HHS Public Access

Author manuscript

Dev Biol. Author manuscript; available in PMC 2019 December 01.

Published in final edited form as:

Dev Biol. 2018 December 01; 444(Suppl 1): S79–S97. doi:10.1016/j.ydbio.2018.02.003.

MAPK and PI3K Signaling: at the Crossroads of Neural Crest Development

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Abstract

Receptor tyrosine kinase-mediated growth factor signaling is essential for proper formation and development of the neural crest. The many ligands and receptors implicated in these processes signal through relatively few downstream pathways, frequently converging on the MAPK and PI3K pathways. Despite decades of study, there is still considerable uncertainty about where and when these signaling pathways are required and how they elicit particular responses. This review summarizes our current understanding of growth factor-induced MAPK and PI3K signaling in the neural crest.

Keywords

Receptor Tyrosine Kinases; ERK; AKT; cell signaling

1. Introduction

Ever since Wilhelm His initially described the ‘intermediate cord’, biologists have been fascinated by this population of cells, later dubbed the neural crest (NC). Considering the NC’s eventual dissemination throughout the body and the variety of cell types it can form, it is not hard to understand why. This group of cells, sometimes dubbed the fourth germ layer, is specified shortly after gastrulation at the border between neural and non-neural ectoderm. These cells then undergo an epithelial-to-mesenchymal transition and subsequent migration throughout the developing embryo where their descendants proliferate and subsequently differentiate into a panoply of cell types and tissues. These cell types include the bones, connective tissue, and pericytes in the face, melanocytes throughout the skin, smooth muscle in the outflow tract of the heart, and numerous neural derivatives such as the facial nerves, dorsal root ganglia, peripheral sensory, sympathetic, and parasympathetic neurons and their associated Schwann cells, the adrenal medulla, and the enteric nervous system, which innervates the digestive tract (Mayor and Theveneau, 2013; Simões-Costa and Bronner, 2015). For a more thorough review of the NC and multipotency, see reviews from LeDouarin

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and Dupin (LeDouarin, 2018; Dupin, 2018) in this issue. A natural question relates to the signals that direct neural crest specification, survival, growth, migration, and ultimate differentiation. One family of signaling molecules particularly relevant to all of these steps are growth factor ligands and the receptor tyrosine kinases (RTKs) they activate (Fantauzzo and Soriano, 2015). The link between NC and RTKs is in fact quite old. Mouse fanciers across the globe have prized mice with unique coat patterns for centuries, and classic alleles such as Dominant Spotting (*W*-locus) and Steel (*Sl*-locus) are caused by mutations in the genes *Kit* and *Kitl*, respectively, that encode the RTK KIT and its ligand Stem Cell Factor (SCF) (Chabot et al., 1988; Copeland et al., 1990; Flanagan and Leder, 1990; Geissler et al., 1988; Silver, 1995; Silvers, 1979; Tokuda, 1935; Zsebo et al., 1990). The importance of RTKs in NC during development was dramatically illustrated by Hans Grüneberg's studies of the Patch mouse in the 1960s (Grueneberg and Truslove, 1960), which turned out to have defects in two RTK genes, *Pdgfra* (Smith et al., 1991; Stephenson et al., 1991) and *Kit* (Wehrle-Haller et al., 1996).

Neural crest RTK signaling is relevant to human disease. Mutations in RTK genes, their effectors, and the signaling pathways they activate cause both congenital malformations and neoplasms attributed to defects in NC, collectively known as neurocristopathies (Bolande, 1974). These include congenital malformations such as craniofrontonasal dysplasia (*EFNB1*), craniosynostosis (*FGFR1*, *FGFR2*, *FGFR3*), Hirschsprung disease (*RET*, *GDNF*), piebaldism (*KIT)*, and a group of syndromes known as the neuro-cardio-facio-cutaneous syndromes that involve NC and non-NC cells (*MAPK1*, *MAPK3*, *PTPN11*, *KRAS*, *HRAS*, *SOS1*, *BRAF, RAF1, MEK1*, *MEK2*) as well as neoplasms such as melanoma (*KIT*, *BRAF*) (Bolande, 1997; Etchevers et al., 2006; Samuels et al., 2009; Watt and Trainor, 2013).

RTKs are a diverse group of membrane-spanning receptor proteins that engage multiple intracellular signaling cascades. Typically, receptors dimerize in response to ligand-binding. Dimerization stimulates the receptors' intrinsic tyrosine kinase activity that in turn activates a number of downstream signaling cascades. This is achieved through autophosphorylation of the cytoplasmic domain of the RTK itself, thus creating docking sites for signaling proteins, and through direct phosphorylation of signaling effectors. RTK signaling has been shown to regulate a broad suite of cellular responses including proliferation, differentiation, migration, and apoptosis. In the human, there are at least 58 RTKs, organized into twenty subfamilies, which are activated by an even greater number of ligands (Lemmon and Schlessinger, 2010). So far, twelve of these families have been clearly implicated in NC development (Fantauzzo and Soriano, 2015). Despite the hundreds of unique receptor-ligand complexes RTKs have been shown to form, they converge on a comparatively limited number of intracellular signaling pathways. These include the mitogen-activated protein kinases (MAPK) such as the extracellular-signal regulated kinases (ERK) 1/2, p38, the c-JUN N-terminal kinases (JNK), and ERK5, as well as by phosphatidylinositol 3-kinase (PI3K), phospholipase-c gamma (PLCγ), protein kinase C (PKC), Src-family tyrosine kinases, and activation of several Signal Transducer and Activator of Transcription (STAT) proteins.

