Transcriptional Hallmarks of Noonan Syndrome and Noonan-Like Syndrome with Loose Anagen Hair

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ABSTRACT: Noonan syndrome (NS) is among the most common nonchromosomal disorders affecting development and growth. NS is genetically heterogeneous, being caused by germline mutations affecting various genes implicated in the RAS signaling network. This network transduces extracellular signals into intracellular biochemical and transcriptional responses controlling cell proliferation, differentiation, metabolism, and senescence. To explore the transcriptional consequences of NS-causing mutations, we performed global mRNA expression profiling on peripheral blood mononuclear cells obtained from 23 NS patients carrying heterozygous mutations in PTPN11 or SOS1. Gene expression profiling was also resolved in five subjects with Noonan-like syndrome with loose anagen hair (NS/LAH), a condition clinically related to NS and caused by an invariant mutation in SHOC2. Robust transcriptional signatures were found to specifically discriminate each of the three mutation groups from 21 age- and sex-matched controls. Despite the only partial overlap in terms of gene composition, the three signatures showed a notable concordance in terms of biological processes and regulatory circuits affected. These data establish expression profiling of peripheral blood mononuclear cells as a powerful tool to appreciate differential perturbations driven by germline mutations of transducers involved in RAS signaling and to dissect molecular mechanisms underlying NS and other RASopathies.

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KEYWORDS: Noonan syndrome; RASopathies; PTPN11; SOS1; SHOC2

Introduction

Dysregulation of RAS signaling has recently been recognized to underlie a group of clinically related disorders affecting development and growth [Schubbert et al., 2007; Tartaglia and Gelb, 2010; Tidyman and Rauen, 2009]. Most of these conditions, which are collectively named as RASopathies, share facial dysmorphism, a wide spectrum of heart disease, reduced postnatal growth, variable cognitive defects, and susceptibility to certain malignancies. In these Mendelian traits, heterozygous germline mutations affect various genes coding for members of the small subfamily of RAS GTPases, signal relay proteins that function as modulators of RAS function, RAS effectors, and downstream signal transducers. Despite the majority of mutations appear to enhance signal traffic through the RAS-mitogen-activated protein kinase (MAPK) axis, each syndrome maintains indeed distinctive phenotypic features. In some of these disorders, a further level of complexity is due to genetic heterogeneity, which explains, in part, the observed clinical variability. Noonan syndrome (NS, OMIM 163950), which is the most common among these diseases, occurring approximately in 1:1000–1:2500 live births, represents a paradigmatic condition [Allanson, 2007; Tartaglia et al., 2010; Van der Burgt, 2007]. NS is genetically heterogeneous, with activating mutations in PTPN11, SOS1, KRAS, NRAS, RAF1, and BRAF occurring in approximately 75% of affected individuals [Tartaglia et al., 2011]. NS is a clinically variable disorder, and recent studies have established clinically relevant genotype–phenotype correlations, such as a high prevalence of pulmonary stenosis among subjects with a mutated PTPN11 allele, occurrence of hypertrophic cardiomyopathy in individuals heterozygous for a mutation in RAF1, or generally normal growth and cognition in subjects carrying a mutated SOS1 gene [Tartaglia et al., 2010]. In contrast to what observed in NS, other RASopathies exhibit a relatively homogeneous phenotype that generally reflects an underlying genetic homogeneity. This is the case of Noonan-like syndrome with loose anagen hair (NL/LAH), a rare condition with clinical features partially overlapping those occurring in NS [Mazzanti et al., 2003], and caused by the invariant c.4A>G missense change (p.Ser2Gly) in SHOC2 [Cordueto et al., 2009], a scaffold protein with regulatory function that positively modulate RAS signaling [Matsunaga-Udagawa et al., 2010; Rodriguez-Viciana et al., 2006].
To provide first insights on the pathogenetic mechanisms underlying NS and related RASopathies, a number of studies have been directed to investigate the consequences of panels of disease-causing mutations on protein structure and function, and their perturbing effects on intracellular signaling [Tartaglia et al., 2010]. No attempt has been directed, however, to investigate the consequences of the aberrant activation of the RAS signaling network driven by the different disease-causing molecular lesions on the control of gene expression. Here, we explored the global gene expression profile of peripheral blood mononuclear cells (PBMCs) collected from two cohorts of subjects with mutations in the two most common NS disease genes (PTPN11 and SOS1), and a third group representative of the genetically homogeneous NS/LAH (SHOC2) in order to identify transcriptional signatures specifically associated with aberrant PTPN11/SHP2, SOS1, and SHOC2 function, as well as to evaluate the extent and branching of intracellular signaling dysregulation associated with these specific pathological conditions.

