in the original tandem affinity purification paper (Rigaut et al., 1999) or the components of the DNA-dependent protein kinase holoenzyme purified from HEK293 cells in the subsequent paper detailing the mammalian-optimized GS-TAP system. Numerous rounds of troubleshooting suggested that while expression was important to reach a minimum level of detection by mass spectrometry, the more critical factors turned out to be related to

time and processing. The priority on specificity and 'cleanliness' favored by the tandem approached turned out to work against the recovery of these delicate complexes, as did the instability of phosphotyrosines to extended processing times and the extremely non-stoichiometric relationship between enzyme and substrate. However our eventual identification of parafibromin by mass spectrometry, and our subsequent validation of that interaction between the endogenous protein and the substrate-trapping SHP-2 mutant is noteworthy, particularly as it was also identified as a SHP-2 substrate by Takahashi et al (Takahashi et al., 2011), who also worked out in great detail a mechanism involving the stabilization and nuclear accumulation of β-catenin (Fig. 3). Although their investigation was performed in a heterologous system involving fibroblasts and a SHP-2 mutant identified from hepatocellular carcinoma, our systems have in common the goal of identifying mechanisms important for transformation, as their mutant was selected based on its ability to transform 3T3 cells, much as the JMML-associated E76K mutant has been

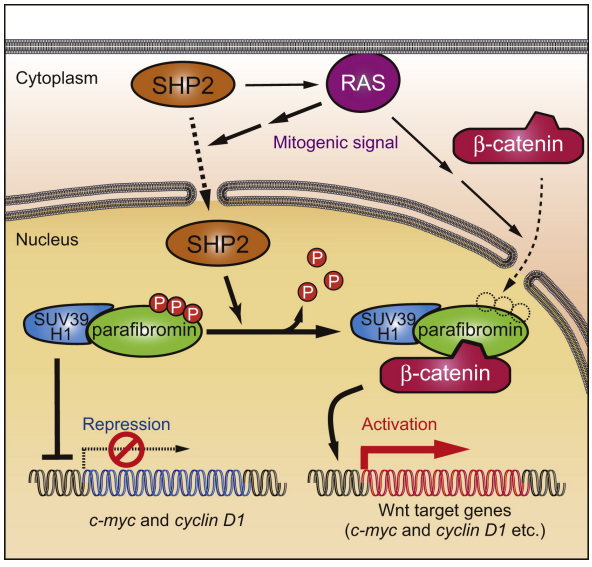

Figure 3. Model of SHP-2-mediated transformation via deregulation of both Ras and Wnt pathways. Adapted from Takahashi et al (Takahashi et al., 2011).

shown to transform the human TF-1 leukemia cell line. These data suggest that, on a larger scale, the relationship between Ras/MAPK and Wnt signaling may warrant further investigation as a means to leukemic transformation.

One of the most compelling points made by Tiganis and Bennett in their review on PTP substrate identification was the idea that ultimately the validation of a genuine substrate would require demonstrating that its functional modulation by dephosphorylation could account for the biological function in question (Tiganis and Bennett, 2007). Perhaps the best example of this is the role of SHP-2 in the pathogenesis of *Helicobacter pylori* infection. *H. pylori* is a gram-negative microaerophilic bacterium that colonizes at least half of the world's population. Chronic infection is known to be a risk factor for the development of gastric diseases ranging from atrophic gastritis to distal adenocarcinoma of the stomach. Chronic gastritis is the strongest known risk factor for this type of cancer, causing *H. pylori* to be classified by the International Agency for Research on Cancer (IARC) as a group I carcinogen. While *H. pylori* infection is common, only a small fraction of patients ever develop gastric cancer. A strain-specific cytotoxin-associated gene (*cag*) pathogenicity island encodes a type IV secretion system that delivers a virulence factor, CagA, into gastric epithelial cells. Patients with *cagA*$^{+}$ *H. pylori* have an increased incidence of severe gastritis, gastric carcinoma, and mucosal-associated lymphoid tissue (MALT) lymphoma. *cagA*$^{+}$ *H. pylori* binds directly to gastric epithelium and causes cells to undergo a dramatic cytoskeletal change in which they adopt a morphology referred to as a 'hummingbird' phenotype, a response similar to that induced by hepatocyte growth factor (HGF).