There are multiple explanations for the superficial disconnect between the number of receptor-ligand pairs and the limited number of signaling pathways they activate. First,

RTKs and their ligands are not ubiquitously expressed, so the large number of genes allows for temporal, spatial, and cell-type specificity of signaling activity. Second, not every receptor activates every potential downstream pathway. Thus, specific receptor dimers or receptor-ligand complexes may activate unique combinations of downstream signaling pathways that, when activated together, elicit a unique cellular response (Lemmon and Schlessinger, 2010; Vasudevan et al., 2015). Finally, even receptors that activate the same subset of signaling pathways do not necessarily activate these pathways to the same degree or with the same temporal dynamics, which in itself can lead to specific gene expression and fate decisions (Amit et al., 2007; J.-Y. Chen et al., 2012; Marshall, 1995; Purvis and Lahav, 2013; Toettcher et al., 2013; Wilson et al., 2017). The importance of proper signaling strength and duration for development is attested by the striking phenotypes in mouse mutants for positive and negative feedback proteins, the focus of a recent review (Neben et al., 2017). The mechanisms by which RTKs achieve specificity have been described (Lemmon and Schlessinger, 2010; Tan and S. K. Kim, 1999; Vasudevan and Soriano, 2016; Volinsky and Kholodenko, 2013). Nevertheless, there is considerable overlap in expression of RTKs and members of the signaling pathways they engage in the neural crest, making their study a challenging objective (Fantauzzo and Soriano, 2015). Complicating matters, these signaling cascades are not exclusively activated by RTKs, but can also be activated, directly or indirectly, downstream of other receptors such as transforming growth factor beta (TGF-β) receptors, bone morphogenetic protein (BMP) receptors, and G-Protein Coupled Receptors (GPCRs) or in response to environmental stress (Cargnello and Roux, 2011; Fruman et al., 2017; Rahman et al., 2015).

One approach to demystify the question of how signaling regulates development is to study the problem at the level of the receptor and its ligands. Expression analysis coupled to gain-of-function (GoF) and loss-of-function (LoF) studies of ligands and receptors is an essential component in advancing our knowledge of RTK signaling and development. The observed effect (or lack thereof) of manipulating a particular receptor or ligand subsequently raises two issues. First, which downstream pathway(s) is responsible for the observed effect? Because RTKs engage multiple downstream signaling cascades, attributing mutant phenotypes to a particular pathway(s) is rarely easy. This can be tested *in vitro* using chemical agonists and antagonists, although their specificity may be an issue. It can be even more challenging to relate an effect to a specific upstream RTK *in vivo* without further genetic manipulation, for instance by making mutations in the RTKs that prevent docking of particular signaling effectors (Tallquist et al, 2003; Klinghoffer et al. 2002; Brewer et al. 2015; (Jain et al., 2006; Jijiwa et al., 2004; Kimura et al., 2004; Maina et al., 2001; Wong et al., 2005). Second, to what extent is the system buffered by redundancy through other RTKs or non-RTK mechanisms that stimulate the same downstream pathways? Cells *in vivo* often simultaneously express multiple RTKs of the same and different families (for instance we observe cranial neural crest cells (cNCCs) expressing PDGFRα, PDGFRβ, FGFR1, and FGFR2) and loss of multiple receptors can enhance a mutant phenotype (Fantauzzo and Soriano, 2016; Klinghoffer et al., 2002; Park et al., 2006). It is therefore likely that cells integrate multiple receptor signaling inputs at the level of the downstream signaling cascade (Kholodenko et al., 2010). Thus, studying development at the level of the intracellular signaling pathway is an equally important task.

This review focuses on MAPK and PI3K signaling and their role in neural crest development, using mainly genetic data from the mouse, but also from the chick, frog, and zebrafish. Both MAPK and PI3K are commonly activated by RTKs and there is extensive published research linking them to NC. In some cases these pathways have not been studied by conditional genetic approaches, so we discuss global knockouts and whether there is a likely NC defect. We also review how MAPK and PI3K signaling regulate the transcription factor Serum Response Factor (SRF), which is critical to induce rapid expression of immediate early genes (IEGs), one of the classic functions of growth factor signaling. For a discussion of RTK signaling generally or the role of the specific RTK families themselves in neural crest development, we direct the reader to these reviews (Fantauzzo and Soriano, 2015; Lemmon and Schlessinger, 2010).

