

## Heterozygous Germline Mutations in the *CBL* Tumor-Suppressor Gene Cause a Noonan Syndrome-like Phenotype

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RAS signaling plays a key role in controlling appropriate cell responses to extracellular stimuli and participates in early and late developmental processes. Although enhanced flow through this pathway has been established as a major contributor to oncogenesis, recent discoveries have revealed that aberrant RAS activation causes a group of clinically related developmental disorders characterized by facial dysmorphism, a wide spectrum of cardiac disease, reduced growth, variable cognitive deficits, ectodermal and musculoskeletal anomalies, and increased risk for certain malignancies. Here, we report that heterozygous germline mutations in *CBL*, a tumor-suppressor gene that is mutated in myeloid malignancies and encodes a multivalent adaptor protein with E3 ubiquitin ligase activity, can underlie a phenotype with clinical features fitting or partially overlapping Noonan syndrome (NS), the most common condition of this disease family. Independent *CBL* mutations were identified in two sporadic cases and two families from among 365 unrelated subjects who had NS or suggestive features and were negative for mutations in previously identified disease genes. Phenotypic heterogeneity and variable expressivity were documented. Mutations were missense changes altering evolutionarily conserved residues located in the RING finger domain or the linker connecting this domain to the N-terminal tyrosine kinase binding domain, a known mutational hot spot in myeloid malignancies. Mutations were shown to affect CBL-mediated receptor ubiquitylation and dysregulate signal flow through RAS. These findings document that germline mutations in *CBL* alter development to cause a clinically variable condition that resembles NS and that possibly predisposes to malignancies.

RAS signaling plays a crucial role in cell proliferation, migration, survival, and cell fate determination and differentiation and participates in early and late developmental processes, including organogenesis, morphology determination, and growth.<sup>1</sup> Because of its nodal role in signal transduction, signal traffic through RAS is tightly controlled, and enhanced flow through it contributes to oncogenesis.<sup>2,3</sup> There are activating somatic RAS gene mutations in approximately 30% of human cancers, but the upregulation of this signaling pathway can also result from enhanced function of upstream signal transducers or RAS effectors, as well as from inefficient function of feedback mechanisms. However, the recent, unpredicted discovery of germline mutations in a number of genes encoding proteins involved in RAS signaling has established a novel scenario in which aberrant signal flow through RAS is causally linked to a group of clinically related developmental disorders, namely the neuro-cardio-facial-cutaneous syndromes (NCFCS), or RAS-opathies.<sup>3–5</sup>

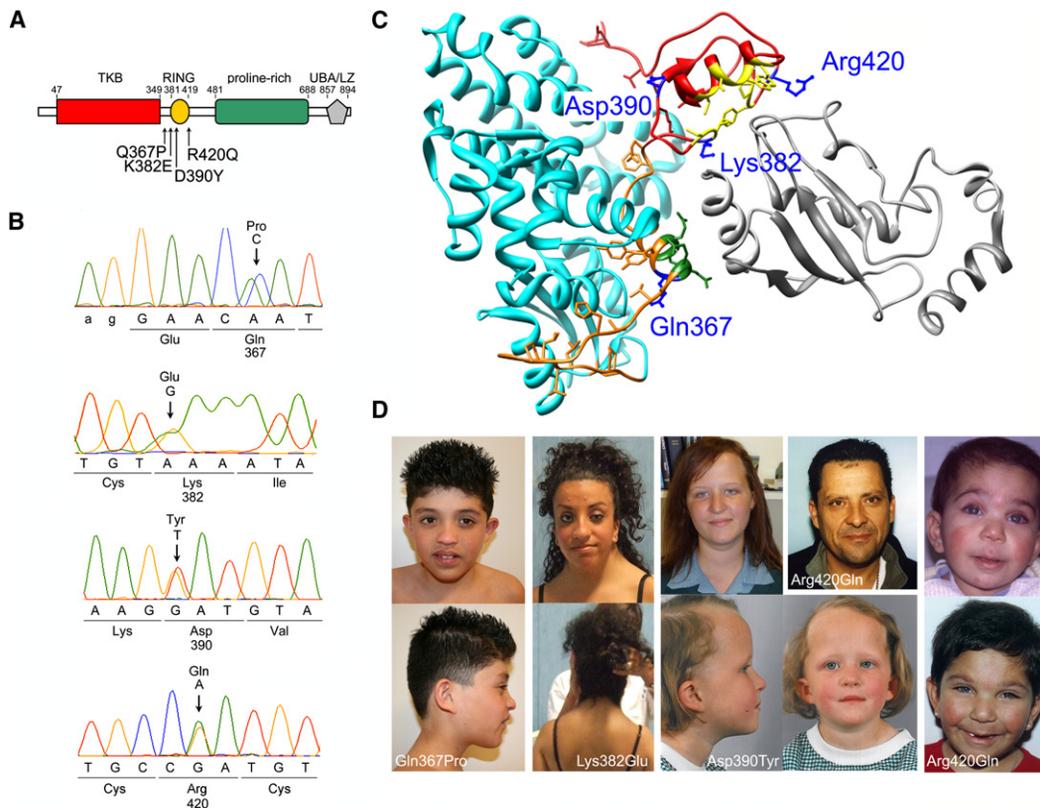
Noonan syndrome (NS [MIM 163950]), the most common among NCFCS, is a clinically variable condition

characterized by facial dysmorphism, a wide spectrum of cardiac disease, reduced postnatal growth, and ectodermal and skeletal defects.<sup>6–8</sup> Distinctive NS facial features consist of a broad forehead, hypertelorism, down-slanting palpebral fissures, and low-set, posteriorly rotated ears. Additional, relatively recurrent features include a webbed and/or short neck, variable cognitive deficits, cryptorchidism, lymphatic dysplasias, bleeding tendency, and, rarely, predisposition to childhood hematologic malignancies, particularly juvenile myelomonocytic leukemia (JMML [MIM 607785]). NS is genetically heterogeneous, and mutations in the *PTPN11* (MIM 176876),<sup>9</sup> *SOS1* (MIM 182530),<sup>10,11</sup> *KRAS* (MIM 190070),<sup>12,13</sup> *NRAS* (MIM 164790),<sup>14</sup> *RAF1* (MIM 164760),<sup>15,16</sup> *BRAF* (MIM 164757),<sup>17</sup> *SHOC2* (MIM 602775),<sup>18</sup> and *MAP2K1*<sup>19</sup> (MIM 176872) genes have been documented to account for 70%–75% of affected individuals. These genes encode transducers that positively contribute to RAS-MAPK signaling. Although some (*i.e.*, *NRAS*, *KRAS*, and *BRAF*) had previously been recognized as proto-oncogenes, the finding of the causal link to NS provided the basis for