CagA is injected directly into the host cell where it localizes to the inner surface of the plasma membrane and undergoes tyrosine phosphorylation by Src family (SFKs) at conserved Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs (Selbach et al., 2002). This phosphorylation is required for adoption of the 'hummingbird' phenotype, as replacement of all the tyrosine residues present in the five conserved EPIYA sequences with alanine resulted in a phosphorylation-resistant mutant that was unable to induce this phenotype in human AGS gastric epithelial cells (Higashi et al., 2002). In light of the similarity of this morphological change to that caused by exposure to HGF, and the demonstrated role of SHP-2 in modulating the activity of Rho downstream of the HGF receptor, c-Met, to regulate the assembly and disassembly of stress fibers and focal adhesions responsible for HGF-induced cell scattering of Madin-Darby canine kidney (MDCK) cells (Kodama et al., 2000), Higashi et al. investigated whether CagA might also interact with SHP-2. They found that wild-type but not phosphorylation-resistant CagA could bind SHP-2 in both AGS and COS-7 cells and that this complex formation was required for the hummingbird phenotype. Tsutsumi et al subsequently found that CagA results in decreased phosphorylation of focal adhesion kinase (FAK) and identified FAK as a SHP-2 substrate. SHP-2-mediated dephosphorylation and inhibition of FAK kinase activity was shown to be required for adoption of the hummingbird phenotype, and the resulting impairment in cell adhesion and increase in cell motility is thought to explain the increased propensity of infected epithelia to succumb to gastric disease (Tsutsumi et al., 2006).

This model is particularly compelling for its direct links between 1) genetics of a human disease (in this case of the *cagA*$^{+}$ virulent strain of *H. pylori*); 2) an in vitro phenotype relating to changes in cell biology (hummingbird phenotype); 3) a biochemical pathway regulating that phenotype (focal adhesion signaling mediated by SHP-2, Rho, FAK); and finally, 4) a biochemical mechanism identifying a substrate (FAK) whose dephosphorylation explains the effect of SHP-2 on each of the above layers, on the signaling, cell biology, and ultimately the human disease initiated by *cagA*. The same could be argued for the potential tools at our disposal, as we have: 1) a disease, JMML, that is even more directly linked to SHP-2 and specifically its catalytic activity than *cagA*$^{+}$ *H. pylori* infection; 2) a cell biological phenotype of growth factor hypersensitivity to a specific cytokine, GM-CSF, in an in vitro colony-forming CFU-GM assay that is specific enough to be considered a 'hallmark' and diagnostic tool in JMML; 3) a signaling axis of interest, the Ras/MAPK pathway, that has been both mutationally implicated in JMML and epistatically linked to SHP-2 function in growth factor signaling and development—all that is lacking is 4) the specific biochemical mechanism that links dephosphorylation of a specific substrate to the aforementioned layers of knowledge we possess.

It is entirely possible and, in fact likely, that the development of therapeutic agents for this disease will not hinge exclusively on the elucidation of this last criteria, however, all the evidence suggests that this goal is achievable, though it has remained elusive for over a decade. Of course, a clear distinction between the two cases lies in the fact that *H. pylori* infection and CagA activity are caused quite literally by the direct 'injection' of an exogenous agent into the host cell, as opposed

to mutation of an endogenous protein that likely has already pleiotropic effects relative to the signaling pathway (Ras/MAPK) of interest as in the case of SHP-2 in JMML. However, the parallels between infectious disease and cancer are longstanding, as many of the known human oncogenes such as *c-myc*, *c-src*, and *c-cbl* were identified first as viral oncogenes, with the 'c-' indicating the cellular homolog of the viral 'v-' counterpart. In a recent Perspective article in *Cell*, Michael Glickman and Charles Sawyers discussed this relationship and its implications for the emerging paradigm of resistance to single-agent targeted therapy in cancer (Glickman and Sawyers, 2012). They note that despite the differing concerns regarding toxicity and population effects, these diseases are fundamentally similar in that the treatment strategies in both strive to exploit the unique dependencies of the disease, whether it be oncogene- or microbe-specific. Although infectious agents can often co-opt host mechanisms for their own purposes, this dependency is much narrower of a window in the neoplastic setting, separated, in the case of JMML, by substitution of a single amino acid in the coding sequence of SHP-2. In light of this and the diversity of SHP-2 functions even in physiologic settings, elucidation of the role of SHP-2 E76K in JMML will require close containment of the investigation to the role of the mutant in hematopoietic cells, similar to the introduction of a microbial pathogen like CagA into gastric epithelial cells, and to the modulation of a definitive phenotype like factor-independence of the TF-1 cell line or CFU-GM colony formation, similar to the adoption of the hummingbird phenotype in CagA infected cells.