2. MAPK Pathways

There are four canonical MAPK pathways in mammals: the ERK1/2, JNK, p38, and ERK5 pathways. All four pathways consist of a three-tiered kinase cascade including an upstream MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK), and the MAPK itself. MAPKs are proline-directed Ser/Thr kinases that phosphorylate hundreds of substrates in the cytoplasm and nucleus. MAPKs are accordingly implicated in numerous cellular behaviors such as proliferation, survival, apoptosis, migration, and differentiation, as well as in human disease (E. K. Kim and Choi, 2010). There are also three non-canonical MAPK pathways, ERK 3/4, ERK7/8, and NLK, that are outside the scope of this review (Cargnello and Roux, 2011). Most RTKs seem to activate MAPK signaling to some degree, though the intensity, duration, and importance of this activity varies considerably by RTK (McKay and D. K. Morrison, 2007). The four canonical MAPK cascades share a consensus phosphorylation site (P-X-S/T-P), but show only partial overlap in their known substrates and elicit particular responses through the activation of unique ensembles of effectors, spatial and dynamic regulation, feedback mechanisms, and scaffolding proteins (Cargnello and Roux, 2011; Casar and Crespo, 2016; Keshet and Seger, 2010; Sheridan et al., 2008; Wortzel and Seger, 2011).

2a. ERK1/2 Pathway

The ERK1/2 MAPK pathway is activated in response to a broad range of stimuli including growth factor-induced RTK signaling, GPCRs, non-canonical TGF-β and BMP signaling, and cellular stress (Cargnello and Roux, 2011). ERK1/2 signaling is strongly associated with the FGF pathway, but is also activated in response to NC-expressed PDGF, EPH, ERBB, RET, MET, KIT, and TRK family RTKs (Fantauzzo and Soriano, 2015). Receptors activate the cascade by recruitment of adapter molecules such as FRS2/3 (Kouhara et al., 1997), SHB (Cross et al., 2002), SHC (Pelicci et al., 1992), SHP2 (Kazlauskas et al., 1993; Lechleider et al., 1993), and CRK/CRKL (CRK) (Brewer et al., 2016; Lemmon and Schlessinger, 2010). These molecules recruit the guanine nucleotide exchange factor (GEF) Son-of-Sevenless (SOS) through the adapter protein GRB2, which is itself also sometimes recruited to RTKs directly (N. Li et al., 1993). SOS mediates activation of the membrane-associated GTP-binding protein RAS. GTP-bound RAS activates the ERK1/2 signaling cascade. This consists of the MAPKKKs A-RAF, B-RAF, and RAF-1/C-RAF; the MAPKKs

MEK1 and MEK2; and the MAPKs ERK1 and ERK2 (Figure 1A). Once phosphorylated by MEK, ERK1/2 can then act on targets in the cytoplasm, such as a broad group of Ser/Thr kinases known as the MAPK-activated protein kinases (MAPKAPs) (Cargnello and Roux, 2011), and in the nucleus, primarily transcription factors such as ELK1, FOS, MYC, and MEF2 (Plotnikov et al., 2011). ERK1/2 activity has been linked in various contexts to the processes of proliferation, survival, migration and fate choice (Cargnello and Roux, 2011; Wortzel and Seger, 2011).

ERK1/2 and Neural Crest Developmental Phenotypes—The ERK1/2 pathway has long been of interest in regard to both development and disease, which has resulted in a wealth of mouse null and conditional alleles available to study the pathway's *in vivo* functions. The mouse models discussed in the review and their phenotypes are summarized in Table 1. These models highlight the neural crest as particularly sensitive to ERK1/2 perturbation. ERK1 and ERK2 are encoded by the genes *Mapk3* and *Mapk1*, respectively. $Mapk3^{-/-}$ mice are viable and fertile (Pages et al., 1999), with a background-dependent hyperactivity phenotype (Mazzucchelli et al., 2002), whereas $Mapk1^{-/-}$ mice die shortly after implantation with a defect in extraembryonic tissue (Hatano et al., 2003; Saba-El-Leil et al., 2003). Similarly, mice lacking one of the two kinases upstream of ERK1/2, MEK2, are viable and fertile, while mice lacking MEK1 die around E10.5 with placental and vascular defects (Belanger et al., 2003; Giroux et al., 1999).

Fremin et al. deleted *Mapk1* throughout the embryo proper using the epiblast-specific *Sox2-Cre* in order to bypass the placental defect. Embryos survived to E16.5, lacked a mandible and tongue, had a shortened maxilla, and had cardiovascular defects such as VSD and PSA. Embryos were also smaller than controls, had shortened forelimbs, and lacked hindlimbs (Frémin et al., 2015). These phenotypes correlate with domains of ERK1/2 activity detected in E9.5 and E10.5 mouse embryos by whole-mount immunohistochemistry for double-phosphorylated ERK1/2 (ppERK1/2), where high levels of ERK activity were observed in the pharyngeal arches, frontonasal prominence, and limb buds, as well as the heart, forebrain, midbrain-hindbrain boundary, liver primordia, and tailbud (Corson et al., 2003; Frémin et al., 2015). Loss of one copy of *Mapk3* in addition to both copies of *Mapk1* led to growth arrest at E10.5. Intriguingly, mice completely lacking both *Mapk1* and *Mapk3* in the embryo proper can be rescued by a ubiquitously expressed *Mapk3* transgene (Frémin et al., 2015). Together the results argue that ERK1 and ERK2 are functionally redundant, act in a dose-dependent manner during development, and that NCCs (and limb) are particularly sensitive to ERK1/2 depletion. This study, however, could not distinguish between NCC autonomous and non-autonomous roles for ERK1/2.