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**Figure 1. Germline *CBL* Mutations Underlying Noonan Syndrome and Related Phenotypes**

(A) Location of predicted amino acid substitutions associated with disease is shown below the *CBL* structure scheme. *CBL* comprises an N-terminal tyrosine kinase binding domain (TKB) connected by a linker to the RING-finger domain (RING) implicated in E2 enzyme binding. These domains are followed by a proline-rich region and a C-terminal portion containing sites of tyrosine phosphorylation (not shown) and a sequence homologous to both the ubiquitin-associated and leucine zipper domain (UBA/LZ). Numbers above the domain structure indicate the amino acid boundaries of those domains.

(B) Electropherograms showing the identified heterozygous missense mutations.

(C) Mutated amino acid residues in the three-dimensional structure of human *CBL* complexed with UBE2L3.<sup>40</sup>  $\alpha$  ribbon trace of the *CBL* tyrosine kinase binding domain (cyan), linker (orange), and RING-finger domain (red) of *CBL* are shown together with the interacting portion of UBE2L3 (gray). Mutated residues are indicated with their side chains as blue sticks. Residues of the linker and RING domain interact with the tyrosine kinase binding domain (orange and red respectively) or bind UBE2L3 (green and yellow, respectively); their side chains are also shown. Visualization and analysis of the molecular structure was performed with the program UCSF Chimera.<sup>54</sup>

(D) Facial features of the affected subjects carrying the c.1100A > C (Gln367Pro), c.1144A > G (Lys382Glu), c.1168G > T (Asp390Tyr) (at 5 years and 15 years), and c.1259G > A (Arg420Gln) (father and daughter) mutations.

the discovery of *PTPN11*'s involvement in leukemogenesis.<sup>20,21</sup> Here, we provide evidence that germline mutations in the *Cas-Br-M* (murine) *ecotropic retroviral transforming sequence* (*CBL* [MIM 165360]) gene, which encodes a multivalent adaptor protein with an established role in myeloid malignancies, can underlie a clinically variable condition with features fitting or partially overlapping NS.

*CBL* is a member of a small family of E3 ubiquitin ligases that negatively regulate intracellular signaling downstream of receptor tyrosine kinases (RTKs), but it also contributes to signal traffic through its adaptor function.<sup>22</sup> *CBL* is ubiquitously expressed and characterized by an N-terminal tyrosine kinase-binding (TKB) domain that is involved in protein-protein interaction and is connected by a short linker to a zinc-binding RING-finger domain mediating the E3 ubiquitin ligase activity (Figure 1A). The C-terminal portion of the protein includes an exten-

sive proline-rich region containing a number of putative SH3-binding motifs, and this region is followed by multiple SH2-binding tyrosine phosphorylation sites and a ubiquitin-associated (UBA) domain overlapping with a leucine zipper (LZ) motif involved in ubiquitin binding and protein dimerization, respectively.<sup>23</sup> *CBL* mediates the conjugation of ubiquitin to activated RTKs; this conjugation is required for receptor internalization, endocytic sorting, and switching off signaling via receptor degradation or recycling.<sup>24</sup> Somatically acquired, mostly homozygous *CBL* mutations have recently been found to occur with variable prevalence in myeloproliferative disorders, including JMML, and myeloid leukemias.<sup>25–33</sup> They are largely small in-frame deletions or missense changes affecting the RING-finger domain and/or the adjacent linker region, and they appear to act in a dominant-negative fashion by uncoupling *CBL* binding to activated RTKs from their ubiquitylation and degradation.

Given the link between the functions of CBL and RAS,<sup>34</sup> CBL's ubiquitous expression, and the negative modulatory role on signaling elicited by several RTKs—including the epidermal growth factor receptor (EGFR [MIM 131550])—that have roles in developmental processes,<sup>35–37</sup> as well as the contribution of impaired CBL function to leukemogenesis, particularly JMML, we hypothesized that this gene might be implicated in the pathogenesis of NS or related phenotypes. Three hundred and sixty-five subjects with NS or a phenotype suggestive of this disorder and without mutation in most of the previously identified disease genes (M.T., B.D., C.R., M.Z., and H.Y., unpublished data) were included in the study. The clinical features in most individuals satisfied the diagnostic criteria for NS,<sup>8</sup> but subjects lacking sufficient characteristics to allow a definitive diagnosis were also considered. DNA samples and clinical data were collected under research projects approved by an institutional review board, and informed consent for genetic analyses was obtained from all subjects included in the study. Genomic DNA was isolated from peripheral blood leukocytes, and the entire *CBL* coding sequence (NM\_005188.2) was scanned for mutations. Primer pairs designed to amplify exons and their intron boundaries are listed in Table S1. PCR amplifications were carried out with the high-fidelity Optimase Polymerase (Transgenomic) at conditions indicated by Optimase ProtocolWriter software (Transgenomic). Mutation analysis of the amplicons was performed via denaturing high-performance liquid chromatography (DHPLC) with the Wave 2100 System (Transgenomic) at column temperatures recommended by Navigator software, version 1.6.4.12 (Transgenomic). Amplicons with abnormal denaturing profiles were purified (Microcon PCR [Millipore]) and then sequenced bidirectionally with the ABI BigDye Terminator Sequencing Kit v.1.1 (Applied Biosystems) and ABI Prism 3100 and 3730 Genetic Analyzers (Applied Biosystems). Mutation analysis allowed the identification of heterozygosity for a *CBL* mutation in four unrelated individuals (Figure 1B). The c.1100A > C, c.1144A > G and c.1168G > T missense changes, predicting the Gln367Pro, Lys382Glu, and Asp390Tyr amino acid substitutions, respectively, were identified in three apparently sporadic cases. Parental DNA sequencing of the relevant exon demonstrated the de novo origin of the c.1100A > C and c.1168G > T transversions, and STR genotyping (AmpF/STR Identifier PCR Amplification Kit [Applied Biosystems]) confirmed paternity. In these cases, the defects were documented in hair bulb (c.1168G > T) and buccal epithelial (c.1100A > C and c.1168G > T) cell specimens, which excluded a somatic event restricted to hematopoietic cells (Figure S1A available online). In case BO5149, the c.1144A > G change was documented as having been inherited from the father, who was originally deemed unaffected. Review of his clinical status, however, revealed minor signs and Chiari type 1 malformation (see below), the latter known to recur in NCFCS, which suggested markedly variable expressivity of the mutation. In the family transmitting the trait, the c.1259G > A transi-

