Hyperactive Ras in developmental disorders and cancer

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Abstract | Ras genes are the most common targets for somatic gain-of-function mutations in human cancer. Recently, germline mutations that affect components of the Ras–Raf–mitogen-activated and extracellular-signal regulated kinase kinase (MEK)–extracellular signal-regulated kinase (ERK) pathway were shown to cause several developmental disorders, including Noonan, Costello and cardio-facio-cutaneous syndromes. Many of these mutant alleles encode proteins with aberrant biochemical and functional properties. Here we will discuss the implications of germline mutations in the Ras–Raf–MEK–ERK pathway for understanding normal developmental processes and cancer pathogenesis.

Pulmonic stenosis
A common form of congenital heart disease that frequently requires surgical correction. Pulmonic stenosis is a common feature of Noonan syndrome.

Phaeochromocytoma
A malignant tumour that arises in chromaffin cells within the adrenal medulla.

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Ras proteins are signal switch molecules that regulate cell fates by coupling receptor activation to downstream effector pathways that control diverse cellular responses including proliferation, differentiation and survival[1–3]. Human cancers frequently express mutant Ras proteins, termed 'oncogenic Ras'. The discoveries of germline mutations in the neurofibromin 1 (*NF1*) and *PTPN11* (which encodes SHP2) genes provided the first indication that aberrant Ras signalling might also contribute to the pathogenesis of some human developmental disorders (reviewed in REFS 4,5) (TABLE 1). Recently, germline mutations in *HRAS* and *KRAS* and in genes encoding other molecules in the Ras–Raf–mitogen-activated and extracellular-signal regulated kinase kinase (MEK)–extracellular signal-regulated kinase (ERK) cascade were shown to cause Noonan, LEOPARD (multiple lentigines, electrocardiographic conduction abnormalities, ocular hypertelorism, pulmonic stenosis, abnormal genitalia, retardation of growth and sensorineural deafness), cardio-facio-cutaneous (CFC) and Costello syndromes (TABLE 1). Many of these mutations encode novel SHP2, SOS1, HRAS, KRAS, BRAF and MEK proteins. The developmental disorders associated with Ras pathway mutations share phenotypic features that include facial abnormalities, heart defects, impaired growth and development, and, in some instances, a predisposition to specific cancers[5,6]. These mutations perturb the intrinsic biochemical properties of the encoded proteins, which can result in abnormal cellular signalling, proliferation, survival and responses to growth factors[7–14]. These new data support the general idea that the degree and/or duration of Ras signalling regulates developmental programmes in specific cell types, and provide a new perspective for considering the role of hyperactive Ras signalling in tumorigenesis.

Ras signalling

Ras proteins are small GTPases that cycle between inactive guanosine diphosphate (GDP)-bound and active guanosine triphosphate (GTP)-bound conformations (Ras–GDP and Ras–GTP, respectively)[15,16]. Ras proteins regulate cellular responses to many extracellular stimuli, including soluble growth factors. Growth factor binding to cell-surface receptors creates intracellular docking sites for adaptor molecules and signal-relay proteins that recruit and activate guanine nucleotide-exchange factors (GNEFs) such as members of the SOS family. GNEFs displace guanine nucleotides from Ras and permit passive binding to GTP, which is abundant in the cytosol. GTP-bound Ras can interact productively with more than 20 effectors, including Raf, phosphatidylinositol 3-kinase (PI3K) and Ral guanine nucleotide-dissociation stimulator (RALGDS), to regulate various cellular responses including proliferation, survival and differentiation[17,18] (FIG. 1). The degree and duration of Ras activation can have profound effects on cell fate decisions. For example, in PC-12 rat adrenal phaeochromocytoma cells, the transient activation of Ras through epidermal growth factor (EGF) signalling stimulates proliferation, whereas nerve growth factor (NGF)-dependent sustained activation of Ras induces differentiation[19–23]. Ras proteins are negatively regulated by GTPase activating proteins (GAPs), which markedly stimulate intrinsic GTPase activity by stabilizing a high-energy transition state that occurs during the Ras–GTP hydrolysis reaction (BOX 1).

At a glance

- Ras proteins regulate signalling pathways that control many cellular responses such as proliferation, survival and differentiation.
- Ras proteins are activated when guanosine triphosphate (GTP) is bound. SOS1, and other exchange factors stimulate guanine nucleotide dissociation from Ras, which results in increased levels of Ras–GTP.
- Ras–GTP signalling is terminated by hydrolysis to Ras–guanosine diphosphate (Ras–GDP), a reaction catalysed by the GTPase-activating proteins (GAPs), including p120GAP and neurofibromin.
- Ras–GTP binds to various effector proteins to stimulate signalling pathways; among these effector pathways is the Raf–mitogen-activated and extracellular-signal regulated kinase kinase (MEK)–extracellular signal-regulated kinase (ERK) cascade.
- Activating somatic mutations in the *Ras* genes and mutations that activate regulators and effectors of Ras proteins are common in tumour development and cancer.
- Germline mutations that affect components of the Ras–Raf–MEK–ERK pathway are now known to underlie a group of developmental disorders, such as Noonan syndrome, Costello syndrome and cardio-facio-cutaneous syndrome.
- Germline mutations in human syndromes frequently encode novel mutant proteins. Studies performed to date suggest that strength and/or duration of signalling through the Ras–Raf–MEK–ERK pathway regulates developmental programmes. Further structural, biochemical and functional analyses of these mutant proteins will extend our understanding of Ras signalling in development and cancer.

Ras-GTP regulates a complex signalling network that modulates cell behaviour by binding to and activating distinct classes of effector molecules. The Raf–MEK–ERK cascade is the best characterized Ras effector pathway[18]. There are three Raf serine/threonine kinases (ARAF, BRAF and RAF1) that activate the MEK–ERK kinase cascade. ERK kinases can phosphorylate both cytosolic and nuclear substrates, which include transcription factors such as JUN and ELK1, which is an E26 transformation-specific sequence (ETS) family member that forms part of the serum response factor that regulates *FOS* expression[24]. JUN and FOS proteins form the activator protein 1 (AP1) transcription factor. Activation of these transcriptional regulators can lead to the expression of proteins that control cell-cycle progression, such as cyclin D[25]. Ras–GTP also binds the catalytic subunit of type I PI3Ks[26,27]. This binding results in the translocation of PI3K to the plasma membrane and subsequent activation. PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4,5-triphosphate, which activates downstream kinases such as 3-phosphoinositide-dependent protein kinase 1 (PDK1) and Akt[28]. Akt is a kinase that promotes survival in many cell types by phosphorylating and therefore inactivating several pro-apoptotic proteins including BAD and Forkhead (FKHR) transcription factors[29,30]. PI3K also activates Rac, a Rho family GTPase, which has been shown to be important for transformation by oncogenic Ras in some cellular contexts[31]. Ras can also activate a family of exchange factors for the Ral small GTPases, which include RALGDS, RALGDS-like gene (RGL) and RGL2 (REF. 32). These exchange factors activate Ral, which can stimulate phospholipase D. A recent study showed that RALGDS is required for tumour formation in a mouse model of Ras-dependent skin carcinogenesis[33]. In addition, signalling through RALA seems to have a crucial role in the transformation and tumorigenesis of other human cell types[34].

Many other effector pathways are directly coupled to Ras–GTP, several of which have known roles in regulating cellular responses. Ras–GTP directly binds to the Rac exchange factor tumour invasion and metastasis inducing protein 1 (TIAM1), leading to increased levels of Rac and subsequent actin reorganization[35]. Evidence that this effector pathway is important for Ras-dependent transformation comes from a study showing that TIAM1 is necessary for the development of Ras-dependent skin tumours[31]. Phospholipase Cε also binds directly to Ras–GTP, and the consequent hydrolysis of PIP2 to diacylglycerol and inositol-1,4,5-triphosphate serves to release calcium and activate protein kinase C[36–38].