**Methods**

**Patients Selection**

The study was approved by the Local Ethics Committee of the Regina Margherita Children’s Hospital, Torino, Italy. Informed consent was obtained from parents or guardians of all participants. Patients were enrolled in the study between March 2006 and May 2008. Controls are children with staturo-ponderal and neuromotor development within normal limits. The diagnosis of NS was established according to Van der Burgt clinical criteria [van der Burgt et al., 1994] and confirmed by molecular analysis on genomic DNA isolated from peripheral blood by the QiAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The 15 coding exons and exon/intron junctions of PTPN11 were amplified by PCR with FastStart Taq DNA Polymerase (Roche Diagnostics Corporation, Indianapolis, IN) under standard conditions with the primers listed in Tartaglia et al. [2002], SOS1 analysis was carried out by amplification and sequencing of the 23 exons as previously described [Tartaglia et al., 2007] and SHOC2 gene was studied as reported in Cordeddu et al. [2009]. The study cohort included 23 subjects with a diagnosis of NS associated with a germline mutation in PTPN11 (N = 17) or SOS1 (N = 6), and five individuals with NS/LAH due to the invariant c.4A>G missense change in SHOC2. Mutation data (location of affected residues and type of amino acid substitution) are resumed in Supp. Table S1. Additional 21 samples were obtained from age- and sex-matched controls. Informed consent was obtained from all subjects included in the study.

**RNA Extraction and Processing for Microarray**

RNA was extracted from PBMCs, isolated from fresh blood samples within 2 hr from collection, using the TRizol Plus RNA purification system (Invitrogen Corp., Carlsbad, CA) according to the manufacturer’s protocol. The quantification and quality analysis of RNA was performed on a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). Synthesis of cDNA and biotinylated cRNA was performed using the Illumina TotalPrep RNA Amplification Kit (Ambion, Foster City, CA; Cat. n. IL1791), according to the manufacturer’s protocol. Quality assessment and quantification of cRNAs were performed with Agilent RNA kits on Bioanalyser 2100. Hybridization of cRNAs (750 ng) was carried out on HumanRef8_V2 BeadChips (Illumina Inc., San Diego, CA). Array washing was performed using Illumina High stringency wash buffer for 10 min at 55°C, followed by staining using streptavidin-Cy3 dyes (Amersham Biosciences, Buckinghamshire, UK), according to standard Illumina protocols.

**Data Analysis**

Cubic spline-normalized probe intensity data, together with detection P-values, were obtained using the BeadStudio 3.1 software (Illumina). Subsequent data processing, carried out with Excel (Microsoft Corp., Redmond, WA) included: (1) scaling, Log, transformation, and detection filtering; (2) removal of genes correlated with age, sex, or differential leukocyte count; (3) Log2 Ratio transformation and selection of genes differentially expressed between controls and mutated groups; (4) Monte carlo simulation for false discovery rate estimation; (5) full leave-one-out classification analysis. All procedures are described in detail in Supp. Methods. Log2 Ratio expression data were clustered and visualized using the GEDAS software [Fu and Medico, 2007].