tion, predicting the Arg420Gln substitution, cosegregated with disease. The inherited c.1144A > G and c.1259G > A changes were unobserved in more than 400 population-matched unaffected individuals scanned by DHPLC analysis and direct sequencing, strongly arguing against the possibility that these variants were disease-unrelated polymorphisms occurring in the population. As expected, the two de novo *CBL* missense changes were not observed in the controls, further evidence that these variants were mutations.

The four mutations affected residues evolutionarily conserved in *CBL* orthologs and paralogs (Figure S1B) that were located within the RING finger domain (Lys382, Asp390, and Arg420) or the adjacent linker connecting this domain to the N-terminal TKB domain (Gln367), a region that is known to represent the mutational hot spot for somatically acquired lesions occurring in malignancies.<sup>25–27,29–32,38,39</sup> According to the crystal structure of the TKB-linker-RING portion of *CBL* complexed with the ubiquitin-conjugating enzyme E2L3 (UBE2L3 [MIM 603721]),<sup>40</sup> Lys382 and Arg420 are positioned on the RING surface involved in binding to UBE2L3 (Figure 1C). These residues are adjacent or close to four of the invariant cysteine residues that participate in the stabilization of the domain structure by binding the two tetrahedrally coordinated zinc ions. Lys382 is adjacent to residues involved in UBE2L3 binding, and Arg420 directly participates in this binding network. Missense mutations affecting Arg420, including the Arg420Gln substitution, or residues flanking Lys382 (Leu380, Cys381, Ile383, and Cys384) had previously been documented in chronic myelomonocytic leukemia (CMML) and acute myeloid leukemia (AML) (Figure S2).<sup>26,27,30–32,38,39</sup> On the other hand, Asp390 is adjacent to a residue (Lys389) contributing to the intramolecular interaction involving the linker and RING domain with the TKB domain, which is critical for proper *CBL* function.<sup>40,41</sup> Consistent with the hypothesis that the Asp390Tyr substitution affects the integrity of the RING-TKB interface, a missense change (Asp390Val) altering Asp390 has been reported in myeloid malignancies.<sup>39</sup> Similarly, Gln367 is located in a region of the linker that includes residues contributing to the intramolecular interaction with the TKB domain and UBE2L3 binding (Figure 1C).<sup>40</sup> The Gln367Pro substitution and a nonconservative amino acid change Gln367Lys affecting the same residue have been identified in CMML.<sup>31</sup>

Detailed clinical information was obtained for the six identified subjects harboring *CBL* mutations (Figure 1D). The patient heterozygous for the c.1100A > C transversion (NMC-NS076) was referred because of developmental delay and congenital heart disease. His parents were non-consanguineous, apparently healthy, and had no features suggestive for NS. Clinical examination at 9 years showed a triangular face with hypertelorism, mild palpebral ptosis, as well as large, slightly low-set ears. He had normal stature, a thorax with widely spaced nipples, and pectus

excavatum. He had several café-au-lait spots, but no other ectodermal abnormalities were apparent. Cardiologic evaluation revealed an enlarged left atrium and transient chaotic ventricular dysrhythmias, an uncommon feature in NS. A head MRI scan showed delayed myelination without structural abnormalities. Overall, his features were suggestive of NS but did not fulfill the clinical criteria for a definitive diagnosis.

The child heterozygous for the de novo c.1168G > T transversion (case ISS-S1N51) was initially referred at age 3 years. She displayed an unusual facial appearance (frontal bossing, wide nasal bridge, low-set and posteriorly rotated ears, and mild left-sided ptosis) and mild global developmental delay. She had generalized hypotonia in the newborn and infancy periods and severe feeding problems. Her early milestones were delayed; she achieved walking at age 21 months and had significant language delay. There was no organomegaly, and her cardiovascular system was normal. Clinical examination at 5 years showed still slightly delayed speech but improved motor skills. There was mild ectodermal involvement; she had fine and sparse scalp hair, widely spaced nipples, and a mild pectus excavatum anteriorly. Her height was at the tenth percentile. She had joint laxity at the elbows, shoulders, and fingers. At 15 years, dysmorphic features appeared less evident, and growth and IQ were in the normal range, but she continued to have low muscle tone generally and hyperextensible joints.