The three cellular *Ras* genes encode four highly homologous 21 kD proteins: HRAS, NRAS, KRAS4A and KRAS4B (FIG. 2). KRAS4A and KRAS4B result from alternative splicing at the C terminus. The N-terminal portion (residues 1–165) of HRAS, KRAS and NRAS comprise a highly conserved G domain that has a common structure. Ras proteins diverge substantially at the C-terminal end, which is known as the hypervariable region. This region contains residues that specify post-translational protein modifications that are essential for targeting Ras proteins to the cytosolic leaflet of cellular membranes. All Ras proteins are farnesylated at a terminal CAAX motif, in which C is cysteine, A is usually an aliphatic amino acid and X is any amino acid. NRAS, HRAS and KRAS4A are additionally modified by one or two palmitic acids just upstream of the CAAX motif. The addition of the hydrophobic farnesyl moiety is complemented by the hydrophobic palmitates (the so-called 'second signal') to firmly anchor these Ras proteins in the membrane. By contrast, KRAS4B, the predominant splice variant referred to from now on as KRAS, contains an alternative second signal that is composed of a polybasic stretch of lysine residues. In this case, membrane anchoring is mediated by the electropositive lysines that form ionic bonds to the predominantly electronegative lipid head groups of the inner leaflet of the plasma membrane. Farnesyltransferase inhibitors were developed as cancer therapeutics based on data showing that post-translational processing by prenyl transferases is essential for the biological activity of normal and oncogenic Ras proteins[39]. However, KRAS and NRAS escape functional inactivation by undergoing geranylgeranylation, which, as reviewed elsewhere[40], probably explains the disappointing clinical efficacy of these agents.

Deciphering how specific Ras isoforms function in normal cellular processes and how their mutant counterparts contribute to disease are important and challenging questions. The pattern of *Ras* gene mutations in cancer is skewed with respect to both tissue type and isoform, with *KRAS* accounting for most somatic cancer-associated mutations (TABLE 2 and following section). Moreover, as discussed later in this article, distinct types of *HRAS* and *KRAS* germline mutations are found in some patients with Costello, Noonan and CFC syndromes. Genetic studies in mice suggest that Ras proteins have both unique and overlapping roles in development. KRAS is essential for mouse embryonic development[41,42]. KRAS-deficient mice die of anaemia

Farnesylation
Farnesyltransferases add farnesyl (15-carbon isoprene) groups to Ras and many other cellular proteins. This post-translational modification is essential for targeting proteins to the plasma membrane and other subcellular compartments.

Prenyl transferases
A general class of enzymes that includes both farnesyltransferases and geranylgeranyl transferases, which transfer prenyl moieties (for example, farnesyl or geranylgeranyl groups) to cellular proteins.

Geranylgeranylation
Geranylgeranyl tranferases add geranylgeranyl (20-carbon isoprene) groups to cellular proteins in order to direct membrane localization. Ras proteins are not normally geranylgeranylated, but KRAS and NRAS are processed by geranylgeranyl transferases in the absence of farnesylation.

Table 1 | **Developmental disorders associated with aberrant Ras signalling**

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

and defective fetal liver erythropoiesis after about 12–14 days of gestation[41,43]. By contrast, mice deficient in HRAS and NRAS, both alone or in combination, develop normally and are viable and fertile[44,45]. Taken together, observations in human patients and gene-targeting studies in mice support the idea that HRAS, KRAS and NRAS have both unique and redundant roles in development and tissue homeostasis.

Variable expression levels, particularly in tissue stem cell and progenitor populations, could explain the non-redundant requirements for specific Ras isoforms. However, this question has not yet been investigated systematically. Alternatively, the biological differences between Ras isoforms might be explained in part by the differential subcellular compartmentalization of these proteins. Specific Ras proteins are processed differently and therefore follow distinct trafficking pathways[46,47]. NRAS and HRAS undergo a palmitoylation–depalmitoylation cycle in which they move back and forth between the plasma membrane and Golgi through vesicle transport. A significant portion of NRAS and HRAS reside in the Golgi, and studies have shown that these proteins can signal on this organelle as well as on other endomembrane compartments, such as the endoplasmic reticulum[46,48]. Chiu *et al.*[49] developed a green fluorescent protein (GFP)-tagged RAF1 Ras-binding domain (RBD) fusion construct to report activated Ras in cells. They discovered that oncogenic HRAS and NRAS colocalized with the reporter on the endoplasmic reticulum and Golgi. In addition, an oncogenic form of HRAS that was restricted to the endoplasmic reticulum with a transmembrane tether retained transforming activity in a focus-forming assay[49]. However, oncogenic HRAS that is restricted to the Golgi is unable to promote transformation[50]. Farnesylated KRAS is targeted to the plasma membrane by an uncharacterized pathway. KRAS can return to endomembrane compartments following phosphorylation of the hypervariable region[51]. Interestingly, the phosphorylation of oncogenic KRAS induced apoptosis in transformed NIH3T3 fibroblasts in a BCL-X_L-dependent manner[51]. Ras isoforms also localize to distinct microdomains in the plasma membrane. HRAS is associated with cholesterol-rich regions on the plasma membrane known as lipid rafts, whereas KRAS is distributed throughout the plasma membrane[52]. Interestingly, GDP-bound HRAS preferentially associated with lipid rafts, but GTP loading redistributed HRAS from rafts into the bulk plasma membrane, and this release from rafts is required for efficient effector activation[52]. However, in more recent studies, computational analysis and immunogold electron microscopy suggest that oncogenic HRAS and KRAS proteins are recruited to small (10–12 nm), dynamic clusters in the inner plasma membrane, referred to as nanoclusters[53]. A recent study using a probe targeted to endogenous Ras–GTP suggests that the plasma membrane is the predominant site of growth-factor-induced activation of Ras[54]. Ras isoforms are also differentially ubiquitylated, which might modulate signalling properties[55]. Although there is great interest in deciphering the compartmentalization of Ras signalling and how subcellular localization

Palmitoylation
The post-translational addition of palmitate (16-carbon fatty acid) to cysteine residues on proteins to modulate membrane affinity.