Newbern et al. took a complementary approach and combined null and conditional alleles to interrogate the ERK1/2 pathway requirement in the neural crest (Newbern et al., 2008). The investigation was prompted by the discovery that human patients with craniofacial and cardiovascular defects similar to DiGeorge Syndrome carried a deletion that spanned *MAPK1*. In an extensive mouse genetic analysis, Newbern et al. used the *Wnt1-Cre* driver, active in early NCCs (discussed further below), to analyze $Mapk1^{flox/flox}$; $Mapk3^{-/-}$; *Wnt1-Cre* embryos, $Mek1^{flox/flox}$; $Mek2^{-/-}$; *Wnt1-Cre* embryos, and $Braf^{flox/flox}$; $Raf1^{flox/flox}$; *Wnt1-Cre* embryos, thereby interrogating the ERK1/2 cascade at all three levels. Conditional

loss of *Mapk1* alone resulted in perinatal lethality. Embryos showed significant craniofacial and cardiovascular defects including cleft palate, a shortened maxilla, a drastically shortened mandible, and variably penetrant cardiac outflow tract defects such as double outlet right ventricle (DORV), persistent truncus arteriosus (PTA), and ventricular septal defect (VSD). Global disruption of *Mapk3* in addition to conditional loss of *Mapk1* enhanced the severity of the craniofacial phenotypes, the penetrance of cardiovascular defects, and produced novel defects such as absence of the tongue, absence of the external ear, failure of thymus development, and a misplaced thyroid gland. Double mutant mice also showed hypoplastic pharyngeal arches at E10.5, suggesting NC migration, proliferation, or survival defects. $Braf^{flox/flox}$; $Raf1^{flox/flox}$; *Wnt1-Cre* and $Mek1^{flox/flox}$; $Mek2^{-/-}$; *Wnt1-Cre* mice largely phenocopied the *Mapk1/3* mice, emphasizing the linear nature of this cascade (Newbern et al., 2008).

NC-specific loss of *Mapk1/3* or *Mek1/2* also affected the peripheral nervous system (PNS). These mice were completely devoid of NC-derived Schwann cells (Newbern et al., 2011). The dorsal root ganglia (DRG) were specified correctly and motor and sensory nerves initially projected axons peripherally, but the DRG later underwent cell death and peripheral nerve projections degenerated, both apparently caused by the lack of Schwann cells. Conditional deletion of these genes in Schwann Precursor Cells slightly later in development (E12-13) using *Dhh-Cre* did not affect Schwann cell number but inhibited myelination and these mice died by four weeks of age (Newbern et al., 2011).

ERK1/2 activity has also been manipulated in the NC through the phosphatase SHP2, which promotes RTK-mediated ERK1/2 activation through recruitment of GRB2 (Araki et al., 2003; Tajan et al., 2015), and can also regulate the PI3K pathway and dephosphorylate activated RTKs (Bennett et al., 1994; Edouard et al., 2010; Tajan et al., 2015). Deletion of *Ptpn11*, the gene encoding SHP2, in NC using *Wnt1-Cre* causes reduced ppERK1/2 levels in NCCs, embryonic lethality between E15.5 and birth, and developmental defects similar to the ERK1/2 pathway mutants discussed above, including a hypoplastic maxilla, vastly reduced mandible and tongue, smaller ears, misplaced eyes, PTA and VSD (Nakamura et al., 2009a). Conversely, expression of a Q79R GoF *Shp2* mutant allele in NC leads to increased ppERK1/2 levels (Nakamura et al., 2009b). A subset of these mice was born with a cleft lip and palate and quickly succumbed, while the rest survived but exhibited growth retardation, a domed skull, a shortened but correctly-patterned mandible, and premature fusion of the frontal bones. These phenotypes were ameliorated by injecting pregnant dams with the MEK1/2 inhibitor U01216, indicating that the observed phenotypes are in fact due to ERK1/2 hyperactivation and demonstrating the potential for therapeutic modulation of growth factor signaling *in utero* (Nakamura et al., 2009a). NC-specific loss of SHP-2 also caused a failure in induction of the lacrimal gland from the ectoderm overlying the NC-derived peri-ocular mesenchyme (Garg et al., 2017). A similar phenotype was seen in $Mapk1^{flox/flox}$; $Mapk3^{-/-}$; *Wnt1-Cre* embryos, $Mek1^{flox/flox}$; $Mek2^{-/-}$; *Wnt1-Cre* embryos, and $Fgfr1^{flox/flox}$; $Fgfr2^{flox/flox}$; *Wnt1-Cre* embryos, showing that FGFR-SHP2-ERK1/2 signaling is required in the NC for lacrimal gland induction (Garg et al., 2017).