**Results**

**PBMC Gene Expression Profiling of NS and NS/LAH Patients**

PTPN11, SOS1, and SHOC2 gene expression in human PBMCs was preliminarily verified by in silico analysis on a published PBMC gene expression dataset [Burczynski et al., 2006]. The analysis indicated that these and other disease genes known to be implicated in RASopathies are expressed, at varying levels, in human PBMCs (Supp. Fig. S1). For gene expression profiling (GEP), we selected 23 NS patients including 17 subjects carrying a mutation in PTPN11 and six with a SOS1 lesion, five NS/LAH subjects with the invariant c.4A>G SHOC2 mutation, and 21 age- and sex-matched controls (Supp. Table S1). Total RNA extracted from PBMCs was processed for GEP on Illumina Beadarrays. We verified expression of PTPN11, SOS1, and SHOC2 mRNA in our samples by checking microarray probe signal intensities for PTPN11 and SOS1, and quantitative real-time PCR signals for SHOC2 that was not represented on the arrays (Supp. Fig. S2). Out of the 20,589 probes analyzed on the array, 5,605 passed filtering for reliable signal detection and for not being correlated with age, sex, or differential leukocyte count (Supp. Fig. S3 and Supp. Methods). Unsupervised hierarchical clustering of all samples based on these probes revealed four major transcriptional subgroups, two of which were enriched, respectively, in NS/LAH and NS samples (Supp. Fig. S4). For supervised statistical detection of genes differentially expressed between NS and NS/LAH samples and controls, a multiple test including fold change (absolute Log2 ratio > 0.5), t-test (P < 0.01), and signal-to-noise ratio (SNR > 0.3; see also Supp. Methods) was applied to the following comparisons: (1) NS+NS/LAH samples versus controls; (2) PTPN11 mutation-positive samples versus controls; (3) SOS1 mutation-positive samples versus controls; and (4) SHOC2 mutation-positive samples versus controls. Four signatures were thus identified, composed of 125, 225, 73, and 1407 probes, respectively (Supp. Table S2). A Monte Carlo simulation considering 2,000 random sample permutations was performed that allowed estimating the fraction of false positive hits as acceptably low (0.001–5.5%; Supp. Table S3).

**NS and NS/LAH Gene-Specific Transcriptional Signatures in Human PBMCs**

Expression of genes belonging to the four signatures is shown in Figure 1. Of note, the signatures obtained separately for the PTPN11,
PBMC transcriptional signatures discriminating NS and NS/LAH patients from unaffected individuals. Heatmap representing Log2 ratio expression for gene probes (rows) across samples (columns). Higher than average (red) and lower than average (green) expression levels are indicated according to the color bar reported below the diagram. Samples are subdivided in four groups, from left to right: controls (C-001–C-021), NS with a mutated PTPN11 allele (PT-001–PT-017), NS with a SOS1 mutation (SO-001–SO-006), and NS/LAH with the c.4A>G change (SH-001–SH-005).

Four major transcriptional signatures composed of genes that significantly discriminate control samples from (1) PTPN11, SOS1, and SHOC2 mutation-positive samples (NS+NS/LAH signature, 125 gene probes), (2) PTPN11 mutation-associated signature (PTPN11 signature, 225 probes), (3) SOS1 mutation-associated samples (SOS1 signature, 73 probes), and (4) SHOC2 mutation-positive samples (SHOC2 signature, 1,407 probes) are shown.

SOS1, and SHOC2 mutations were found to discriminate more efficiently the individual mutation groups from controls, compared to the signature characterizing the entire “RASopathy” cohort of PBMCs with mutated PTPN11, SOS1, and SHOC2 alleles, indicating occurrence of significant heterogeneity among subgroups. Indeed, the three disease gene-specific signatures displayed detectable, but only partial overlaps (Supp. Table S2E). The signature characterizing NS/LAH was the largest, including 1,394 genes. Within this group, the expression profiles were highly homogeneous, possibly because of the invariant occurrence of the SHOC2 c.4A>G mutation underlying this disorder, and appeared to be oppositely modulated within both the PTPN11 and SOS1 mutation-associated NS groups. A robust signature, characterized by 223 differently expressed genes was also attained for the PTPN11 mutation group. This signature was shared, in part, with the SHOC2 mutation group, while it noticeably diverged in the SOS1 mutation group. Differently from what observed in the PTPN11 and SHOC2 mutation cohorts, the SOS1 mutation group shared a signature with restricted size, which, however, appeared to efficiently discriminate this group from controls. The SOS1 mutation-associated signature appeared oppositely modulated in the SHOC2 mutation group, while it was relatively conserved among samples of the PTPN11 mutation group.

Overall, these findings document that the PTPN11, SOS1, and SHOC2 mutations induce detectable gene expression changes in PBMCs, and suggest that the specific perturbation in gene expression modulation occurring within each subgroup cannot be simply ascribed to a differential perturbing effect of mutations in individual disease genes on the extent of signal flow through a common signal transduction pathway (i.e., the RAS-MAPK cascade).