To that end, a more careful interrogation of the genetic and proteomic effects of introducing this 'exogenous' mutant could yield valuable information. We have already begun a collaboration with the laboratory of Dr. Jonathan Weissman at UCSF to apply recent advances in genome-wide RNAi screening technology to identify candidate genes capable of modulating the factor-independence conferred by SHP-2 E76K in TF-1 cells, in addition to undertaking more conventional microarray analysis to look at changes in gene expression secondary to transformation by SHP-2 E76K. Despite the challenges we faced with the tandem affinity purification approach, the use of immunoprecipitation with a substrate-trapping mutant combined with mass spectrometry for protein identification is still a valuable tool, particularly in a hematopoietic context. All of these studies have the advantage of being unbiased in nature and could easily be translated into a primary cell setting and performed in short-term culture of bone marrow- or fetal liver-derived hematopoietic progenitors in order to eliminate confounding effects arising from additional cell-line specific genetic alterations, as well as artifacts arising from the use of long-passaged, immortalized cell lines. These could be performed in retrovirally transduced cells or in hematopoietic cells isolated from transgenic SHP-2 knock-in or knockout murine models. Given our experience with downregulated expression of SHP-2 E76K over time, retroviral transduction should be undertaken under a tetracycline-inducible promoter for maximal expression, and given the experience of Dr. Art Weiss (UCSF) in studying the PTP CD45, use of a complete genetic null such as the SHP-2 *fl/fl* or *Ex3*$^{-/-}$ should be opted for over RNAi-mediated knockdown, as PTPs often demonstrate different activities depending on their level

of expression (Zikherman et al., 2010), and low-level residual expression could either mask meaningful differences or result in altogether unexpected, paradoxical effects on signaling.

Additionally, one important observation from our experience with the TAP tag was the difficulty in comparing peptide counts between samples, particularly given the subtle nature of the difference between the substrate-trapping 'AS' and the non-substrate binding 'M' mutants that we sought to exploit. Our difficulty in elucidating differences between these mutants was likely more reflective of the non-quantitative nature of the peptide recovery and identification process, rather than the lack of biological difference between these mutants. The biochemical distinction between these mutants is valuable and likely to be highly informative if combined with a properly quantitative detection method such as stable isotope labeling by amino acids in cell culture (SILAC). SILAC is an in vivo 'labeling' strategy that involves the metabolic incorporation of 'light' or 'heavy' isotopes of a given amino acid into the protein complement of a given cell line and permits their differential detection by mass spectrometry, thus enabling the quantification of differences in protein expression between different conditions. This strategy has been used to compare protein secretion by pancreatic cancer-derived cells with that of non-neoplastic pancreatic ductal cells (Gronborg et al., 2006), to similarly compare the 'secretomes' of human retinal pigment epithelium cultures derived from patients with age-related macular degeneration and age-matched healthy donors (An et al., 2006), as well as to compare surface protein expression between normal and malignant breast cancer cells isolated from the same patient (Liang et al., 2006).

The combination of SILAC with an affinity purification substrate-trapping strategy would be highly instructive.

SILAC has also been used for phosphotyrosine (pY) profiling, as that method is highly reliant on the ability to detect small quantitative differences, and while global pY profiling could be informative on its own if performed under the correct circumstances, comparing, for instance, the pY content of SHP-2 E76K-expressing (transduced or transgenic) hematopoietic progenitors with that of wild-type, the addition of phosphotyrosine enrichment prior to affinity purification could provide a more specific means of increasing specificity. That is to say, rather than using two unrelated affinity tags, replacement of the first of these sequential rounds of purification with pY-specific immunoprecipitation would increase the specificity for a particular class of protein (tyrosine-phosphorylated proteins) that is of particular biological relevance for the type of complex we are seeking to isolate. Furthermore, the pY-specific antibodies like 4G10 or Y100 antibody are known to result in a fair amount of non-specific binding and high degree of background, especially compared with the highly specific interactions between Protein A/G and IgG or between calmodulin/streptavidin and calmodulin-/streptavidin-binding peptide, which works to our advantage in this case as we would favor 'dirtiness' in favor of maximal recovery. To that end, the extensive washing undertaken between rounds of purification could be dispensed with in favor of speed, together promoting the most efficient recovery of a pre-selected population of interest. The first half of this approach, involving MS analysis of pY-immunoprecipitated lysates, has been described and dubbed 'tandem immunoprecipitation of phosphotyrosine-mass

spectrometry,' or 'TIPY-MS' (Tong et al., 2008). We propose the incorporation of this technology into that of the tandem affinity purification approach, thus resulting in a pY-specific tandem affinity purification approach tentatively referred to here as 'TIPY-TAP.'