Together, these studies show that precise ERK1/2 signaling is required for proper NC development. This is seen in multiple NC populations including the cranial and cardiac

crest, as well as trunk NC-derived Schwann cells. These studies did not evaluate melanocytes or enteric crest, but both require RTKs capable of inducing ERK1/2 (e.g. KIT and RET, respectively (Fantauzzo and Soriano, 2015)) so defects in these populations would not be surprising.

ERK1/2 in Neural Crest Specification—Neural crest cells are induced in the border region between neural and non-neural ectoderm. Work primarily in chick, frog, and zebrafish has shown that a balance of WNT, FGF, and BMP signals are important for specifying the NC. Several recent reviews address NC specification in more detail (Milet and Monsoro-Burq, 2012; Pegoraro and Monsoro-Burq, 2012; Sauka-Spengler and Bronner-Fraser, 2008; Stuhlmiller and García-Castro, 2012a). Because FGF is a strong inducer of ERK1/2 signaling in frog, zebrafish, and mouse (Christen and Slack, 1999; Corson et al., 2003; Curran and Grainger, 2000; Shinya et al., 2001), this pathway seemed a good candidate for regulating neural crest induction. In chick, FGF-dependent ppERK1/2 is detected at the presumptive neural plate border region prior to NC induction, with higher levels in the more medial neural plate region and lower levels in the more lateral non-neural ectoderm. Inhibition of ERK1/2 activity via overexpression of an ERK1/2 phosphatase, DUSP6, reduced expression of the NC marker PAX7 in its endogenous domain, but upregulated it ectopically in the neural plate, where ppERK1/2 levels were initially higher. This result was confirmed by culturing explants from different lateral regions with a MEK1/2 inhibitor (Stuhlmiller and García-Castro, 2012b).

Xenopus embryos expressing a dominant negative FGFR4 showed reduced NC gene expression at mid-neurula stages. Animal cap explants initially had high ERK1/2 activity, but transitioned to a low ERK1/2, high PI3K state as they differentiated. Both signaling pathways were inhibited by dominant negative FGFR4 expression. However, caps directed to a NC fate (via *Pax3* and *Zic3* expression) show persistent high ERK1/2 and low PI3K activity, inhibiting MEK or stimulating PI3K signaling in caps interferes with their adoption of a NC fate, and MEK-inhibited animal caps show reduced NC gene expression. This suggests ERK1/2 downstream of FGFR4 is important for multipotent animal cap cells to adopt a NC identity (Geary and LaBonne, 2018).

The mechanism(s) by which ERK1/2 signaling promotes NC induction remains unresolved. Studies from chick demonstrate FGF can act directly on presumptive NC (Stuhlmiller and García-Castro, 2012b). Work in cultured cells and Xenopus indicate it may function by antagonizing BMP activity through inhibitory phosphorylation of SMAD1 by ERK1/2 (Kretzschmar et al., 1997; Pera et al., 2003; Sater et al., 2003). However, knock-in mice expressing a mutant form of SMAD1 in which the linker MAPK phospho-sites are mutated do not have NC defects, so this pathway may not be conserved in the mouse, MAPK may regulate SMAD1 at other sites, other kinases may act in a redundant fashion at other sites, and/or defects in SMAD1 may be compensated for by the related SMAD5 and SMAD8 proteins (Aubin et al., 2004). Indeed, regulation of SMAD1 by MAPK is likely to be complex as there is evidence that other kinases can phosphorylate these linker residues and MAPK may phosphorylate SMAD1 outside the linker region (Eivers et al., 2009; Sapkota et al., 2007).

Data in *Xenopus* shows that FGF signals to underlying mesoderm, which induces NC-promoting Wnt signals (Monsoro-Burq et al., 2003). These mechanisms are not mutually exclusive nor do they preclude further roles for ERK1/2 activity. Verifying these mechanisms in the mouse has been challenging due to a combination of genetic redundancy, methodological approaches, and possible species differences (Barriga et al., 2015). Of particular utility would be a Cre driver that is active in the neural plate border region or E7.0-E8.0 ectoderm, allowing for genetic manipulation prior to NC induction while circumventing implantation and gastrulation defects. The widely used *Wnt1-Cre* may only turn on after neural crest specification has already begun (Barriga et al., 2015). *Wnt1-Cre* mediated reporter activity is first detected around E8.0 (6 somites) in the midbrain and soon expands to other regions of NC, as well as portions of the neuroepithelium (G. Chen et al., 2017), roughly coinciding with the appearance of NC markers, but 0.5–1.0 days after the neural plate border region from which NC arises is specified (Stuhlmiller and García-Castro, 2012a). Indeed, Chen et al. noted that some SOX9-positive NC cells at the level of the hindbrain escape reporter labeling, suggesting *Wnt1-Cre* is expressed as NC emerges, but may miss the earliest patterning steps and first-specified cells (G. Chen et al., 2017). It would also be informative to assess NC specification in previously generated $Mapk3^{\Delta/flox}$; $Mapk1^{+/-}$; *Sox2-Cre* embryos (Frémin et al., 2015), although as discussed above this experiment would not distinguish between autonomous versus non-autonomous requirements for ERK1/2 as *Sox2-Cre* deletes throughout the epiblast.