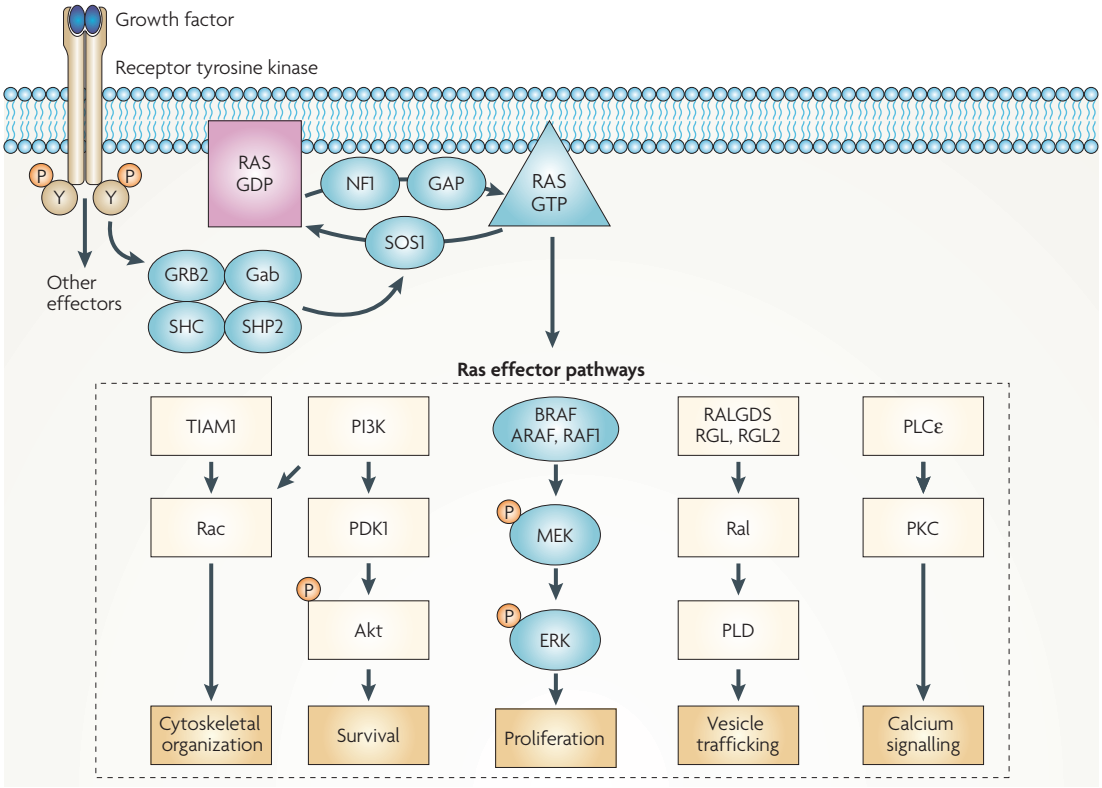

Figure 1 | **The Ras signalling pathway.** This illustration of the Ras signalling pathway highlights proteins affected by mutations in developmental disorders and cancer. Growth factor binding to cell-surface receptors results in activated receptor complexes, which contain adaptors such as SHC (SH2-containing protein), GRB2 (growth-factor-receptor bound protein 2) and Gab (GRB2-associated binding) proteins. These proteins recruit SHP2 and SOS1, the latter increasing Ras–guanosine triphosphate (Ras–GTP) levels by catalysing nucleotide exchange on Ras. The GTPase-activating protein (GAP) neurofibromin (NF1) binds to Ras–GTP and accelerates the conversion of Ras–GTP to Ras–GDP (guanosine diphosphate), which terminates signalling. Several Ras–GTP effector pathways have been described, and some of the key effectors are depicted here. The BRAF–mitogen-activated and extracellular-signal regulated kinase kinase (MEK)–extracellular signal-regulated kinase (ERK) cascade often determines proliferation and becomes deregulated in certain cancers and in developmental disorders such as cardio-facio-cutaneous syndrome. Ras also activates the phosphatidylinositol 3-kinase (PI3K)– 3-phosphoinositide-dependent protein kinase 1 (PDK1)–Akt pathway that frequently determines cellular survival. RALGDS, RALGDS-like gene (RGL), RGL2 and TIAM1 are exchange factors of Ral and Rac, respectively. Among the effectors of Ral is phospholipase D (PLD) an enzyme that regulates vesicle trafficking. Rac regulates actin dynamics and, therefore, the cytoskeleton. Ras also binds and activates the enzyme phospholipase Cε (PLCε), the hydrolytic products of which regulate calcium signalling and the protein kinase C (PKC) family. P, phosphate; Y, receptor tyrosine.

modulates signal output, an important caveat is that data obtained in transduced cells that express high levels of Ras and/or exogenous sequences that are used to report activated Ras might not accurately reflect what happens in untransduced cells.

Biochemical effects of oncogenic *Ras* mutations

Activating *Ras* mutations occur in ~30% of human cancers. Specific *Ras* genes are mutated in different malignancies: *KRAS* mutations are prevalent in pancreatic, colorectal, endometrial, biliary tract, lung and cervical cancers; *KRAS* and *NRAS* mutations are found in myeloid malignancies; and *NRAS* and *HRAS* mutations predominate in melanoma and bladder cancer, respectively[40,56] (TABLE 2). In most cases, the somatic missense *Ras* mutations found in cancer cells introduce amino-acid substitutions at positions 12, 13 and 61. These changes impair the intrinsic GTPase activity and confer resistance to GAPs, thereby causing cancer-associated mutant Ras proteins to accumulate in the active, GTP-bound conformation[57]. The three-dimensional structure of HRAS bound to the catalytic domain of p120GAP, known as the GAP-related domain (GRD), has been solved and provides a structural understanding of the biochemical activation of oncogenic Ras proteins[58] (FIG. 3). GAP proteins have a highly conserved arginine finger that interacts with the phosphate-binding loop (P loop) of Ras (FIG. 2). This interaction stimulates catalysis by stabilizing a transition state of the Ras–GTP hydrolysis reaction. Glutamine 61 is essential for GTP hydrolysis, and substituting any amino acid at this position except glutamic acid blocks hydrolysis[59]. In the Ras–GAP complex, glutamine 61 points towards the phosphate chain of bound guanine nucleotide and forms a hydrogen bond with the backbone carbonyl group of the catalytic arginine residue, which thereby contributes to

Arginine finger
A highly conserved residue in GTPase-activating proteins that directly interacts with the P loop of Ras proteins and is essential for accelerating intrinsic Ras GTPase activity.

Box 1 | **General principles of Ras regulation**

Intrinsic nucleotide binding and hydrolysis

Ras proteins bind guanine nucleotides with high affinity. In the basal state, Ras is bound to guanosine diphosphate (Ras–GDP). Occasionally, GDP will spontaneously dissociate from the Ras–GDP complex (about once per hour)[138,139]. As cellular guanine-nucleotide concentrations are high, Ras will quickly rebind nucleotide. Cellular levels of guanosine triphosphate (GTP) are generally higher than GDP (often 10-fold higher), so the re-bound nucleotide is usually GTP. GTP binding induces a conformational shift in Ras, which increases its affinity for effectors and switches on downstream signalling pathways. Ras hydrolyses the terminal phosphate of GTP at a slow rate (about once per hour)[138–140]. This intrinsic hydrolysis rate is sufficient to terminate signalling, even in the absence of other regulators. The deregulation of intrinsic nucleotide dissociation and hydrolysis is thought to be sufficient to cause disease. However, these intrinsic properties are regulated by additional proteins, which also have a role in disease.

Activation

Growth factor binding to cell-surface receptors results in activated receptor complexes, which contain adaptor proteins and other molecules. These include direct activators of Ras, such as the guanine nucleotide-exchange factors (GNEFs) SOS1, SOS2 and a family of guanine nucleotide-releasing proteins (GRPs) (RASGRP1, RASGRP2, RASGRP3 and RASGRP4). The basic mechanism by which these proteins stimulate exchange has been proposed based on structural characterization of the binding of Ras and SOS1 (REF. 141). SOS1 opens up the nucleotide-binding site by inserting an α-helix that causes displacement of switches I and II in Ras, which consequently disrupts nucleotide binding. Recently, it has been shown that SOS1 also contains an allosteric Ras–GTP binding site that stimulates binding of Ras–GDP to the catalytic site[133]. This positive-feedback loop amplifies Ras activation.

Inactivation

The slow intrinsic Ras–GTP hydrolysis rate is augmented several thousand-fold by GTPase-activating proteins (GAPs). The two key GAP proteins are p120GAP and neurofibromin. The proposed mechanism by which GAPs catalyse Ras–GTP hydrolysis is based on structural analysis of the complex between Ras and the catalytic domain of p120GAP[58]. The structure reveals a key arginine residue (arginine 789 in p120GAP, known as the arginine finger) that putatively stimulates GTP hydrolysis by stabilizing the transition state. This arginine is also conserved in neurofibromin (NF1).

the stability of the GTP–GDP transition state. Replacing glycine 12 of Ras with any other amino acid except proline also biochemically activates Ras. These substitutions are thought to be unfavourable in the GTP–GDP transition state because of a steric clash of side chains with the catalytic arginine and with the side chain of glutamine 61. Substituting proline for glycine 12 renders Ras resistant to GAPs, but has increased intrinsic GTP hydrolysis[60,61]. Importantly, this mutant does not transform cells, which suggests that levels of intrinsic GTPase activity are biologically relevant even though GAPs can accelerate GTP hydrolysis several thousand-fold. Consistent with this idea, the transforming potential of HRAS proteins with different codon 61 substitutions is inversely related to intrinsic GTPase activities[16].

Oncogenic Ras proteins deregulate downstream effector pathways to confer the abnormal functional properties of cancer cells: deregulated cell growth, survival and differentiation. Oncogenic Ras proteins with second site substitutions in amino acids that result in impaired activation of specific effectors were constructed to determine requirements for transformation[18,62–67]. Although this approach is limited by the inability to engineer clean mutant proteins that efficiently interact with only one effector, the studies performed so far generally support the conclusion that PI3K, Raf–MEK–ERK and RALGDS activation all contribute to Ras-induced transformation[18,63–67]. Glycine 12 point mutants transform rodent cell lines; however, the morphological phenotype can depend on the particular amino-acid substitution at this position. G12V and G12R mutants have robust transformation phenotypes, whereas G12S and G12D, although transforming, have less striking morphological effects[68]. Although much of the pioneering work on oncogenic Ras was based on the ability of retroviruses harbouring *Ras* oncogenes to transform rodent cells, we now appreciate that cell context and levels of expression strongly modulate the functional consequences of mutant Ras expression. The overexpression of oncogenic Ras in primary cells can induce senescence or growth arrest, unless accompanied by other genetic lesions such as loss of *CDKN2A* (also known as $p16^{INK4a}$), *CDKN2D* (also known as $p19^{INK4d}$) or *TP53* (REF. 69).

In an elegant series of experiments, strains of mice were created that express an oncogenic $Kras^{G12D}$ or $Kras^{G12V}$ allele from the endogenous *Kras* locus[70–72]. The initial model, which required a spontaneous recombination event to activate a latent mutant allele, showed conclusively that $Kras^{G12D}$ could initiate lung cancer and T-cell lymphoma *in vivo*[70]. To activate oncogenic *Kras* at defined time points in specific tissues, second generation conditional mutant alleles were created in which loxP-stop-loxP elements were introduced to silence oncogenic *Kras* in the absence of Cre recombinase expression. These studies confirmed that oncogenic *Kras* can initiate lung hyperplasias with 100% penetrance, and also showed that widespread expression of the mutant *Kras* allele is lethal during embryogenesis[71,72]. Use of tissue-specific Cre strains in the developing pancreas induced pancreatic cancer precursor lesions, and some animals developed invasive carcinomas with prolonged latency or in the presence of cooperating tumour-suppressor gene mutations[73,74], such as INK4a deficiency. In *Mx1–Cre* mice, Cre recombinase is under control of the *Mx1* promoter, which drives high-level expression in haematopoietic cells and some other tissues in response to interferon[75]. *Mx1–Cre*; $Kras^{G12D}$

Second site mutations
This refers to the creation of mutant proteins that contain two independent mutations. As applied to Ras, this typically involves mutating an amino acid in the switch I or II domains in the context of an oncogenic V12 or D12 protein.