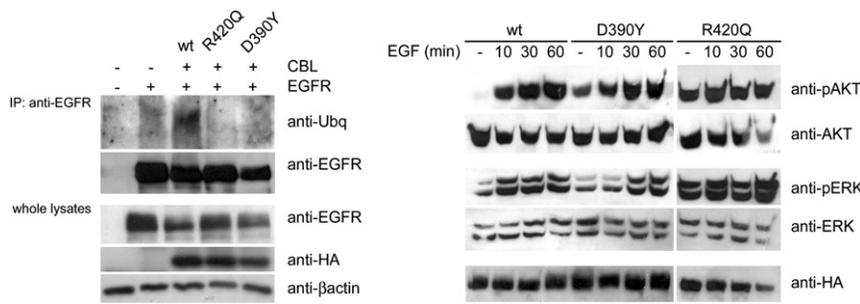
The subject inheriting the c.1144A > G substitution (case BO5149) was an 18-year-old girl who fulfilled the clinical criteria for the diagnosis of NS, exhibiting short stature (<3<sup>rd</sup> percentile), distinctive facial features (hypertelorism, ptosis, downslanting palpebral fissures, epicanthal folds, and low-set, posteriorly rotated ears), a short neck with a marked pterigium colli, and a low posterior hairline. She presented with a bicuspid aortic valve with stenosis requiring surgical intervention. Other features included strabismus, thorax anomalies, cubitus valgus, hyperextensible joints, conductive hearing loss, and unilateral renal agenesis. Her 46-year-old father, who carried the missense change, did not show significant dysmorphic facial features, had a normal height, and showed no evidence of relevant cardiovascular and skeletal anomalies. He did have, however, a broad neck, low posterior hairline, and a Chiari type 1 malformation, a feature recurring in NS and clinically related disorders,<sup>42–45</sup> that was complicated by tri-ventricular hydrocephalus and cervical syringomyelia resulting in weakness and hyperreflexia of the legs, sensory deficits of the trunk and lower right limb, and nystagmus, which required decompressive suboccipital craniectomy and cervical laminectomy.

At six months of age, the girl (case HD316) inheriting the c.1259G > A transition from her affected father had a coarse, dysmorphic face (epicanthal folds, flat nasal bridge, thick lips, dysmorphic ears with thick helices, and a mid-face angioma). She had a short neck with redundant skin, thin hair, dysplastic toenails, dark skin, café-au-lait

spots and streaks on the legs, and muscular hypotonia. Growth was in the normal range. Echocardiography revealed mild-to-moderate mitral valve insufficiency due to dysplastic leaflets. At 8 years of age, facial features and signs included pale-blue eyes, long philtrum, a thick and everted lower lip, and dysmorphic ears. Her hair was thin, dysplastic, and slow growing, and her eyebrows were sparse. Her neck was short, and her chest was broad with wide-spaced nipples. Her skin appeared dark and dry, and she had eczematous lesions on the face, keratosis pilaris, and café-au-lait streaks and spots on her legs. Her joints were hypermobile, and her voice was hoarse. Cognitive development was mildly delayed. A head MRI showed an abnormal corpus callosum with enlargement of the anterior portion and hypoplasia of the posterior region, as well as cerebellar vermis hypoplasia, the latter representing, to our knowledge, an unreported feature in NCFCS. Her 40-year-old father showed a high forehead, sparse eyebrows, down-slanting palpebral fissures, thick lips, thick helices, a short neck, a broad chest, pectus excavatum, and dry and dark skin with hyperpigmented lesions in the sacral region. Bilateral cryptorchidism was operated upon at 8 years of age. Developmental milestones were achieved normally, but learning difficulties were recorded. None of the six subjects heterozygous for a *CBL* mutation exhibited any hematologic anomaly or malignancy.

To explore the consequences of the identified mutations on CBL ubiquitin ligase activity as well as their effects on RAS signal output, we transiently expressed HA-tagged Asp390Tyr and Arg420Gln CBL mutants in COS-1 cells and evaluated the levels of EGFR ubiquitylation and the extent of signal flow through the MAPK and PI3K-AKT cascades (Figure 2). These mutations were selected as representatives of the two classes of lesions affecting residues located at the interface between the TKB and RING-finger domains (Gln367Pro and Asp390Tyr) or facing toward the E2 ubiquitin-conjugating enzyme (Lys382Glu and Arg420Gln). Compared to that of the wild-type CBL protein, expression of each mutant resulted in impaired receptor ubiquitylation in response to EGF stimulation. These findings are in line with previous studies focused on leukemia-associated *CBL* mutations, indicating that these lesions impair CBL ubiquitin ligase function, weaken CBL-mediated degradation of cell-surface receptors, and have a dominant-negative effect on the wild-type protein.<sup>26,31</sup> Transient expression of each mutant was shown to enhance AKT phosphorylation basally, possibly because of inefficient CBL-mediated downregulation of cell-surface receptors. Cells expressing the Arg420Gln mutant also showed constitutive ERK phosphorylation. Overall, our findings indicate that germline disease-associated *CBL* mutations dysregulate intracellular signaling through RAS and that this effect is due, at least in part, to impaired receptor ubiquitylation. These results are consistent with the available data on leukemia-associated CBL mutants.