ERK1/2 and Neural Crest Proliferation—The ERK1/2 pathway is well known for its ability to drive proliferation (Chambard et al., 2007). This is perhaps best exemplified in the number of activating mutations found in this pathway in human cancer (Dhillon et al., 2007). Conversely, mouse embryonic fibroblasts (MEFs) completely devoid of ERK1/2 fail to proliferate (Pages et al., 1993; Voisin et al., 2010). ERK1/2 stimulates cell-cycle progression by directly phosphorylating and activating growth-promoting transcription factors including TCFs, other Ets-family transcription factors, JUN, and MYC. ERK1/2 also activates MAPKAP families such as the Ribosomal Protein S6 Kinases (RSKs) and Mitogen and Stress-Activated Kinases (MSKs). Phosphorylated RSKs translocate to the nucleus and phosphorylate transcriptional targets including SRF, FOS, and the cAMP Response Binding Protein (CREB). MSKs target CREB, Activating Transcription Factor 1 (ATF1) which can form part of the Activating Protein 1 (AP-1) complex, and histones (Cargnello and Roux, 2011). Together, this ensemble directs rapid induction of IEGs and cell cycle genes, such as CyclinD1, in response to growth factors (Albanese et al., 1995; Lavoie et al., 1996; O'Donnell et al., 2012).

It might seem obvious that NC proliferation relies on ERK1/2 pathway activity, but *in vivo* evidence is mixed. It is clear that ERK1/2 activity is necessary for growth-factor induced proliferation in a number of NC-derived cell lines. Classic experiments in rat PC12 pheochromocytoma cells showed that a short pulse of ERK1/2 activity in response to Epidermal Growth Factor (EGF) could stimulate proliferation (Marshall, 1995). Intriguingly, extended ERK1/2 activity in response to Nerve Growth Factor (NGF) stimulated differentiation and neurite outgrowth, highlighting the importance of signaling dynamics in encoding different ERK1/2 responses and suggesting ERK1/2 could be an important node

regulating a balance between proliferation and differentiation (Marshall, 1995). Dynamic encoding of RTK signaling has been described in several non-NC cell lines, as well (Francavilla et al., 2013; Freed et al., 2017). As shown in our own laboratory, FGF stimulates proliferation of NC-derived mouse embryonic palatal mesenchyme cells (MEPMs) and expression of several growth-associated genes is MEK-dependent (Vasudevan et al., 2015). Some *in vivo* GoF studies further support a role for ERK1/2 driven proliferation in NC, while others do not. Activation of RAF in adult Schwann cells stimulated their partial dedifferentiation, reduced myelin gene expression, and increased proliferation (Napoli et al., 2012). On the other hand, expressing a constitutively active MEK in Schwann cells beginning at E16 with *Egr2*^{Cre} or E12-E13 with *Dhh-Cre* activated ERK1/2 and caused hypermyelination and increased translation, but the study did not note differences in proliferation or cell number (Sheean et al., 2014). Whether this discrepancy is caused by activation of RAF versus MEK, the timing of induction, expression differences, or a combination of these is unclear. *Wnt1-Cre*-driven expression of an activated allele of *Shp2* causes upregulated ppERK1/2 but no change in proliferation in the frontal bone (Nakamura et al., 2009b). This is consistent with the observation that hyperactive ERK1/2 activity can block the cell cycle in the absence of additional signaling perturbations (Chambard et al., 2007; Pumiglia and Decker, 1997).

LoF studies are even less clear. Several reports in which ERK1/2 pathway components were deleted in NC failed to detect any defect in proliferation, but rather found striking defects in NC differentiation, discussed in more detail below (Nakamura et al., 2009a; Parada et al., 2015). Mosaic loss of X-linked Ephrin-B1 in female heterozygous mice caused reduced proliferation in the anterior palatal shelf mesenchyme at E13.5 and E14.5. Stimulation of Ephrin-B1 in cultured MEPMs activated ERK1/2 but not p38, JNK, or AKT, and induced MEK-dependent proliferation and IEG expression, suggesting the *in vivo* proliferation defect was due to decreased ERK1/2 activity (Bush and Soriano, 2010). Crucially, NC proliferation has not been assayed *in vivo* in cells lacking all ERK1/2 activity, so proliferation may be supported by low or transient levels of ERK1/2 signaling. Clonal analysis of ERK1/2 null NCCs could resolve this discrepancy.