Latent allele
Conditional mutant alleles in mice are referred to as latent because they are present in the animal and can be inducibly expressed in specific populations of cells.

Hyperplasia
This refers to an increase in the number of cells in a tissue that are generally non-transformed.

Myeloproliferative disorder
A clonal myeloid malignancy in which there are excessive numbers of cells within one or more lineages that retain some capacity to differentiate *in vivo*.

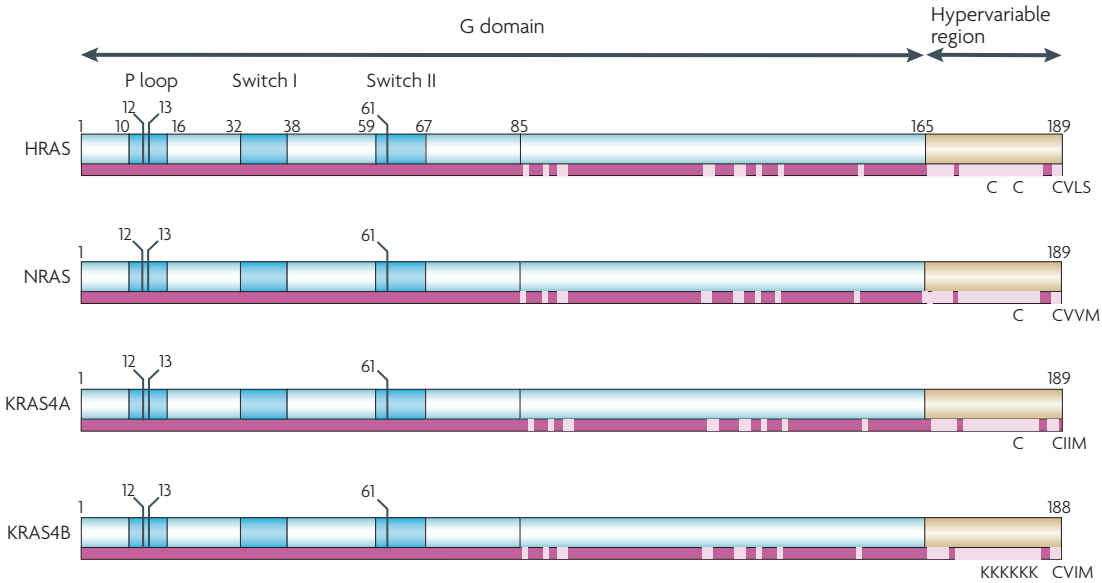

Figure 2 | **The four isoforms of Ras.** HRAS, NRAS, KRAS4A and KRAS4B are highly homologous throughout the G domain (amino acids 1–165). The first 85 amino acids are identical in all four proteins and specify binding to guanosine diphosphate (GDP) and guanosine triphosphate (GTP). This includes the P loop (phosphate-binding loop, amino acids 10–16), which binds the γ-phosphate of GTP, and switch I (amino acids 32–38) and II (amino acids 59–67) which regulate binding to Ras regulators and effectors. The next 80 amino acids (85–165) show ~85–90% sequence identity. The C-terminal hypervariable domain (amino acids 165–188/189) specifies membrane localization through post-translational modifications that include the farnesylation of each isoform on the C-terminal CAAX motif (CVLS, CVVM, CIIM and CVIM, respectively) and palmitoylation of key cysteines on HRAS, NRAS and KRAS4A; these cysteines are highlighted below each representation (C). Membrane localization of KRAS4B is facilitated by a stretch of lysines (KKKKKK) proximal to the CVIM motif. To highlight the degree of homology, a box at the bottom of each isoform representation shows the conserved residues in magenta and the variable residues in pink. Somatic *RAS* mutations found in cancer introduce amino-acid substitutions at positions 12, 13 and 61.

compound mutant mice uniformly develop a myeloproliferative disorder that closely models human juvenile and chronic myelomonocytic leukaemia (JMML and CMML; BOX 2)[76,77]. Oncogenic *Kras* expression in colonic epithelium caused hyperplasia in one model[72], but intestinal crypts remained normal in another[71]. However, oncogenic *Kras* accelerated intestinal tumorigenesis in mice that also carried a mutant allele of the adenomatous polyposis coli (*Apc*) tumour-suppressor gene[78]. In summary, the data from these mouse models are generally consistent with genetic studies of human cancer, and support the idea that oncogenic *Kras* expression can initiate lung, pancreatic and haematological malignancies *in vivo*, and functions as a secondary mutation in the multistep pathogenesis of colon cancer.

Ras in human developmental disorders

Over the past three decades, much has been learned about both the normal Ras GTPase cycle and the functional and biochemical consequences of somatic mutations that occur in cancer[79,80]. The appreciation that the degree and duration of Ras activation has important effects on developmental programmes has emerged more recently. These insights have come from studies of human patients with neurofibromatosis type 1 (NF1) and with Costello, Noonan, CFC and LEOPARD syndromes. The term 'neuro-cardio-facial-cutaneous' (NCFC) syndromes has been proposed based on the phenotypic similarities of these disorders and the inferred role of aberrant Ras signalling in their pathogenesis[6].

***NF1* mutations.** NF1 is a dominant familial cancer syndrome with an incidence of approximately 1 in 3,500 (REFS 81,82). Affected individuals show pigmented skin lesions, Lisch nodules in the iris and a strong propensity to develop benign peripheral nerve sheath tumours called neurofibromas (TABLE 1). Individuals with NF1 frequently display learning disabilities and are predisposed to malignant tumours including neurofibrosarcoma, astrocytoma, phaeochromocytoma and JMML[4,16] (BOX 2). The *NF1* gene was identified in 1990 and encodes neurofibromin, which contains a GAP domain with a conserved arginine finger motif[83–86]. Subsequent biochemical analysis demonstrated potent Ras GAP activity[86,87], and the discovery that inactivating *NF1* mutations cause NF1 implicated hyperactive Ras signalling in the pathogenesis of a human developmental disorder. Whereas the GAP domain of neurofibromin shows significant sequence homology to both mammalian p120GAP and to the yeast proteins IRA1 and IRA2, the overall structure is closer to IRA1 and IRA2. In particular, regions flanking the GAP domain are conserved between IRA1 and IRA2. Moreover, neurofibromin lacks Src-homology 2 (SH2) and SH3 domains that are found in p120GAP.

The consequences of heterozygous and biallelic *NF1* inactivation are complex. Haploinsufficiency appears

Lisch nodules
Hyperpigmented lesions in the eye that are a hallmark of NF1 disease.

Neurofibrosarcoma
A malignant tumour of connective tissue that generally arises in the extremities and is difficult to cure. This cancer is also referred to as a malignant peripheral nerve sheath tumour.

Astrocytoma
A tumour of the central nervous system that shows a range of histological and biological properties from benign (grade I) to highly malignant (grade IV).

Macrocephaly
This term refers to an abnormally large head circumference (> ninety-fifth percentile for age).

Table 2 | ***HRAS*, *KRAS*, *NRAS* and *BRAF* mutations in human cancer**

Cancer type	*HRAS*	*KRAS*	*NRAS*	*BRAF*
Biliary tract	0%	33%	1%	14%
Bladder	11%	4%	3%	0%
Breast	0%	4%	0%	2%
Cervix	9%	9%	1%	0%
Colon	0%	32%	3%	14%
Endometrial	1%	15%	0%	1%
Kidney	0%	1%	0%	0%
Liver	0%	8%	10%	3%
Lung	1%	19%	1%	2%
Melanoma	6%	2%	18%	43%
Myeloid leukaemia	0%	5%	14%	1%
Ovarian	0%	17%	4%	15%
Pancreas	0%	60%	2%	3%
Thyroid	5%	4%	7%	27%

The mutation data was obtained from the Sanger Institute Catalogue of Somatic Mutations in Cancer web site[148].

to play an important role in common NF1-associated developmental phenotypes such as macrocephaly and learning disabilities, and heterozygous inactivation of murine *Nf1* has biochemical and phenotypic consequences in mast cells and melanocytes, two cell types that frequently demonstrate abnormal growth and differentiation in affected individuals[88]. Although the pattern of autosomal dominant inheritance infers that many of the cardinal features of NF1 arise during embryonic development, postnatal hyperplasia may also play an important role in some affected tissues. *NF1* also functions as a classic tumour-suppressor gene as the analysis of tumours from individuals with NF1 and from *Nf1* mutant mice frequently shows biallelic inactivation. Finally, *Nf1* inactivation in Schwann cells induces plexiform neurofibromas in mice only in the presence of heterozygous mutant cells in the microenvironment[89,90]. These exciting data suggest that targeting infiltrating mast cells, and perhaps other cell types, is a new strategy for treating these debilitating lesions.