Beyond potential further improvements to the experiments already undertaken, several of the hits we identified also merit further investigation. As discussed, the relationship between SHP-2, parafibromin, and β-catenin is of particular interest given the substrate identification in a heterologous context and the known role of β-catenin in oncogenic transformation. Wnt proteins are secreted glycoprotein signaling molecules that activate multiple different intracellular pathways. The best understood 'canonical' Wnt pathway results in the activation of nuclear β-catenin, which directly regulates gene expression. In the absence of Wnt ligands, β-catenin is recruited into a large multi-protein 'destruction complex' that includes the tumor suppressor adenomatous polyposis coli (APC) and the scaffolding protein Axin, which facilitate the phosphorylation of β-catenin by casein kinase 1 (CK1) and then the constitutively active glycogen synthase kinase-3β (GSK3β). Prospective target genes are kept in a repressed state by interacting with T-cell factor (TCF) and lymphoid enhancer-binding protein (LEF) transcription factors. Extracellular Wnt ligands are rendered inactive by interactions with secreted antagonists of the Frizzled-related protein (sFRP) and Dickkopf (DKK) protein families until they reach sufficient local concentration to exceed that buffering capacity and bind the Frizzled (FZ) family of receptors and co-receptors of the low-density lipoprotein receptor-related protein family, LRP5 and LRP6. Wnt

binding leads to activation of Dishevelled (DSH), which reduces degradation of β-catenin by directly inhibiting its phosphorylation by GSK3β, and also by recruiting the 'destruction complex' to the plasma membrane, thus resulting in the degradation of Axin. Stabilization of β-catenin leads to its nuclear translocation and interaction with the TCF and LEF transcription factors to activate target genes. Hyperactivation of this pathway has been noted in various human diseases, most notably in colon cancer.

The example of colorectal cancer is interesting in that it also highlights the relationship between the Wnt/β-catenin and Ras/MAPK pathways. The best example of these genes acting in concert to promote oncogenesis comes from colon cancer, where loss of APC is an initiating event, followed by activation of KRAS to promote progression to a late-stage adenoma (Fearon and Vogelstein, 1990). Experiments in zebrafish and human cells have demonstrated that activation of KRAS is required for intestinal cell proliferation and nuclear localization of β-Catenin following APC loss, suggesting synergy between these pathways is required for transformation (Phelps et al., 2009). A recent paper (Singh et al., 2012) found that *KRAS*-dependent colon cancer cell lines were sensitive to inhibition of the TAK1 kinase secondary to hyperactivation of the Wnt signaling pathway, suggesting again, that both of these pathways may be required for progression to full-blown cancer. SHP-2 modulation of β-catenin would be a novel mechanism of crosstalk between these two important signaling pathways. Interestingly, β-catenin has also been shown to play an important role in the progression of chronic *cagA*$^{+}$ *H. pylori* infection to gastric cancer. Increased nuclear accumulation of β-catenin has been

observed in gastric cancer precursor lesions such as gastric adenomas, (Tsukashita et al., 2003) and *H. pylori* infection has been found to increase expression of β-catenin and β-catenin-responsive genes like *c-myc* and *cyclin D1* in colonized gastric mucosa and in gastric epithelial cells vitro (El-Etr et al., 2004; Hirata et al., 2001; Nardone et al., 1999). Franco et al generated an in vivo model of *H. pylori*-induced gastric cancer by using an in vivo 'adaptation' strategy that involved serially passaging *H. pylori* in Mongolian gerbils to increase its carcinogenic potential. They found that this 'adapted' isolate, endowed with the ability to rapidly and reproducibly induce gastric dysplasia and adenocarcinoma selectively activated β-catenin in a CagA dependent manner compared with the parental non-oncogenic strain, (Franco et al., 2005; Higashi et al., 2002). Increased nuclear accumulation of β-catenin was observed in epithelium harvested from these gerbils as well as from people carrying *cagA⁺ H. pylori.* Given the demonstrated importance of SHP-2 in CagA-mediated pathogenesis, as well as in human cancer, these data once again highlight the potential importance of β-catenin in SHP-2 signaling.