ERK1/2 and Neural Crest Survival—The ERK1/2 pathway can act as a survival signal by promoting turnover of the pro-apoptotic protein BIM and antagonizing apoptotic MAPK signaling such as the p38 pathway (Lu and S. Xu, 2006). Stimulation of PC12 cells with NGF results in ERK1/2-dependent phosphorylation (and subsequent turnover) of BIM, promoting survival (Ley et al., 2005). *In vivo*, however, ERK1/2 LoF studies found no defect in NC proliferation or apoptosis, though this observation is subject to the same caveat that ERK1/2 activity was not completely disrupted (Nakamura et al., 2009a; Parada et al., 2015). Loss of SHP2 in NC reduced ppERK1/2 levels, but one study found no increase in apoptosis and another found only a slight increase (Garg et al., 2017). Mutation in *Fgf8*, expressed in the pharyngeal arch epithelium and forebrain, results in a striking increase in NC apoptosis in the underlying mesenchyme without significant changes in proliferation (Abu-Issa et al., 2002; Frank et al., 2002; Griffin et al., 2013; Storm et al., 2006; Trumpp et al., 1999). This argues that ERK1/2, strongly activated by FGF receptors in this region of the mouse embryo (Corson et al., 2003), could be an important survival signal for NC. However, loss of *Fgf8*

must suppress ERK1/2 activity to a greater extent than conditional deletion of *Mapk1* or *Shp2,* or the apoptosis in *Fgf8* mutants must be dependent on additional pathways besides ERK1/2. Finally, a missense mutation in the ERK1/2 substrate *Ets1* underlies the mouse allele *variable spotting* and *Ets1*$^{-/-}$ mice that survive postnatally have a white belly spot due to increased apoptosis in melanoblasts (Gao et al., 2010; Saldana-Caboverde et al., 2015), though whether this process is ERK1/2-dependent is unknown. ERK1/2 may therefore promote NC survival, perhaps in combination with other pathways.

ERK1/2 and Neural Crest Migration—ERK1/2 may regulate cell migration, though its role in this process remains controversial. ERK1/2 can bind the focal adhesion protein PAXILLIN, which can facilitate ERK1/2 activation by simultaneously associating with MEK1/2. ERK1/2 can also phosphorylate PAXILLIN and the cytoskeletal regulatory proteins Focal Adhesion Kinase (FAK) and Myosin Light Chain Kinase (MLCK), and can repress integrin activation (Huang, 2004). Waves of ERK1/2 were recently shown to orient collective cell migration in epithelial cells (Aoki et al., 2017), and elevated ERK downstream of EGFR oriented border cell migration in *Drosophila* (Bianco et al., 2007). It was demonstrated that ‘trailblazing’ cells at the migration front in chick NC migration have a distinct gene expression profile and are enriched for *Fgfr2* relative to the cells migrating behind them (McLennan et al., 2015), though a connection between FGFRs, ERK1/2, and migration-specific gene expression has not been demonstrated. FGF2 and FGF8 have chemotactic activity for mouse mesencephalic neural crest (Kubota and Ito, 2000). It was found in chick that FGF8 could act as a chemoattractant for cardiac crest and this activity was partially blocked by MEK1/2 inhibition, though PI3K inhibition had a stronger effect (Sato et al., 2011). Goto et al. utilized transgenic mice expressing FRET-based biosensors to assess signaling activity in migrating enteric neural crest. The study noted some ERK1/2 activity in these cells but there was no enrichment of activity in cells at the leading edge of migration and MEK1/2 inhibition did not affect migration in this system (Goto et al., 2013). However, a separate study cultured E11.0 small intestine fragments with GDNF-expressing cells, and NCC outgrowth was sensitive to MEK1/2 inhibition (Natarajan et al., 2002). Newbern et al. noted hypoplastic pharyngeal arches in compound ERK1/2 conditional mutants and suggested migration defects as one possible explanation, though this was not directly assessed (Newbern et al., 2008). Fate mapping NCCs in *Shp2* conditional LoF or GoF mutants did not indicate a widespread NC migration defect, though there was a reduced number of NC cells in the cardiac OFT in the LoF model (Garg et al., 2017; Nakamura et al., 2009b; 2009a). The transcription factor ETS1 is a known ERK1/2 substrate and *Ets1*$^{-/-}$ mice have deficient migration of cardiac NCCs and melanocytes as assessed by *Wnt1-Cre*-mediated fate-mapping, although cell migration was not directly imaged or evaluated in culture and the importance of ERK1/2 upstream of ETS1 was not tested in regard to migration (Gao et al., 2010).

Several mutant RTK models draw a link between ERK1/2 and migration. Homozygous null mutations in *Erbb2* or *Erbb3* or in their ligand *Nrg1* results in migration defects of the NC-derived sympathetic neurons with no defects in apoptosis (Britsch et al., 1998). Conditional loss of *Erbb3* or its downstream effector *Shp2* in Schwann cells causes myelination defects that can be rescued by expressing an activated *Mek* allele (Sheean et al., 2014). If ERBB2/3

functions similarly in the two cell types, it would be logical that ERK1/2 controls migration downstream of ERBB2/3 in sympathetic neurons, though it remains possible that ERBB2/3 is utilizing different pathways in E10 neurons compared to later Schwann cells. *Epha4*$^{-/-}$; *Twist*$^{+/-}$ mice show craniosynostosis of the coronal suture, staining indicated reduced ppERK1/2 levels, and the primary cellular defect was ectopic migration of osteogenic cells that would normally contribute to the frontal and parietal bones into the coronal suture (Ting et al., 2009). Finally, mice with homozygous Y567F and Y569F mutations in *Kit*, which disrupts binding site for SFKs, SHP-1, and SHP-2, completely lack melanocytes in adult mice. Mutant KIT protein no longer activates ERK1/2 in response to the KIT ligand SCF, while AKT activation is normal (Kimura et al., 2004). Cellular behavior was not analyzed in this model, however KIT null mice initially specify melanoblasts, which then fail to migrate and are subsequently lost (Bernex et al., 1996). Therefore ERK1/2 may regulate NCC migration in some situations but this effect seems to be highly context-dependent. Precisely when, where, and how this occurs needs to be studied in more detail.