PTPN11 mutations. Noonan syndrome is a dominant developmental disorder characterized by short stature, facial dysmorphism, skeletal abnormalities, cardiac defects, learning disabilities and a predisposition to haematological abnormalities including JMML[5] (BOX 2). NF1 and Noonan syndrome have some common clinical features that include cutaneous abnormalities and an increased incidence of learning disabilities (TABLE 1). Indeed, a group of patients with extensive overlap have been described with neurofibromatosis–Noonan syndrome[5]. *PTPN11* was identified as the causative gene in ~50% of Noonan syndrome cases through a positional cloning and candidate gene approach[91]. *PTPN11* encodes SHP2, an SH2-domain-containing non-receptor tyrosine phosphatase (PTPase) that relays signals from activated growth-factor-receptor complexes to regulate responses such as proliferation, differentiation and migration[92–94]. SHP2 generally has a positive role in signal transduction, and is required for the full activation of the Ras–Raf–MEK–ERK pathway in most, if not all, cellular contexts[92,93,95–98]. Upon cytokine or growth-factor binding, SHP2 binds directly to some receptors, such as the platelet-derived growth-factor receptor (PDGFR), and associates with various adaptor proteins including growth-factor-receptor bound protein 2 (GRB2), fibroblast growth-factor-receptor substrate, insulin-receptor substrate and GRB2-associated binding (Gab) proteins[93,99]. Although SHP2 phosphatase activity is required for most known signalling functions, the key substrates that are required to activate the Ras–Raf–MEK–ERK pathway remain uncertain[92,93].

Several SHP2 substrates have been proposed that might mediate Ras activation. One involves a p120GAP docking site on growth-factor receptors or adaptors such as Gab; by dephosphorylating such sites, SHP2 prevents the membrane localization of p120GAP, therefore preventing the downregulation of Ras[100–102]. In addition, SHP2 has been shown to dephosphorylate the tyrosine-phosphorylated feedback inhibitor Sprouty 1. By inactivating this negative regulator, SHP2 would have a generalized positive effect on growth–factor-dependent signalling[103,104]. Finally, the inactivation of the negative regulatory CSK pathway has also been proposed to mediate SHP2-dependent Ras activation[105,106].

The *PTPN11* mutations identified in patients with Noonan syndrome are gain-of-function alleles, which encode SHP2 proteins that variably deregulate phosphatase activity, the affinity of the SH2 domains for phosphotyrosyl ligands and/or substrate specificities[107]. The important role of SHP2 in Ras signalling and the association of Noonan syndrome and JMML suggested that *PTPN11* might be activated by oncogenic mutations in human cancer. Indeed, somatic *PTPN11* mutations occur in ~35% of JMML samples (BOX 2) and in a small percentage of other haematological malignancies, but are rare in solid tumours[108–111]. Araki and colleagues[112] created a mouse model of Noonan syndrome by constitutively expressing D61G SHP2 from the endogenous *Ptpn11* locus. These mice show many features of Noonan syndrome, including craniofacial abnormalities, cardiac and haematological abnormalities. Importantly, $Ptpn11^{D61G/+}$ embryos show increased ERK activation, but only in tissues where developmental abnormalities were observed, which suggests that mutant SHP2 expression has lineage-specific effects.

LEOPARD syndrome is a rare disorder characterized by multiple lentigines (flat, pigmented spots on the skin), electrocardiographic conduction abnormalities, ocular hypertelorism, pulmonic stenosis, abnormal genitalia, growth retardation and deafness[113] (TABLE 1). Those affected have germline *PTPN11* mutations that alter specific amino acids in the PTPase domain, including tyrosine 279, threonine 468, arginine 498 and glycine 510. These mutations are correlated with specific phenotypic features, including the development of lentigines, café-au-lait patches, hypertrophic cardiomyopathy, conduction abnormalities and sensineural deafness. However,

Mast cells
Specialized haematopoietic cells derived from myeloid progenitors that are abundant in tissues and mediate local inflammatory and immunological responses.

Melanocytes
Specialized cells within the skin that produce the pigment melanin. Melanocyte precursors are the cells of origin in melanoma.

Schwann cells
Specialized neural crest cells within peripheral nerves that have a central role in myelination. Compelling genetic evidence supports the idea that NF1 inactivation in Schwann cells is essential for neurofibroma formation.

Plexiform neurofibroma
A developmental lesion in individuals with NF1 that is frequently disfiguring and may cause substantial morbidity by impinging on normal anatomic structures. Plexiform neurofibromas can acquire additional genetic lesions and progress to malignant peripheral nerve-sheath tumours.

Box 2 | **Ras, developmental disorders and cancer intersect at JMML**

Juvenile myelomonocytic leukaemia (JMML) is an aggressive myeloproliferative disorder (MPD) characterized by the over-production of myeloid lineage cells that infiltrate into several organs (reviewed in REF. 142). Children with neurofibromatosis type 1 (NF1) and Noonan syndrome are at increased risk of developing JMML. *NF1* functions as a tumour-suppressor gene in JMML, and the tumour cells of affected children frequently show the loss of the normal *NF1* allele. JMML samples from children with NF1 show decreased neurofibromin GTPase-activating protein (GAP) activity, increased levels of Ras–guanosine triphosphate (Ras–GTP), and extracellular signal-regulated kinase (ERK) activation. Somatic *NRAS* and *KRAS* mutations are found in ~25% of JMML bone marrows, but are restricted to children who do not have NF1.

The discovery of germline missense *PTPN11* mutations as the cause of ~50% of Noonan syndrome cases led to the surprising finding that *PTPN11* is also the most common target of somatic mutations in JMML (reviewed in REFS 143,144). Overall, ~85% of JMML specimens show largely mutually exclusive mutations with either *KRAS*, *NRAS*, *NF1* or *PTPN11*, which is consistent with the idea that they encode components of the same growth-control pathway. The spectrum and distribution of somatic *PTPN11* mutations identified in JMML differ from the germline mutations that cause Noonan syndrome. Leukaemia-associated *PTPN11* mutations encode stronger gain-of-function mutant proteins. The most common leukaemia-associated substitution (E76K) induces a hypersensitive pattern of myeloid progenitor colony growth that is dependent on SHP2 catalytic activity[145,146]. Murine bone-marrow cells that expressed mutant SHP2 caused anaemia and a JMML-like disorder in lethally irradiated recipient mice[145,146].

The distinct spectrum of germline and somatic mutations found in JMML strongly supports the hypothesis that hyperactive Ras has a central role in leukaemogenesis. This idea is consistent with studies of *Nf1*, *Kras* and *Ptpn11* mutant mice, all of which develop myeloid disorders that resemble JMML[76,77,112,147]. Understanding the association of JMML with NF1 and Noonan syndrome has helped to uncover genes and proteins that are crucial for normal growth control and, when deregulated, contribute to cancer.

surprisingly these mutant alleles encode proteins with defective phosphatase activities that show dominant-negative effects on EGF-stimulated ERK activation[8,14,114]. An interesting paradox is how mutations in *PTPN11* that either impair or activate the SHP2 phosphatase cause distinct developmental disorders with similar clinical characteristics. Molecular modelling suggests that LEOPARD syndrome mutations result in open forms of SHP2 (REF. 8), and it is possible that these proteins have gain-of-function effects in the absence of phosphatase activity, perhaps through an adaptor function. It is also possible that these mutant proteins affect different cell types during development. In summary, whereas *in vitro* studies suggest that SHP2 proteins encoded by LEOPARD syndrome-associated *PTPN11* mutations result in loss of function, the complex architecture of Ras signalling networks leaves the possibility open that the mutant proteins aberrantly activate Ras.