Mutations of genes coding for transducers implicated in RAS-MAPK signaling have recently been recognized as the



**Figure 2. Functional Consequences of the Disease-Causing Asp390Tyr and Arg420Gln CBL Mutant Proteins**

(Left) EGFR ubiquitylation in COS-1 cells transiently expressing the Asp390Tyr, Arg420Gln, and wild-type CBL proteins after EGF stimulation (100 ng/ml, 1 hr). EGFR ubiquitylation (anti-ubiquitin, Santa Cruz Biotech, sc-8017) was determined on immunoprecipitated EGFR (Cell Signaling, #4267). Immunoprecipitated EGFR, and expression levels of exogenous CBL (anti-HA antibody, Sigma, H3663) and EGFR

proteins and  $\beta$ -actin (Sigma, A5441) in cell lysates are also shown. Blots are representative of three experiments performed. (Right) AKT and ERK phosphorylation assays in COS-1 cells transiently expressing the Asp390Tyr, Arg420Gln, and wild-type CBL proteins. Protein phosphorylation status (pAKT, Cell Signaling, #9271; pERK1/2, Cell Signaling, #9106) was evaluated in time-course experiments basally or after EGF stimulation. AKT (Cell Signaling, #9272), ERK1/2 (Cell Signaling, #9102) and CBL (anti-HA, see above) expression levels are also shown.

molecular cause underlying a group of clinically related developmental disorders of which NS represents the most recurrent condition. These Mendelian traits are caused by mutations in genes encoding RAS proteins (KRAS, HRAS, and NRAS), downstream transducers (RAF1, BRAF, MAP2K1, and MAP2K2 [MIM 601263]), or pathway regulators (PTPN11, SOS1, NF1 [MIM 613113], SPRED1 [MIM 609291], and SHOC2). Here, we report that germline missense mutations affecting the linker region or the RING finger domain of CBL, a known negative regulator of activated RTKs, can underlie a condition resembling NS or conditions with clinical features partially overlapping it, and we thereby identify a previously unpredicted consequence of dysregulated CBL function in human disease. One mechanism by which intracellular signaling is switched off is RTK downregulation through receptor internalization and degradation. CBL contributes to this process as an adaptor molecule with ubiquitin ligase activity.<sup>23</sup> It has been shown that direct or GRB2-mediated binding of CBL to EGFR is required for multiple mono-ubiquitylation of the activated receptor. Ubiquitylation of EGFR is followed by its internalization in clathrin-coated vesicles that fuse with internal vesicles to form endosomes, from where ubiquitylated EGFR is sorted to lysosomes for degradation while nonubiquitylated EGFR is recycled back to the plasma membrane. In this process, CBL function is required in the sorting step for efficient EGFR downregulation.<sup>46,47</sup> On the basis of these considerations, mutations impairing the ubiquitin ligase activity of CBL are expected to promote enhanced intracellular signal flow through inefficient receptor degradation, which is consistent with the present findings and recently published data.<sup>26,31</sup> Hinting at the present findings, acquired CBL mutations affecting the RING-finger domain and the adjacent linker stretch had been shown to contribute to the pathogenesis of myeloid leukemias and myeloproliferative disorders, including JMML. In JMML, CBL mutations are largely missense; residue Tyr371 is mutated in the majority of cases.<sup>27,29</sup> These lesions are mutually exclusive from PTPN11, KRAS, NRAS, and NF1 mutations, which also occur in this myeloproliferative disorder. This finding

supports the functional equivalence of CBL defects in causing enhanced RAS signaling, at least in precursor cells of the myeloid lineage.<sup>27,29</sup>

Although the location and type of identified mutations are compatible with the idea that this newly identified condition results from the dominant-negative effect of one mutated CBL allele, it should be considered that CBL is also believed to regulate signaling positively, for example as part of the MAPK and PI3K/AKT pathways, via its adaptor function.<sup>48–50</sup> This positive modulatory role is attained through binding to a plethora of proteins and functioning as a scaffold allowing the formation of multi-protein complexes mediating signal flow. Although we cannot formally exclude effects on this still poorly characterized positive modulatory function, the fact that the disease-causing mutations are far from the known binding sites makes this unlikely.

In JMML and other myeloid malignancies, CBL mutations generally occur as homozygous lesions as a result of acquired isodisomy, which suggests that the contribution of a CBL mutant to leukemogenesis requires either loss of function of the wild-type protein or increased dosage of the mutated allele.<sup>27,31,32</sup> Although no hematological malignancy was documented in the six subjects carrying a germline CBL mutation, this consideration supports the idea that the identified heterozygous condition for a germline CBL mutation might represent a condition that predisposes to malignancies. A similar scenario within the NCFCS family is observed in neurofibromatosis type I (NF1 [MIM 162200]), in which a second, somatic loss-of-function mutation or deletion of the wild-type NF1 allele is associated with the development of malignancies.<sup>51</sup> NF1 is a relatively common autosomal-dominant disorder characterized by café-au-lait spots, neurofibromas, Lisch nodules, axillary and inguinal freckling, and recurrent features comprising reduced growth, skeletal defects, deficits in cognitive function, and increased risk for certain malignancies, including JMML. Similar to findings of the present study, a markedly wide phenotypic spectrum is associated with germline mutations in the NF1 gene; even within the same family, some patients are mildly

affected, and others have severe manifestations of disease. Neurofibromin, the *NF1* gene product, is a negative modulator of RAS function, which partly explains the overlap with NS in some patients and the resulting so-called neurofibromatosis-Noonan syndrome (NFNS [MIM 601321]).<sup>52,53</sup>

Although analysis of wider cohorts will be required to characterize more precisely the phenotypic spectrum associated with germline *CBL* mutations, the present findings indicate that these lesions are associated with a strikingly variable phenotype and that clinical features might be quite subtle. Several lines of evidence, however, support the idea that the heterozygous condition for a germline *CBL* mutation underlies a previously unrecognized disorder resembling or partially overlapping NS. First, no missense change affecting the linker region and the adjacent RING-finger domain was identified in more than 400 unaffected population-matched subjects. Such a nonrandom distribution of sequence variants is statistically significant (Fisher's exact probability < 0.05). Second, two mutations arose de novo in the germline. Third, the mutations altered highly conserved residues in *CBL* orthologs and paralogs located in protein domains functionally required for *CBL*-mediated signaling to be properly switched off. Moreover, they overlapped in position and, in some cases, with the precise substitution of somatic *CBL* mutations observed in human cancers. Fourth, the four germline *CBL* missense mutations have functional effects on signal transduction. Previous and present characterization of three of the mutants identified in this study showed impairment of *CBL* ubiquitin ligase activity and derangement of signaling through ERK and AKT.