To verify whether the disease gene-specific signatures could reliably distinguish samples with mutations in the respective disease genes from control samples in a diagnostic setting, we performed full leave-one-out cross-validation analysis. Briefly, each sample (either mutated or not) was individually removed from the dataset, and the remaining samples were used to select again significant genes and redefine the four signatures. The left-out sample was then classified by calculating a weighted average score for each signature (NS+NS/LAH, PTPN11, SOS1, and SHOC2; see Supp. Methods). Finally, the four classification scores for each sample were displayed in dot plots (Fig. 2) and used for t-test-based statistics. Overall, the
RASopathy-associated score was significantly different between control and NS+NS/LAH samples (t-test P-value < 0.001). It correctly classified all the SHOC2 samples as well as the majority of PTEN11 samples (P < 0.001), but failed to discern SOS1 mutation-positive samples from controls (P = 0.29). The PTEN11 mutation-associated score was documented to discriminate efficiently samples with a mutated PTEN11 allele from healthy controls (P < 0.001) and those with SOS1 mutations (P < 0.005), but not from samples with mutated SHOC2 (P = 0.706). The SOS1 mutation score maintained a significant discrimination efficacy against control samples (P < 0.001), but displayed low specificity. Finally, the SHOC2 mutation score, despite being derived from just four samples in the “leave-one out” approach, displayed very good sensitivity and specificity against both controls (P < 0.001) and other mutated samples (SHOC2 vs PTEN11: P < 0.001; SHOC2 vs SOS1 P < 0.001). Overall, these results represent proof of concept that PBMC-derived transcriptional signatures are sufficiently robust to be considered as distinctive for each of the different conditions and to eventually be used for diagnostic purposes.

In silico Data Mining Reveals Biological Significance of NS and NS/LAH Mutation-Specific Signatures

To functionally characterize genes transcriptionally associated to NS and NS/LAH causative mutations, we tested the PTEN11, SOS1, and SHOC2 mutation-specific signatures for enrichment in functional annotation keywords using DAVID [Huang da W et al., 2009; see Supp. Methods]. This analysis was conducted first using all the genes of each signature, then using subgroups of only up- or down-regulated genes. As shown in Supp. Table S4, the PTEN11 signature was found to be enriched in genes encoding proteins with SH2 domains (P < 0.001) and tyrosine-specific protein kinases (P < 0.01). The SOS1 signature did not show significant enrichments, whereas the downregulated genes of the SHOC2 signature were strongly enriched for genes having regulatory role in transcription (P < 10^-7). These results prompted additional data mining focused on genes implicated in signal transduction and transcriptional control. The three signatures were therefore assessed for significant enrichment in kinase targets via the web-based “Kinase Enrichment Analysis” tool [Lachmann et al., 2009] (Table 1). Interestingly, this analysis documented that the PTEN11 signature displayed highly significant enrichment in targets of tyrosine kinases, particularly SRC family kinases (FYN, LYN, LCK, SRC) and SRC family interacting kinases (CSK, SYK, ZAP70). Despite its small size, the SOS1 signature displayed significant enrichment in substrates of LCK, while the SHOC2 signature was enriched in targets of MAPK and SRC family members and their interacting kinases. These data show that a significant percentage of genes transcriptionally modulated by NS and NS/LAH disease-causing alleles are themselves known targets of tyrosine kinases involved in signal transduction.

Subsequently, we focused on protein–protein interactions, using the Gather [Chang and Nevins, 2006] and Genes2networks [Berger et al., 2007; see Supp. Methods] web-based tools. Of note, the only protein displaying significant interactor enrichment in both PTEN11 and SHOC2 signatures with both tools was CBL, recently found to be mutated in a condition partially overlapping NS [Martinelli et al., 2010]. These data suggest that protein–protein interaction in silico analysis of gene expression signatures referred to the different RASopathies can represent an informative tool to identify new candidate disease genes for these disorders.