Germline HRAS and KRAS mutations. Noonan syndrome overlaps phenotypically with other disorders, including Costello and CFC syndromes, which are also characterized by facial dysmorphism, heart abnormalities, short stature and a high incidence of mental retardation (TABLE 1). Patients with Costello syndrome are predisposed to specific cancers, including rhabdomyosarcoma, ganglioneuroblastoma and bladder cancer (TABLE 1). These individuals do not have mutations in *PTPN11*, and Aoki and colleagues[7] proposed that Costello syndrome may be caused by mutations in genes that encode proteins involved in SHP2 signalling pathways. They identified *de novo* germline mutations in *HRAS* in 12 of 13 individuals with Costello syndrome, which was corroborated by other groups[115–118]. Remarkably, almost all *HRAS* alleles identified in Costello syndrome introduce amino-acid substitutions at codons 12 and 13 that also occur as somatic mutations in tumours. However, the most common substitution identified in Costello syndrome (G12S), is uncommon in cancer. Importantly, biological data indicate that the transforming properties of G12S HRAS are attenuated relative to G12V HRAS, which is the most common cancer-associated substitution[68]. Germline mutations that encode K117R and A146T HRAS substitutions have also been reported in Costello syndrome[117,118]. Lysine 117 resides in a conserved guanine nucleotide-binding sequence, NKXD (residues 116–119), and substitutions at these positions have increased rates of guanine nucleotide dissociation[59]. As the GTP:GDP ratio is ~9:1 in cells[119], an increased rate of dissociation favours the active, GTP-bound state of Ras. Indeed, K117R HRAS shows an increased rate of guanine nucleotide dissociation and transforms rodent fibroblasts[59]. Structural analysis of HRAS bound to guanine nucleotides suggests that alanine 146 is involved in binding to the guanine base, and substitution to threonine might similarly increase guanine nucleotide dissociation[118]. Consistent with the oncogenic potential of the *HRAS* mutations described above, cultured fibroblasts from individuals with Costello syndrome show increased proliferation[7].

Ras genes were also considered good candidate disease genes in the ~50% of patients with Noonan syndrome without *PTPN11* mutations, and studies from several laboratories have identified germline mutations in *KRAS* in 2–4% of affected individuals[11,120,121]. Importantly, these mutations introduce new amino acid substitutions that are not found in cancer. Biochemical and functional analyses of Noonan syndrome-associated KRAS proteins show that they are gain-of-function mutants, but are less activated than oncogenic KRAS proteins. Specifically, the intrinsic GTPase activities of the Noonan syndrome-associated V14I and T58I KRAS recombinant proteins are lower than wild-type KRAS, but less impaired than oncogenic G12D KRAS[11]. Furthermore, both mutant proteins also show intermediate levels of GTP hydrolysis in response to the GAP-related domains of p120GAP and neurofibromin, compared with wild-type and G12D KRAS. A particularly interesting feature of the T58I mutant protein is its differential responsiveness to p120GAP and neurofibromin. Studies of V14I and T58I KRAS in haematopoietic progenitors, macrophages and COS-7 monkey kidney cells showed that these alleles are activated, and support the idea that cell context modulates their functional and biochemical effects[11].

Amino acid substitutions in the α-5 helix of the KRAS4B isoform were identified in some patients with Noonan syndrome, including V152G, D153V and F156I[11,120,121]. These residues are located far from the GTP-binding region of the protein. Based on structural modelling, Carta and colleagues[120] proposed that the V152G and D153V substitutions destabilize regions of

Facial dysmorphism and craniofacial abnormalities
These terms refer to phenotypic abnormalities of the skull and face that result from an abnormal pattern of bone and cartilage development.

the KRAS protein that contribute to guanine nucleotide binding, and they predicted that these substitutions increase nucleotide dissociation in a manner similar to K117R HRAS. Although in-depth biochemical analysis of the V152G and D153V KRAS mutants has not yet been reported, an F156L substitution in HRAS results in a rapid rate of nucleotide dissociation, modestly increases Ras–GTP levels, and transforms rodent fibroblasts[122]. Germline *KRAS* mutations discovered in individuals with Noonan syndrome and CFC syndrome (see below) comprise a unique allele series for assessing the relative importance of the intrinsic KRAS GTPase, GAPs and GNEFs in regulating developmental fates.

BRAF–MEK–ERK pathway mutations. CFC syndrome is a rare sporadic disorder characterized by distinctive craniofacial features, heart defects and mental retardation[123] (TABLE 1). As these systems are also affected in Noonan syndrome, it has been uncertain whether Noonan syndrome and CFC are related entities or distinct disorders[5]. Indeed, individuals who show clinical features of both Noonan syndrome and CFC syndrome are sometimes designated Noonan syndrome/CFC or 'severe Noonan syndrome'. Based on these clinical observations, it is perhaps not surprising that germline *KRAS* mutations were identified in some individuals with CFC and Noonan syndrome/CFC[10,11,120,121]. Whether individuals with germline *KRAS* mutations should be classified clinically as having CFC syndrome, severe Noonan syndrome or as a distinct pathological entity is unclear.

As researchers expanded their candidate gene searches to include other molecular components of Ras effector pathways, two groups independently identified heterozygous missense *BRAF* mutations as the major cause of CFC syndrome[9,10]. Germline *MEK1* and *MEK2* mutations were also discovered in some individuals without *BRAF* mutations[9]. The finding that germline *BRAF* mutations underlie most cases of CFC syndrome is of exceptional interest to cancer biologists because, as discussed below, a V600E substitution in BRAF is one of the most common molecular lesions found in human cancer[124–126]. The *BRAF* mutations that occur in CFC syndrome introduce amino acid substitutions that are more widely distributed across the BRAF protein than substitutions found in cancer, and only a few individuals with CFC syndrome had a substitution that has been reported in tumours (FIG. 4). Most CFC-associated BRAF proteins have increased kinase activity, including some (Q257R, S467A, L485F and K499E) that are strongly activated[9]. However, some mutants show impaired BRAF kinase activity and did not activate ERK in human 293T kidney epithelial cells. These initial biochemical data argue against a simplistic hypothesis in which the CFC-associated mutations lead to intermediate strength and/or duration of signalling compared with the robust signalling instigated by oncogenic *BRAF* mutations. However, one potential confounding issue in interpreting these studies is that the levels of oncogenic V600E BRAF kinase activity relative to wild-type BRAF that were measured in this report were substantially lower than levels that others have observed[127]. This raises the formal possibility that the germline *BRAF* mutant alleles found in CFC syndrome encode mutant BRAF proteins with significantly lower kinase activity than V600E BRAF .

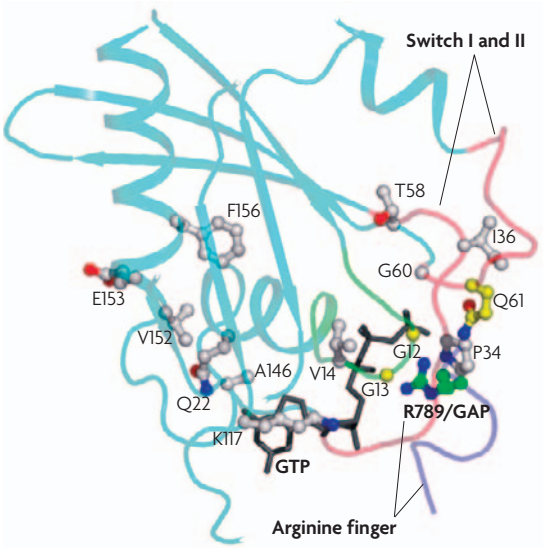

Figure 3 | **The structure of Ras.** Three-dimensional structure of Ras–guanosine triphosphate (Ras–GTP), highlighting key residues involved in disease. Amino acids that are altered by somatic mutations in human tumours are represented using yellow carbons (glycine 12, glycine 13 and glutamine 61). The residues affected by germline mutations found in individuals with Noonan, Costello and cardio-facio-cutaneous (CFC) syndromes are represented using white carbons (valine 14, glutamine 22, proline 34, isoleucine 36, threonine 58, glycine 60, lysine 117, alanine 146, valine 152, glutamate 153 and phenylalanine 156). The loops that are important in binding to effectors, switch I and switch II, are shown as pink ropes. Also shown is the arginine finger of GTPase-activating proteins (GAPs), which stimulates the GTP hydrolysis reaction (shown as a blue rope). Arginine 789 from the proximal loop of the p120GAP arginine finger is represented using blue carbons. The guanine nucleotide is shown as a stick representation coloured in black. Figure courtesy of K. Zhang.