For our purposes of discovering the mechanism by which SHP-2 E76K promotes leukemic transformation of hematopoietic progenitors, the immediate initial questions revolve around whether JMML patient samples similarly demonstrate nuclear accumulation of β-catenin, and whether exogenous expression of SHP-2 E76K and/or K-Ras G12D is necessary and/or sufficient to do the same. Validation of parafibromin as the SHP-2 substrate mediating this effect would require use of the Y290/293/315F mutant described by Takahashi et al to see if its expression in hematopoietic cells induces nuclear accumulation of β-catenin as it

did in AGS cells, and whether its expression increases transcription of β-catenin-responsive genes. More importantly, the most biologically relevant test would be whether expression of the parafibromin mutant in a CFU-GM colony-forming assay reproduces the factor-independent growth and GM-CSF hypersensitivity characteristic of SHP-2 E76K-mediated transformation. Our experience with the activated SFK alleles speaks to the specificity of this assay, as even expression of constitutively active Src or Lyn, both of which are normally expressed in hematopoietic cells, did not result in factor independent colony growth or GM-CSF hypersensitivity.

The more global question of whether b-catenin plays a role in JMML-like transformation could be addressed by performing the same CFU-GM assay with an activated allele of b-catenin. Similar to our interrogation of the role of SFKs in SHP-2 mediated transformation, expression of SHP-2 E76K in b-catenin null bone marrow would provide valuable information as to whether loss of β-catenin would prevent transformation by SHP-2 E76K in a CFU-GM assay. Adoptive transfer could also be used to assess the leukemogenic potential of these cells in vivo. A genetic cross could also be performed using β-catenin conditional knockout (Zhao et al., 2007) and the E76K conditional knock-in mice (Xu et al., 2011), both involving 'floxed' alleles of their respective genes, under a hematopoietic-specific Cre to look for modulation of disease phenotype. The implication of this pathway would provide a potential therapeutic avenue to be used in conjunction with inhibition of the Ras/MAPK signaling pathway. 'Druggable' targets in the Wnt pathway have been historically elusive, but Huang et al. several years ago described their use of a

chemical genetics screen to identify a small molecule inhibitor XAV939 that selectively inhibited β-catenin-mediated transcription. They found that this molecule specifically targeted the poly-ADP-ribosylating enzymes tankyrase 1 and tankyrase 2, which stimulate the ubiquitination and degradation of Axin, a critical component of the β-catenin degradation complex, thus inhibiting Wnt signaling by stabilizing Axin (Huang et al., 2009).

Interestingly, the role of β-catenin in hematopoiesis has been interrogated to some detail, and with compelling results. β-catenin null (*Ctnnb1*$^{-/-}$) mice die in utero (Haegel et al., 1995), so Brault et al generated mice with a conditional allele of β-catenin with required exons flanked by loxP recombination sequences (Brault et al., 2001). In this initial report they sought to investigate the role of β-catenin during brain morphogenesis, so these mice were crossed with those expressing Cre under control of Wnt1 regulatory sequences, but more recently, Zhao et al crossed them with vav-Cre mice to examine the role of β-catenin in adult hematopoietic stem cell function and leukemia progression (Zhao et al., 2007). Loss of β-Catenin was found to affect renewal of both normal and malignant hematopoietic cells in vivo. In particular, HSCs from these mice demonstrated decreased ability to promote long-term growth and maintenance following transplantation, and retroviral transduction of kit+ cells from these mice demonstrated a dramatic reduction in BCR-ABL-induced CML development. Conversely, expression of a constitutively active β-catenin in HSCs led to increased self-renewal in vitro and increased reconstitution efficiency in transplanted lethally irradiated recipients (Reya et al., 2003). However, crossing of the same conditional null model to an Mx1-

Cre transgenic animal to induce expression in the hematopoietic compartment led to no discernable phenotype, suggesting β-catenin was dispensable for hematopoiesis (Cobas et al., 2004). Furthermore, in vivo conditional expression of a constitutively active β-catenin in the hematopoietic compartment disrupted the long-term repopulating ability of HSCs and caused multilineage cytopenias due to premature cell cycling and differentiation arrest, suggesting that too much or too little β-catenin activity might result in impaired hematopoiesis (Scheller et al., 2006). Such nuanced distinctions are unfortunately all too common, reminiscent of the largely differing phenotypes between Cbl loss-of-function and Cbl loss-of-RING finger-function mice, and the largely similarly phenotypes of the Lyn gain- and loss-of-function mice, reinforcing the idea that many of these signaling molecules, and likely SHP-2 as well, play both positive and negative roles that are highly