Overall, the present data indicate that *CBL* gene mutations underlie a clinically variable condition that can resemble NS phenotypically. *CBL* mutations are likely to account for a small portion of subjects with features fitting NS (<1%), but they might be more common among subjects with clinical features partially overlapping NS or a phenotype that is suggestive of this disorder, and particularly among subjects with NS/JMML without mutations in the *PTPN11*, *NF1*, or *RAS* genes. These findings provide evidence that *CBL* functional dysregulation can significantly perturb a wider range of cellular processes than was previously known and have a direct impact on development. Molecular genetic as well as structural and functional data support the view that disease-associated *CBL* mutations perturb intracellular signaling by affecting receptor metabolism (i.e., recycling versus degradation) rather than directly altering signal flow at the level of RAS or its downstream effectors, which represents the first evidence for disorders of the NCFCS family. Additional work will need to provide a more precise delineation of the phenotypic spectrum associated with germline mutations in *CBL* as well as their molecular diversity and functional consequences in intracellular signaling and development.

## Supplemental Data

Supplemental Data include two figures and one table.

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## Web Resources

Accession numbers and the URLs for data are as follows:

Entrez Gene, <http://www.ncbi.nlm.nih.gov/gene/>

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/>

Optimase ProtocolWriter software, <http://www.MutationDiscovery.com>

Protein Data Bank (PDB), <http://www.rcsb.org/pdb/home/home.do>

UCSF Chimera, <http://www.cgl.ucsf.edu/chimera/>

## References

1. Karnoub, A.E., and Weinberg, R.A. (2008). Ras oncogenes: Split personalities. *Nat. Rev. Mol. Cell Biol.* 9, 517–531.
2. Rajalingam, K., Schreck, R., Rapp, U.R., and Albert, S. (2007). Ras oncogenes and their downstream targets. *Biochim. Biophys. Acta* 1773, 1177–1195.
3. Schubbert, S., Shannon, K., and Bollag, G. (2007). Hyperactive Ras in developmental disorders and cancer. *Nat. Rev. Cancer* 7, 295–308.
4. Tidyman, W.E., and Rauen, K.A. (2009). The RASopathies: Developmental syndromes of Ras/MAPK pathway dysregulation. *Curr. Opin. Genet. Dev.* 19, 230–236.
5. Tartaglia, M., Zampino, G., and Gelb, B.D. (2010). Noonan syndrome: Clinical aspects and molecular pathogenesis. *Molecular Syndromology* 1, 2–26.
6. Noonan, J.A. (1994). Noonan syndrome. An update and review for the primary pediatrician. *Clin. Pediatr. (Phila.)* 33, 548–555.
7. Allanson, J.E. (2007). Noonan syndrome. *Am. J. Med. Genet. C. Semin. Med. Genet.* 145C, 274–279.
8. van der Burgt, I. (2007). Noonan syndrome. *Orphanet J. Rare Dis.* 2, 4.