Somatic missense *BRAF* mutations occur in most malignant melanomas (TABLE 2). *BRAF* mutations are also found frequently in thyroid (30–50%), colorectal (5–20%) and ovarian cancers (30%)[124,126]. Most of the cancer-associated mutations are in the kinase domain, and the V600E substitution accounts for ~90% of mutations[125,126]. The crystal structure of the BRAF kinase domain has been solved and has provided an improved understanding of BRAF regulation[127]. Most cancer-associated *BRAF* mutations encode gain-of-function mutants that constitutively activate the kinase and the MEK–ERK pathway. The mutations seem to disrupt the interaction of the glycine loop and activation segment, which destabilizes the inactive conformation of the protein. The cancer-associated mutants display a wide range of kinase activities, including oncogenic proteins that show impaired kinase activity compared with wild-type BRAF. Interestingly, these proteins still activate ERK through a mechanism involving RAF1 (REF. 127), raising an additional mechanistic avenue

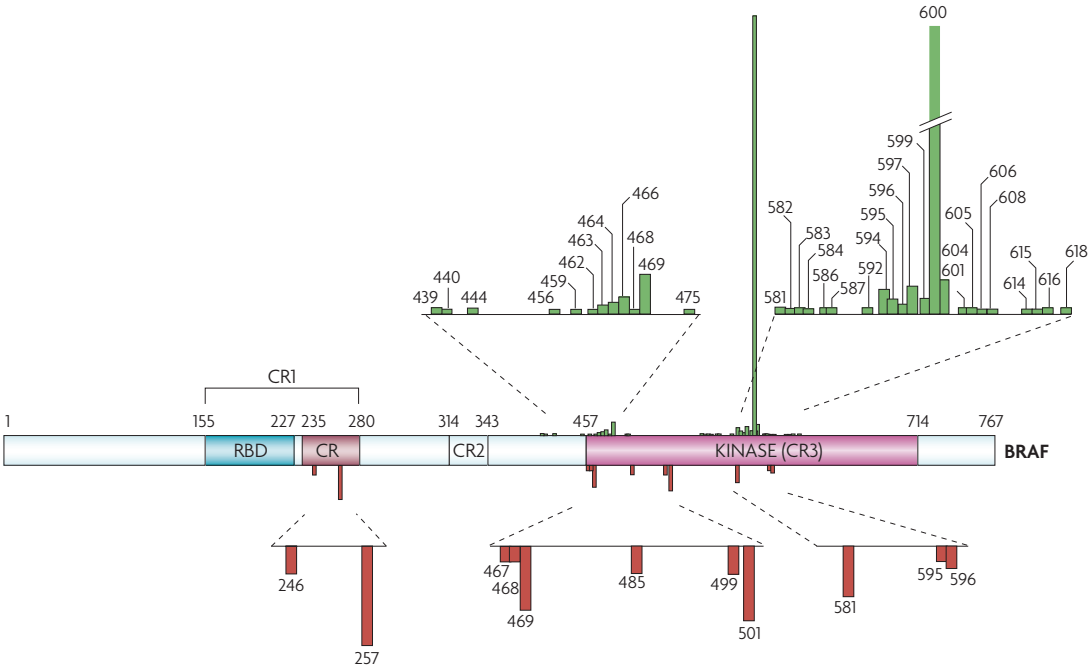

Figure 4 | **Disease mutations in BRAF.** The distribution of amino acid substitutions in BRAF that are encoded by germline and somatic disease-associated mutations. Green bars show the locations of somatic mutations found in human tumours. The length of the bars represents the relative proportion of mutations at that residue; substitution of valine 600 occurs in over 90% of the tumours. Red bars show the locations of germline mutations found in individuals with cardio-facio-cutaneous syndrome. The three conserved Raf domains are indicated: CR1 is comprised of the Ras-binding domain (RBD) and an adjacent cysteine-rich domain (CR); CR2 is a serine- and threonine-rich regulatory domain; and CR3 comprises the catalytic kinase domain. Note that *BRAF* mutations are much more frequent, so red bars cannot be compared to green bars.

that might also be exploited by CFC-associated mutations. The V600E mutation is also detected in a large percentage of nevi, which are benign skin lesions of melanocytes that are thought to be senescent[128,129]. In addition, mice that express endogenous levels of $Braf^{V600E}$ develop benign lung tumours that rarely progress to adenocarcinoma and show features of senescence[130]. These data support the idea that *BRAF* mutations are an early event in tumorigenesis, but are insufficient to cause cancer. Cooperating mutations that enable malignancy are likely to further increase dependence on the BRAF pathway, as chemical inhibitors of BRAF signalling selectively block the proliferation of tumour cell lines with *BRAF* mutations[131,132]. Perhaps the absence of cooperating mutations is a primary discriminator between CFC-associated and oncogenic BRAF pathway activation.

In contrast to *BRAF*, *MEK1* and *MEK2* mutations have not been reported in cancer or in any other human disease. Expressing CFC syndrome-associated MEK mutant proteins in cells resulted in levels of phosphorylated ERK that were increased compared with cells that expressed wild-type MEK, but lower than cells that expressed constitutively active MEK mutants[9]. An important implication of the *BRAF* and *MEK* mutations that cause CFC syndrome is that the Raf–MEK–ERK kinase cascade is a crucial downstream effector pathway of Ras–GTP in regulating developmental programmes. The clinical features of CFC syndrome are generally more severe than other disorders of the Noonan syndrome spectrum. It is intriguing that mutations in the BRAF–MEK–ERK pathway cause more severe developmental defects, whereas the Noonan syndrome -associated mutations discovered so far, which alter upstream signalling molecules, cause a less profound clinical phenotype even though they could potentially deregulate many effector pathways. This implies that the BRAF pathway also has a dominant role downstream of Ras–GTP in Noonan syndrome and other developmental disorders, but perhaps some of the additional pathways regulated by Ras–GTP (FIG. 1) actually serve to mitigate BRAF–MEK–ERK activation.

SOS1 mutations. Germline mutations in *PTPN11* and *KRAS* cause slightly over half of Noonan syndrome cases. Based on these data, two groups screened other upstream components of the Ras–Raf–MEK–ERK pathway and uncovered germline missense mutations in *SOS1* in ~10% of Noonan syndrome cases[12,13]. SOS1 is a complex, multidomain protein that is a major Ras guanine nucleotide-exchange factor. A tandem Dbl homology and pleckstrin homology (DH and PH) domain serves to mask the catalytic CDC25 homology domain (also called the Ras–GEF domain). Many of the mutations identified in Noonan syndrome alter amino acids in the DH and PH domain that are thought to contribute to this autoinhibition. Therefore, these mutant SOS1 proteins are thought to enable the increased access of Ras to the catalytic site, thereby increasing the rate of guanine nucleotide exchange[133,134]. Interestingly, the regulation of SOS1 is highly sensitized to Ras–GTP levels — a second, allosteric, binding site on SOS1 allows

Ras–GTP to further activate SOS1 activity through a positive-feedback mechanism. Therefore, Noonan syndrome-associated *SOS1* mutations encode proteins that are primed to activate the Ras pathway. Consistent with this model, expressing mutant SOS1 proteins in cultured cell lines resulted in the sustained activation of Ras and ERK in response to epidermal growth factor. One group reported genotype–phenotype correlations, including an increased incidence of abnormal ectodermal features (facial keratosis pilaris (excessive growth of horny tissue around the hair follicles) and curly hair), in affected individuals with a *SOS1* mutation compared with the general Noonan syndrome population[13]. Further investigation is required to firmly establish consistent genotype–phenotype correlations in Noonan syndrome, which might also relate to the biochemical properties of specific SOS1 mutant proteins.

Conclusions and future directions

The germline mutations that cause developmental disorders comprise a novel allele series for furthering our current understanding of how the Ras–Raf–MEK–ERK pathway regulates developmental programmes and becomes deregulated in human disease. Remarkably, malignant tumours are relatively uncommon in these disorders, which is in marked contrast to the highly penetrant cancer phenotypes seen in individuals with germline mutations of tumour-suppressor genes such as *TP53*, *RB1* (retinoblastoma 1), *BRCA1* and *BRCA2*. One potential explanation for this observation is that the degree and/or duration of Ras activation are insufficient to initiate tumorigenesis in most tissues. Consistent with this idea, the mutant SHP2 and KRAS proteins encoded by germline mutations have attenuated biochemical and cellular phenotypes compared with the corresponding oncoproteins. The example of JMML is particularly intriguing in this respect. Whereas cases that are associated with somatic *PTPN11* or *KRAS* mutations are clinically aggressive, JMML-like myeloproliferative disorders that occur in infants with Noonan syndrome frequently regress without treatment. These data suggest that the strength and duration of hyperactive Ras signalling influences the probability of cancer formation. With the exception of *NF1*, these developmental disorders are caused by dominant gain-of-function alleles that must be tolerated in the germline. The precedent that widespread $Kras^{G12D}$ expression leads to embryonic lethality in mice[72] suggests that some, if not most, of the dominant gain-of-function mutations found in cancer are incompatible with normal development.