The fact that the SHOC2 mutation was found to downregulate a large number of transcription factors (TFs) prompted us to an in-depth analysis of circuits of transcriptional regulation within the NS and NS/LAH signatures. To this aim, we searched for cases of concomitant presence within the same signature of TFs and their predicted targets. The results of this analysis, performed by the Opossum tool [Ho Sui et al., 2005], highlighted four cases of concomitant and significant coregulation (Supp. Table S5 and Supp. Methods). In PTEN11-mutated samples, GFI1 was negatively regulated with respect to controls (P < 0.001) and its targets were preferentially downmodulated. In SOS1-mutated samples, GABPA was significantly upregulated (P < 0.01) and its targets were preferentially downregulated. Finally, SHOC2-mutated samples displayed higher expression of CREB1 (P < 0.001) and SP1 (P < 0.001), while the respective targets were preferentially downregulated. Overall, these results indicate the presence of at least one transcriptional regulation circuit in each signature (Fig. 3).

Discussion

Transcriptome analysis is a key tool to explore biological complexity of human diseases. We applied this approach to RASopathies with the aim of finding molecular correlates of the mutational status in PBMCs, focusing on the two genes most frequently mutated in NS, PTEN11, and SOS1, and on SHOC2, which has been recently discovered to cause NS/LAH, a disorder with clinical overlap with the former [Cordeddu et al., 2009].

When grouped together and compared to age-matched unaffected individuals, NS and NS/LAH-derived samples yielded a transcriptional signature of 123 genes that correctly classified most samples. Such a signature, however, was not representative for the samples heterozygous for a mutated SOS1 allele and a fraction of subjects with mutations in PTEN11. When the overall cohort of NS patients was subdivided on the basis of the genetic lesion in the three gene-specific subgroups, larger and more homogeneous signatures emerged despite the lower sizes of subgroups. These results show that, although germline mutations in PTEN11, SOS1, and

### Table 1. Enrichment of the PTEN11, SOS1, and SHOC2 Signatures for Substrates of Kinases

| Kinase | Number of substrates in signature | Enrichment P-value |
|--------|----------------------------------|--------------------|
| **PTEN11 signature** | | |
| INSR   | 9 | 4.09E-03 |
| PDGFRB | 5 | 5.81E-03 |
| ERBB3  | 6 | 1.57E-03 |
| ERBB4  | 4 | 8.43E-03 |
| SRC    | 12 | 2.53E-03 |
| LCK    | 13 | 3.72E-06 |
| FYN    | 12 | 2.10E-04 |
| LYN    | 10 | 2.28E-04 |
| SYK    | 1 | 1.15E-03 |
| CSK    | 5 | 2.31E-03 |
| ZAP70  | 5 | 3.49E-03 |
| ITK    | 4 | 3.31E-03 |
| BTK    | 3 | 6.64E-03 |
| AXL    | 3 | 9.35E-03 |
| **SOS1 signature** | | |
| LCK    | 5 | 1.84E-03 |
| PRKAA2 | 2 | 2.81E-04 |
| **SHOC2 signature** | | |
| PDGFRB | 12 | 6.23E-03 |
| SYK    | 17 | 1.19E-03 |
| CSK    | 10 | 9.09E-03 |
| FYN    | 26 | 9.19E-03 |
| ZAP70  | 11 | 6.67E-03 |
| ITK    | 8 | 7.00E-03 |
| MAPK11 | 6 | 6.40E-03 |
| MAPK14 | 52 | 3.97E-04 |

Gene lists from each of the three signatures were tested on the KEA web-based tool for enrichment in substrates of kinases. The table reports only kinases whose substrates were significantly enriched (P < 0.01).
SHOC2 deregulate the RAS-MAPK pathway, each mutated gene drives specific perturbations in intracellular signaling leading to different transcriptomic changes. Leave-one-out analysis confirmed that such gene-specific signatures correctly classified most NS and NS/LAH patients, which opens the way to potential clinical diagnostic application of this approach. Partial overlap was observed between the PTPN11 mutation-associated transcriptome and in a mutually exclusive manner, those associated with SOS1 and SHOC2 gene mutations, allowing to define two PTPN11 subgroups, whose biological significance remains to be elucidated. SOS1 and SHOC2 mutations appeared to drive anticorrelated transcriptional changes. Interestingly, there was significantly more transcriptome perturbation in SHOC2-mutated specimens (1,394 genes) as compared to PTPN11 and SOS1-mutated samples (223 and 73 genes, respectively). Within the PTPN11 subgroup, no significant associations were found between transcriptional profiles and the clinical scoring system developed by van der Burgt [van der Burgt et al., 1994], possibly due to the small number of cases analyzed.