Where signaling and cell biology could further intersect is in the role of SHP-2 in megakaryopoeisis. Our data suggest that SFKs are not required for SHP-2 E76K-mediated transformation, but the myeloid specific SFK family members still appeared to demonstrate an important role downstream of the GM-CSF receptor. In particular, expression of SHP-2 E76K in the presence of GM-CSF appeared to induce a megakaryocytic-type morphological change in TF-1 cells, which would be consistent with the apparent SFK inhibition observed in this context. This paradoxical activation/inhibition of SFKs is intriguing and may be explained by the complex heteromeric assembly of the GM-CSF receptor in the absence and presence of ligand, with ligand binding perhaps resulting in recruitment of additional factors to the receptor that would modulate the interaction between SFK and SHP-2.

Further investigation on this front would involve further characterization of the SHP-2 E76K-expressing TF-1 cells for DNA content and specific megakaryocytic markers like vWF and CD41. Similar transduction experiments in primary murine or human hematopoietic progenitors would also be interesting to see whether E76K could be sufficient to promote differentiation down this lineage. If SHP-2 is found to play a role in megakaryopoiesis, cyclin dependent kinase 1 (Cdk1) would be an interesting candidate to investigate, as it is a known SFK target, another putative hit identified by our MS analysis, and has been implicated in the endomitotic behavior of megakaryocytes. MS-based phosphotyrosine analysis performed by another group using cell line xenografts also detected increased Cdk1 T14/Y15 phosphopeptide in SHP-2 knockdown tumors, thus providing indirect confirmation of our TAP tag results (Ren et al., 2010). Of note, Notch has been shown to play an important role in megakaryopoiesis (Mercher et al., 2008), and Notch has been shown to act both downstream and upstream of β-catenin, activated by β-catenin-mediated transcription of the Notch-ligand Jagged1 (Rodilla et al., 2009) as well as responsible for directly binding and negatively regulating post-translational accumulation of active β-catenin (Kwon et al., 2011). Thus, megakaryopoiesis may represent a convergence of the SHP-2, SFK, and β-catenin pathways. These data also warrant further inquiry into the dysregulation of megakaryocytes and platelets in JMML patients, particularly in light of the centrosomal amplification and aneuploidy observed in transgenic SHP-2 E76K knock-in mice (Xu et al., 2011).

Thus, our findings point to several potentially interesting avenues of investigation with regard to SHP-2 function, both in JMML and in normal

hematopoiesis. In particular, the interplay between Ras/MAPK, SFK, and Wnt/β-catenin pathways outlined here could provide novel strategies for therapeutic intervention in JMML, as well as new insights into the basic biology of megakaryocytic differentiation. The substrate question could also be further addressed with additional modifications to our original TAP strategy as discussed. However, even simpler experiments could still be done to address this question of how SHP-2 promotes leukemogenesis. For instance, the similarity between the *motheaten* and the *motheaten viable* mice, where the former is protein-null and the latter is PTP-null, indicates the catalytic-dependence of that phenotype. Although in vitro experiments have been done using catalytically inactive versions of JMML-associated SHP-2 mutants, given that most of those substitutions, including E76K, occur outside the PTP domain, it would be interesting to evaluate the effect ablating PTP function in an in vivo setting. Similarly, crystallization of the mutant oncogenic SHP-2 would be interesting, as it is unclear how the SH2 domains are affected by the destabilization of the N-SH2-PTP interface. It has also been suggested that engagement of the C-SH2 domain alone could result in recruitment of SHP-2 to a membrane receptor without concomitant PTP activation, a possibility that is particularly intriguing in the leukemogenic setting as it suggests non-catalytic functions could be important as well (Barford and Neel, 1998). Even the initial transduction transplantation experiments done by Schubbert et al and Mohi et al demonstrating strain differences in the transforming ability of SHP-2 E76K suggests the possibility of a genetic modifier screen such as that described by Mao et al(Mao et al., 2006) to identify enhancers and suppressors of SHP-2 mediated

leukemogenesis. As is often the case in science, despite the ever-advancing technologies at our disposal, the greatest weapon is still a good question. Despite all the knowledge we have accumulated surrounding the genetics and biochemistry of JMML, finding a cure for this lethal childhood disease will require us to return to the basic questions in order to advance that knowledge beyond the bench.

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