Although the general idea that germline mutant alleles are mild hypermorphs compared with somatic cancer-associated mutations is appealing, the *HRAS* mutations that cause Costello syndrome encode strong gain-of-function proteins and it is therefore not surprising that affected individuals are predisposed to specific benign and malignant tumours. The normal tissues that are perturbed in Costello syndrome (the nervous system and the musculoskeletal system) overlap with the types of malignancies that are observed (rhabdomyosarcoma, neuroblastoma, ganglioneuroblastoma and bladder cancer). Perhaps endogenous levels of activated *HRAS* alleles are only transforming in the context of certain tissues or the gene is expressed at low levels in most types of cancer-initiating cells. *HRAS* mutations account for <1% of all cancer-associated *RAS* mutations[40]. The observation that many individuals with Costello syndrome do not develop cancer provides additional evidence that mutant *HRAS* requires cooperating mutations for tumorigenesis. Interestingly, loss or silencing of the normal *HRAS* allele has been reported in some of the malignancies that arise in the context of Costello syndrome[7,115]. It is also notable that the kinase activities of some of the mutant BRAF proteins encoded by CFC syndrome-associated mutations are comparable to BRAF oncoproteins, yet the germline CFC mutations do not predispose to tumour formation. These observations suggest that the relatively low risk of cancer in many of these developmental syndromes is not entirely due to attenuated biochemical activities of the relevant mutant proteins.

The cancers associated with NF1 and disorders of the Noonan syndrome spectrum arise in specific tissues, suggesting that some cell types are sensitive to changes in Ras activation, whereas others are not. Myeloid, myogenic and neural tumours predominate, and it is remarkable that epithelial cancers that show a high incidence of *KRAS* mutations (that is, carcinomas of the lung, pancreas and colon) have not been reported. As $Kras^{G12D}$ efficiently initiates lung and pancreatic cancer in mice, the absence of these tumours in individuals with Noonan syndrome and CFC syndrome might be due to the fact that strong gain-of-function *KRAS* alleles are not tolerated in the germline (see above). This idea is consistent with the observation that *KRAS*, but not *HRAS* or *NRAS*, is essential for murine development[41,42,44,45]. As the aberrant reactivation of developmental programmes is thought to be integral to malignant transformation in many tissues, it is perhaps not surprising that *KRAS* is altered by somatic mutation in cancer cells far more often than either *HRAS* or *NRAS*. By contrast, germline oncogenic *HRAS* mutations and *Hras* inactivation are compatible with normal development in humans and mice, respectively. The discrete genotype–phenotype associations with respect to the germline *Ras* mutations found in human developmental disorders — strong *HRAS* gain-of-function alleles in Costello syndrome, less potent *KRAS* mutations in Noonan and CFC syndromes, and no alterations in *NRAS* — underscore the importance of understanding the unique functional role that each Ras isoform has in development, normal cellular growth and cancer.

Data from diverse sources such as unbiased genetic screens in yeast, animal models, experiments in which cultured cells or genetically engineered mice were exposed to inhibitors of signalling molecules, and human clinical trials have uncovered unexpected complexity and adaptation in signalling networks[135]. Indeed, the contributions of individual proteins to the output of a particular pathway seem to vary in a continuous manner rather than suggesting the existence of one or a few dominant elements[136]. From this vantage point, it is surprising that mutations of single genes are sufficient to

confer overt clinical phenotypes as seen in individuals with germline mutations that perturb Ras signalling. In considering how and why specific mutations cause disease, it is probable that tissue-specific phenotypes reflect both the primary biochemical properties of a mutant protein and the capacity of the cell to neutralize any deleterious effects by remodelling the pathway. From this perspective, the tissues that are perturbed in NF1 and in Costello, Noonan, CFC and LEOPARD syndromes may not only be highly dependent on Ras–Raf–MEK–ERK signalling to specify cell fates, but may also be relatively inflexible with respect to their ability to modulate this pathway. This idea might explain why either decreasing or increasing SHP2 phosphatase activity has deleterious developmental consequences.

Because the germline mutations that cause NF1, Costello, Noonan, CFC and LEOPARD syndromes are present throughout development, there is a substantial window of time for cells to adapt to them. Moreover, all of the cells in the organism express the same mutant protein, which might activate regulatory feedback loops that involve different cell types. By contrast, cancer-associated *KRAS* and *BRAF* mutations arise somatically in a cell (or in a small field of cells) that is surrounded by normal cells. Given the increasing evidence that cell–cell interactions within tissue microenvironments negatively and positively affect tumour growth, these differences are likely to be important. These recent observations also raise intriguing questions about the relationship between the developmental timing of specific mutations and the resultant phenotype(s) in individual cells. The proliferative potential and plasticity of fetal cells can differ substantially from their counterparts in adult tissues. Perhaps the clearest example of how developmental mechanisms that are cell intrinsic influence the potential for malignant growth can be found in the haematopoietic compartment. Although haematopoiesis is a dynamic and life-long process in which immature stem cell and progenitor populations give rise to differentiated progeny, it is striking that children, but not adults, with NF1 are predisposed to myeloid malignancies. The most straightforward explanation of this clinical observation is that both the 'soil' (a susceptible haematopoietic stem cell) and the 'seed' (loss of the normal *NF1* allele) are essential for leukaemogenesis. This idea is consistent with a provocative study showing that leukeamia-associated *GATA1* mutations, which cause a transient myeloproliferative disease in infants with Down syndrome, are far more potent in fetal than adult haematopoietic cells[137]. These data infer that the phenotypic consequences of specific mutations that perturb Ras signalling are likely to be strongly influenced by the developmental state of the cell in which they occur.

Based on the precedent of *KRAS*, *HRAS*, *BRAF* and *PTPN11*, other components of Ras signalling networks that are mutated in developmental disorders are novel candidate oncogenes. It will be of interest to determine if somatic *SOS1* mutations occur in cancer and, if so, to compare the spectrum of germline and cancer-associated somatic mutations.

Investigating the properties of the proteins encoded by novel series of mutations in *PTPN11*, *HRAS*, *KRAS*, *SOS1*, *BRAF*, *MEK1* and *MEK2* could provide opportunities to better understand the architecture and biochemistry of Ras signalling networks, how cells adapt to hyperactive Ras, and mechanisms of growth control and tumorigenesis in different cell types. Proteins with unexpected behaviour may prove particularly instructive. For example, addressing how excessive SHP2 PTPase activity causes Noonan syndrome whereas the loss of catalytic activity induces LEOPARD syndrome has implications for understanding the general role of SHP2 in regulating cell fates. Along these lines, detailed investigation of the biochemical properties of mutant BRAF proteins found in developmental disorders will probably provide insights into how these molecules are deregulated in cancer. Generating strains of mutant mice that express alleles of interest under the control of endogenous promoters will also be integral for understanding their developmental and tumorigenic consequences *in vivo*. Together, the discoveries of the past few years open new avenues of research that will further our understanding of how Ras signalling networks regulate developmental programmes and the pathological consequences of hyperactive Ras in different cellular contexts. Addressing these questions has important implications for the fields of cancer biology and molecularly targeted therapeutics.

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Competing interests statement
The authors declare competing financial interests: see web version for details.

DATABASES
The following terms in this article are linked online to:
Entrez Gene:
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
Akt | *Apc* | ARAF | BAD | BCL-X_L | BRAF | *BRCA1* | *BRCA2* | *CDK2NA* | CDK2ND | EGF | ELK1 | FKHR | *FOS* | *GATA1* | GRB2 | *HRAS* | JUN | *KRAS* | MEK | *Mx1* | *NF1* | NGF | NRAS | PDGFR | PDK1 | PI3K | Phospholipase Cε | *PTPN11* | RAF1 | RALA | RALGDS | *RB1* | RGL | RGL2 | SOS1 | Sprouty 1 | TIAM1 | TP53
OMIM: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM
cardio-facio-cutaneous syndrome | CMML | Costello syndrome | Down syndrome | JMML | NF1 | Noonan syndrome | LEOPARD syndrome

FURTHER INFORMATION
Kevin Shannon's laboratory homepage: http://www.ucsf.edu/kmslab/
Plexxikon homepage: http://www.plexxikon.com
Sanger Institute Catalogue of Somatic Mutations In Cancer: http://www.sanger.ac.uk/cosmic
Access to this links box is available online.