The differences highlighted by transcriptional profiling were found to be consistent with the different role of SHP2, SOS1, and SHOC2 in modulating intracellular signaling. SHP2 is a nonreceptor protein tyrosine phosphatase [Neel et al., 2003] required for efficient activation of growth factor-induced RAS-MAPK signaling via multiple potential mechanisms [Dance et al., 2008]. Moreover, besides the positive modulatory role on RAS signaling, SHP2 controls additional signal transduction pathways, as those linked to STAT and SRC proteins that are well known to contribute significantly to transcriptional control [Grossmann et al., 2009; Xu and Qu, 2008; Zhang et al., 2004]. SOS1 has instead a narrower role, being a bifunctional guanine nucleotide exchange factor (GEF) for RAS and RAC [Nimnual and Bar-Sagi, 2002]. This difference in function together with the possibility of a cell context specificity of the perturbing effect of mutations on intracellular signaling could explain the only partial overlap between the signatures characterizing the SOS1- and PTPN11-mutation groups. Of particular relevance is the fact that the invariant c.4A>G (p.Ser2Gly) SHOC2 mutation was observed to provoke a profound alteration in the PBMC transcriptome. SHOC2 encodes a widely expressed protein supposed to be required for efficient RAF1 activation following growth factor stimulation by promoting membrane translocation of the catalytic subunit of protein phosphatase 1 (PP1C) that is required for stable RAF1 binding to RAS [Rodriguez-Viciana et al., 2006]. The invariant missense change was demonstrated to introduce an N-myristoylation site that causes stable translocation to the plasma membrane of the mutated protein and enhanced ERK1/2 phosphorylation in a cell context-dependent fashion [Cordeddu et al., 2009]. Being a scaffold protein permanently anchored at the plasma membrane, myristylated SHOC2 may exert still uncharacterized actions leading to massive transcriptional deregulation. Intriguingly, 110 of the 225 genes composing the PTPN11 signature are also present and concordant in the SHOC2 signature, but most of the remaining several hundreds of the SHOC2 signature genes display a SHOC2-specific behavior. This finding strongly suggests that SHOC2 might control not only the RAS-MAPK axis, but also other signaling pathways and/or cellular processes. Consistent with the present findings, it was demonstrated that SHOC2 translocates in the nucleus following growth factor stimulation [Cordeddu et al., 2009], which supports the idea of a possible direct involvement of this protein in the control of processes linked to gene expression.

Another striking finding of this work is the opposite sign of regulation of SOS1 target genes in the SHOC2 mutation group, and vice versa. In this case, despite the fact that both gene products in principle positively regulate the RAS-MAPK axis, gene-specific features of signal transduction apparently drive opposite transcriptional responses. A possible explanation for this paradox is that aberrant signaling by a mutated gene can be counteracted by negative feedback loops that under particular circumstances may account for most of the transcriptional changes observed at the steady-state level. According to this view, the PTPN11, SOS1, and SHOC2 mutation-associated transcriptomes may be considered not only to directly report the grade of activity of the RAS-MAPK axis, but also highlight a more complex transcriptional circuitry that in some cases may result in opposite changes [Amit et al., 2007].