9. Tartaglia, M., Mehler, E.L., Goldberg, R., Zampino, G., Brunner, H.G., Kremer, H., van der Burgt, I., Crosby, A.H., Ion, A., Jeffery, S., et al. (2001). Mutations in *PTPN11*, encoding the protein tyrosine phosphatase SHP-2, cause Noonan syndrome. *Nat. Genet.* *29*, 465–468.
10. Roberts, A.E., Araki, T., Swanson, K.D., Montgomery, K.T., Schiripo, T.A., Joshi, V.A., Li, L., Yassin, Y., Tamburino, A.M., Neel, B.G., and Kucherlapati, R.S. (2007). Germline gain-of-function mutations in *SOS1* cause Noonan syndrome. *Nat. Genet.* *39*, 70–74.
11. Tartaglia, M., Pennacchio, L.A., Zhao, C., Yadav, K.K., Fodale, V., Sarkozy, A., Pandit, B., Oishi, K., Martinelli, S., Schackwitz, W., et al. (2007). Gain-of-function *SOS1* mutations cause a distinctive form of Noonan syndrome. *Nat. Genet.* *39*, 75–79.
12. Carta, C., Pantaleoni, F., Bocchinfuso, G., Stella, L., Vasta, I., Sarkozy, A., Digilio, C., Palleschi, A., Pizzuti, A., Grammatico, P., et al. (2006). Germline missense mutations affecting *KRAS* Isoform B are associated with a severe Noonan syndrome phenotype. *Am. J. Hum. Genet.* *79*, 129–135.
13. Schubert, S., Zenker, M., Rowe, S.L., Böll, S., Klein, C., Bollag, G., van der Burgt, I., Musante, L., Kalscheuer, V., Wehner, L.E., et al. (2006). Germline *KRAS* mutations cause Noonan syndrome. *Nat. Genet.* *38*, 331–336.
14. Cirstea, I.C., Kutsche, K., Dvorsky, R., Gremer, L., Carta, C., Horn, D., Roberts, A.E., Lepri, F., Merbitz-Zahradnik, T., König, R., et al. (2010). A restricted spectrum of *NRAS* mutations causes Noonan syndrome. *Nat. Genet.* *42*, 27–29.
15. Pandit, B., Sarkozy, A., Pennacchio, L.A., Carta, C., Oishi, K., Martinelli, S., Pogna, E.A., Schackwitz, W., Ustaszewska, A., Landstrom, A., et al. (2007). Gain-of-function *RAF1* mutations cause Noonan and LEOPARD syndromes with hypertrophic cardiomyopathy. *Nat. Genet.* *39*, 1007–1012.
16. Razaque, M.A., Nishizawa, T., Komoike, Y., Yagi, H., Furutani, M., Amo, R., Kamisago, M., Momma, K., Katayama, H., Nakagawa, M., et al. (2007). Germline gain-of-function mutations in *RAF1* cause Noonan syndrome. *Nat. Genet.* *39*, 1013–1017.
17. Sarkozy, A., Carta, C., Moretti, S., Zampino, G., Digilio, M.C., Pantaleoni, F., Scioletti, A.P., Esposito, G., Cordeddu, V., Lepri, F., et al. (2009). Germline *BRAF* mutations in Noonan, LEOPARD, and cardiofaciocutaneous syndromes: molecular diversity and associated phenotypic spectrum. *Hum. Mutat.* *30*, 695–702.
18. Cordeddu, V., Di Schiavi, E., Pennacchio, L.A., Ma'ayan, A., Sarkozy, A., Fodale, V., Cecchetti, S., Cardinale, A., Martin, J., Schackwitz, W., et al. (2009). Mutation of *SHOC2* promotes aberrant protein N-myristoylation and causes Noonan-like syndrome with loose anagen hair. *Nat. Genet.* *41*, 1022–1026.
19. Nava, C., Hanna, N., Michot, C., Pereira, S., Pouvreau, N., Niihori, T., Aoki, Y., Matsubara, Y., Arveiler, B., Lacombe, D., et al. (2007). Cardio-facio-cutaneous and Noonan syndromes due to mutations in the *RAS/MAPK* signalling pathway: Genotype-phenotype relationships and overlap with Costello syndrome. *J. Med. Genet.* *44*, 763–771.
20. Tartaglia, M., Niemeyer, C.M., Fragale, A., Song, X., Buechner, J., Jung, A., Hählen, K., Hasle, H., Licht, J.D., and Gelb, B.D. (2003). Somatic mutations in *PTPN11* in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia. *Nat. Genet.* *34*, 148–150.
21. Tartaglia, M., Martinelli, S., Cazzaniga, G., Cordeddu, V., Iavarone, I., Spinelli, M., Palmi, C., Carta, C., Pession, A., Aricò, M., et al. (2004). Genetic evidence for lineage-related and differentiation stage-related contribution of somatic *PTPN11* mutations to leukemogenesis in childhood acute leukemia. *Blood* *104*, 307–313.
22. Dikic, I., and Schmidt, M.H. (2007). Malfunctions within the Cbl interactome uncouple receptor tyrosine kinases from destructive transport. *Eur. J. Cell Biol.* *86*, 505–512.
23. Swaminathan, G., and Tsygankov, A.Y. (2006). The Cbl family proteins: ring leaders in regulation of cell signaling. *J. Cell. Physiol.* *209*, 21–43.
24. Schmidt, M.H., and Dikic, I. (2005). The Cbl interactome and its functions. *Nat. Rev. Mol. Cell Biol.* *6*, 907–918.
25. Caligiuri, M.A., Briesewitz, R., Yu, J., Wang, L., Wei, M., Arnoczky, K.J., Marburger, T.B., Wen, J., Perrotti, D., Bloomfield, C.D., and Whitman, S.P. (2007). Novel c-CBL and CBL-b ubiquitin ligase mutations in human acute myeloid leukemia. *Blood* *110*, 1022–1024.
26. Sargin, B., Choudhary, C., Crosetto, N., Schmidt, M.H., Grundler, R., Rensinghoff, M., Thiessen, C., Tickenbrock, L., Schwäble, J., Brandts, C., et al. (2007). *Flt3*-dependent transformation by inactivating c-Cbl mutations in AML. *Blood* *110*, 1004–1012.
27. Loh, M.L., Sakai, D.S., Flotho, C., Kang, M., Fliegau, M., Archambeault, S., Mullighan, C.G., Chen, L., Bergstraesser, E., Bueso-Ramos, C.E., et al. (2009). Mutations in *CBL* occur frequently in juvenile myelomonocytic leukemia. *Blood* *114*, 1859–1863.
28. Makishima, H., Cazzolli, H., Szpurka, H., Dunbar, A., Tiu, R., Huh, J., Muramatsu, H., O'Keefe, C., Hsi, E., Paquette, R.L., et al. (2009). Mutations of e3 ubiquitin ligase cbl family members constitute a novel common pathogenic lesion in myeloid malignancies. *J. Clin. Oncol.* *27*, 6109–6116.
29. Muramatsu, H., Makishima, H., Jankowska, A.M., Cazzolli, H., O'Keefe, C., Yoshida, N., Xu, Y., Nishio, N., Hama, A., Yagasaki, H., et al. (2010). Mutations of an E3 ubiquitin ligase c-Cbl but not *TET2* mutations are pathogenic in juvenile myelomonocytic leukemia. *Blood* *115*, 1969–1975.
30. Reindl, C., Quentmeier, H., Petropoulos, K., Greif, P.A., Ben-thaus, T., Argiropoulos, B., Mellert, G., Vempati, S., Duyster, J., Buske, C., et al. (2009). *CBL* exon 8/9 mutants activate the *FLT3* pathway and cluster in core binding factor/11q deletion acute myeloid leukemia/myelodysplastic syndrome subtypes. *Clin. Cancer Res.* *15*, 2238–2247.
31. Sanada, M., Suzuki, T., Shih, L.Y., Otsu, M., Kato, M., Yamazaki, S., Tamura, A., Honda, H., Sakata-Yanagimoto, M., Kumano, K., et al. (2009). Gain-of-function of mutated *C-CBL* tumour suppressor in myeloid neoplasms. *Nature* *460*, 904–908.
32. Grand, F.H., Hidalgo-Curtis, C.E., Ernst, T., Zoi, K., Zoi, C., McGuire, C., Kreil, S., Jones, A., Score, J., Metzgeroth, G., et al. (2009). Frequent *CBL* mutations associated with 11q acquired uniparental disomy in myeloproliferative neoplasms. *Blood* *113*, 6182–6192.
33. Sugimoto, Y., Muramatsu, H., Makishima, H., Prince, C., Jankowska, A.M., Yoshida, N., Xu, Y., Nishio, N., Hama, A., Yagasaki, H., et al. (2010). Spectrum of molecular defects in juvenile myelomonocytic leukaemia includes *ASXL1* mutations. *Br. J. Haematol.*, in press.
34. Moghal, N., and Sternberg, P.W. (2003). A component of the transcriptional mediator complex inhibits *RAS*-dependent vulval fate specification in *C. elegans*. *Development* *130*, 57–69.

35. Grøvdal, L.M., Stang, E., Sorkin, A., and Madshus, I.H. (2004). Direct interaction of Cbl with pTyr 1045 of the EGF receptor (EGFR) is required to sort the EGFR to lysosomes for degradation. *Exp. Cell Res.* *300*, 388–395.
36. Visser, G.D., and Lill, N.L. (2005). The Cbl RING finger C-terminal flank controls epidermal growth factor receptor fate downstream of receptor ubiquitination. *Exp. Cell Res.* *311*, 281–293.
37. Umebayashi, K., Stenmark, H., and Yoshimori, T. (2008). Ubc4/5 and c-Cbl continue to ubiquitinate EGF receptor after internalization to facilitate polyubiquitination and degradation. *Mol. Biol. Cell* *19*, 3454–3462.
38. Dunbar, A.J., Gondek, L.P., O’Keefe, C.L., Makishima, H., Rataul, M.S., Szpurka, H., Sekeres, M.A., Wang, X.F., McDevitt, M.A., and Maciejewski, J.P. (2008). 250K single nucleotide polymorphism array karyotyping identifies acquired uniparental disomy and homozygous mutations, including novel missense substitutions of c-Cbl, in myeloid malignancies. *Cancer Res.* *68*, 10349–10357.
39. Bacher, U., Haferlach, C., Schnittger, S., Kohlmann, A., Kern, W., and Haferlach, T. (2010). Mutations of the TET2 and CBL genes: Novel molecular markers in myeloid malignancies. *Ann. Hematol.* *89*, 643–652.
40. Zheng, N., Wang, P., Jeffrey, P.D., and Pavletich, N.P. (2000). Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. *Cell* *102*, 533–539.
41. Kassenbrock, C.K., and Anderson, S.M. (2004). Regulation of ubiquitin protein ligase activity in c-Cbl by phosphorylation-induced conformational change and constitutive activation by tyrosine to glutamate point mutations. *J. Biol. Chem.* *279*, 28017–28027.
42. Holder-Espinasse, M., and Winter, R.M. (2003). Type 1 Arnold-Chiari malformation and Noonan syndrome. A new diagnostic feature? *Clin. Dysmorphol.* *12*, 275.
43. Tubbs, R.S., Rutledge, S.L., Kosentka, A., Bartolucci, A.A., and Oakes, W.J. (2004). Chiari I malformation and neurofibromatosis type 1. *Pediatr. Neurol.* *30*, 278–280.
44. Beier, A.D., Barrett, R.J., Burke, K., Kole, B., and Soo, T.M. (2009). Leopard syndrome and Chiari type I malformation: A case report and review of the literature. *Neurologist* *15*, 37–39.
45. Gripp, K.W., Hopkins, E., Doyle, D., and Dobyns, W.B. (2010). High incidence of progressive postnatal cerebellar enlargement in Costello syndrome: Brain overgrowth associated with HRAS mutations as the likely cause of structural brain and spinal cord abnormalities. *Am. J. Med. Genet. A.* *152A*, 1161–1168.
46. Longva, K.E., Blystad, F.D., Stang, E., Larsen, A.M., Johannesen, L.E., and Madshus, I.H. (2002). Ubiquitination and proteasomal activity is required for transport of the EGF receptor to inner membranes of multivesicular bodies. *J. Cell Biol.* *156*, 843–854.
47. Duan, L., Miura, Y., Dimri, M., Majumder, B., Dodge, I.L., Reddi, A.L., Ghosh, A., Fernandes, N., Zhou, P., Mullane-Robinson, K., et al. (2003). Cbl-mediated ubiquitinylation is required for lysosomal sorting of epidermal growth factor receptor but is dispensable for endocytosis. *J. Biol. Chem.* *278*, 28950–28960.
48. Finkelstein, L.D., and Shimizu, Y. (2000). Role of phosphoinositide 3-kinase and the Cbl adaptor protein in coupling the alpha4beta1 integrin to mitogen-activated protein kinase signalling. *Biochem. J.* *345*, 385–392.
49. Garcia-Guzman, M., Larsen, E., and Vuori, K. (2000). The proto-oncogene c-Cbl is a positive regulator of Met-induced MAP kinase activation: A role for the adaptor protein Crk. *Oncogene* *19*, 4058–4065.
50. Song, J.J., Kim, J.H., Sun, B.K., Alcalá, M.A. Jr., Bartlett, D.L., and Lee, Y.J. (2010). c-Cbl acts as a mediator of Src-induced activation of the PI3K-Akt signal transduction pathway during TRAIL treatment. *Cell. Signal.* *22*, 377–385.
51. Brems, H., Beert, E., de Ravel, T., and Legius, E. (2009). Mechanisms in the pathogenesis of malignant tumours in neurofibromatosis type 1. *Lancet Oncol.* *10*, 508–515.
52. Allanson, J.E., Hall, J.G., and Van Allen, M.I. (1985). Noonan phenotype associated with neurofibromatosis. *Am. J. Med. Genet.* *21*, 457–462.
53. De Luca, A., Bottillo, I., Sarkozy, A., Carta, C., Neri, C., Bellacchio, E., Schirinzi, A., Conti, E., Zampino, G., Battaglia, A., et al. (2005). NF1 gene mutations represent the major molecular event underlying neurofibromatosis-Noonan syndrome. *Am. J. Hum. Genet.* *77*, 1092–1101.
54. Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., and Ferrin, T.E. (2004). UCSF Chimera—A visualization system for exploratory research and analysis. *J. Comput. Chem.* *25*, 1605–1612.