Downstream of the affected signaling pathways, NS and NS/LAH gene mutations ultimately drive functional alterations that result in clinically observable phenotypic traits. Indeed, by looking at the functions of the proteins encoded by genes included in the various above-mentioned signatures, we could reconstruct at least some of the regulatory circuits potentially involved in the molecular pathogenesis of these disorders. Basic functional keyword enrichment analysis revealed that many of the genes regulated by PTPN11, SOS1, and SHOC2 mutations are themselves involved in signal transduction and control of transcription. Subsequent deeper analyses focused on these features highlighted interesting properties of the transcriptional targets of signaling pathways modulated by SHP2,
SOS1, and SHOC2. In particular, a higher than expected representation of substrates of members of the SRC family of tyrosine kinases (FYN, LYN, LCK, SRC) was observed. This finding highlights a complex interplay between mutations in PTPN11, SOS1, and SHOC2, and this family of kinases known to be involved in signaling through the MAPK cascade [Zhang et al., 2004] and to regulate fundamental cellular processes such as growth, shape change, and migration in multiple cell lineages [Parsons and Parsons, 2004]. Based on these findings, it can be speculated that such an interplay could be at the basis of mesenchymal alterations giving rise to skeletal, cardiac, and hemopoietic abnormalities observed in NS and other RASopathies. Through a different approach, based on mining protein–protein interaction databases, we found that a high fraction of PTPN11 and SHOC2 target genes encode proteins interacting with the E3 ubiquitin ligase, CBL. Intriguingly, germine CBL mutations have been recently found in a condition with clinical features partially overlapping NS and with predisposition to hematologic malignancies during childhood, as well as in diverse myeloproliferative disorders and myeloid leukemias as somatically acquired lesions [Martini et al., 2010; Niemeyer et al., 2010; Pêrez et al., 2010]. Altogether, these results reveal a highly integrated genetic program, whereby biochemical activation of the RAS-MAPK axis drives transcriptional regulation of a relevant subset of proteins involved in functionally related signaling networks. In this view, deeper exploration of transcriptome/interaction connections may highlight new candidate genes for RASopathies, not yet molecularly elucidated.

Finally, we focused on TF/target gene circuits modulated by the PTPN11, SOS1, and SHOC2 mutations. The most interesting one involves GFI1 (Growth factor independence 1) that was negatively modulated in samples with mutated PTPN11 and whose predicted targets were concordantly downregulated in the same samples. Notably, children with NS present increased risk of myeloproliferative disorder (MPD) [Kratz et al., 2005], and GFI1 loss of function has been documented to cause MPDs [Khandanpour et al., 2011]. This evidence suggests that GFI1 downmodulation could be causally linked to MPD susceptibility in NS. In principle, genes differentially expressed in PBMCs could also be regulated in other tissues, and, therefore, related to nonhematologic anomalies. As an example, altered gene expression has been found to correlate with Huntington’s disease, a specific neurodegenerative autosomal dominant disorder [Runne et al., 2007]. Indeed, GFI1 is also involved in the development of the inner ear hair cells [Móro T., 2005], and its mRNA was robustly downregulated in two independent murine models of hearing loss [Hertzano et al., 2004, Lewis et al., 2009]. In this respect, PTPN11 mutation-driven GFI1 downregulation could play a key role in hearing abnormalities observed in NS [Scheiber et al., 2009; Qiu et al.,1998]. In the SHOC2 signature, two TFs, CREB1 and SP1 were consistently upregulated, while their targets resulted to be preferentially downregulated. CREB1 encodes a 43-kDa basic/leucine zipper (bZIP) TF known to be a target of the MAPK/ERK pathway [Morgan et al., 2001]. Interestingly, hippocampi deriving from a knockout mouse model of neurofibromatosis presented increased activity of the RAF-ERK axis and of CREB [Goulding et al., 2007], indicating a possible involvement in the pathogenesis of cognitive impairment observed in RASopathies. SP1 belongs to the SP/KLF TF family and is a MAPK target [Benasciutti et al., 2004; Curry et al., 2008]. Interestingly, enhanced SP1 activity has been linked to cardiac hypertrophy, a recurrent cardiac anomaly in NS [Azakie et al., 2006; Hu et al., 2010; Lin et al., 2009]. Finally, the putative GABPA circuit detected in the SOS1 signature is consistent with the fact that GABPA is a known target of the MAPK pathway [Flory et al., 1996; Fromm and Burden, 2001]. Altogether, functional data mining focused on signal transduction and TF activity highlighted genes and modules of transcriptional regulation present in the PTPN11, SOS1, and SHOC2 signatures that provide useful hints on the molecular pathogenesis of NS.

It is likely that current advances in massive sequencing will pave the way to molecular characterization of all germine mutations causing RASopathies. In this perspective, the clinical potential of transcriptional NS signatures will not reside as much on first diagnosis applications, but rather on its utility as a transcriptional readout of the actual functional status of the affected tissue. In this view, the results shown here open the way to exploit PBMC gene signatures as surrogate markers of specific MAPK pathway activation driven by NS gene mutations and, therefore, as a powerful tool to monitor the biological response to molecular targeted drugs.

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