ERK1/2 and Neural Crest Differentiation—ERK1/2 signaling regulates NC differentiation in a variety of contexts. As discussed above, an extended pulse of ERK1/2 activity causes differentiation of neural crest-derived PC12 pheochromocytoma cells into autonomic-nerve like cells (Marshall, 1995). This is reminiscent of the pathway's role in mouse embryonic stem cells, where ERK1/2 inhibition is required to maintain pluripotency (Nichols et al., 2009; Stuhlmiller and García-Castro, 2012a; Yamanaka et al., 2010). On the other hand, FGF treatment of MEPMs led to proliferation at the expense of differentiation down the osteoblast lineage (Vasudevan et al., 2015). Inhibition of MEK promoted differentiation in these cells and reduced expression of genes associated with proliferation. Supporting this, *Fgfr1*$^{flox/flox}$; *Wnt1-Cre* conditional mutant mice showed expanded alkaline phosphatase activity in the face, indicating ectopic or precocious osteoblast differentiation, though the involvement of other pathways downstream of FGFR1 cannot be ruled out (Vasudevan et al., 2015). Parada et al. generated *Mapk1*$^{flox/flox}$; *Wnt1-Cre* mice that had less severe phenotypes than similar mice analyzed by Newbern et al., due to differences in genetic background (Newbern et al., 2008; Parada et al., 2015). These *Mapk1*$^{flox/flox}$; *Wnt1-Cre* mice exhibited cleft palate, maxillary hypoplasia, a small and asymmetric mandible, micrognathia, tongue defects, and perinatal lethality. This study found no defects in NC proliferation or apoptosis, but rather found specific defects in the ability of NC to differentiate down the osteoblast lineage. Consistent with this observation, mice conditionally lacking the phosphatase SHP2 in NC had vastly reduced ppERK1/2 staining and a noted defect in osteoblast differentiation in the frontal bone with no defect in proliferation or apoptosis in the pharyngeal arches or outflow tract. The same study showed that MEK1/2 inhibition reduced NCC expression of differentiation markers TUJ1 and SMA in a cardiac NCC explant assay (Nakamura et al., 2009a). A separate report observed a slight increase in apoptosis in the pharyngeal arches of *Shp2*$^{flox/flox}$; *Wnt1-Cre* embryos, without significant defects in NC specification or migration, but primarily noted a failure to induce *Alx* genes necessary for lacrimal gland induction (Garg et al., 2017). Curiously, expression of a GoF *Shp2* allele in NC increased ppERK1/2 but also resulted in osteoblast differentiation defects in the frontal bone, as assessed by osteopontin expression, without defects in proliferation or survival (Nakamura et al., 2009b). Loss of the ERK1/2 substrate

Ets1 leads to ectopic NC-derived cartilage in the heart, and culture of hearts in the presence of a MEK1/2 inhibitor is sufficient to induce ectopic cartilage (Gao et al., 2010).

Mice mutant for *Rps6ka*, the gene encoding the ERK1/2 substrate RSK2, have craniofacial abnormalities and decreased bone mass (Laugel-Haushalter et al., 2014; X. Yang et al., 2004). *RSK2* mutations in humans cause Coffin-Lowry Syndrome, an inherited disorder distinguished by mental retardation and skeletal abnormalities. Mice deficient for the RSK2 substrate ATF4 show delayed mineralization, like *Rsk2* mice, and *Rsk2* and *Atf4* interact genetically, suggesting an ERK1/2-RSK2-ATF4 signaling axis as one mechanism by which ERK1/2 signaling promotes osteoblast differentiation (X. Yang et al., 2004).

Altogether these studies argue for a critical role for ERK1/2 in the regulation of NC differentiation. Just what that effect is, however, depends on the context. ERK1/2 signaling can promote proliferation and/or antagonize differentiation (in cultured cells, *Fgfr1* conditional mutants, and *Ets1* mutant hearts), promote differentiation with little effect on proliferation (in the various ERK1/2 LoF mouse models), and inhibit differentiation when overactive (in SHP2 GoF experiments). The inner workings of these pathways are not well understood, but the answer may once again lie within the PC12 paradigm that emphasizes the importance of quantitative differences in signaling, rather than a simple on/off model (Marshall, 1995). Proper differentiation may require high or sustained, but still physiological levels of signaling, whereas proliferation may be supported by low or transient levels of signaling. It is also important to remember that many of these genetic experiments may perturb additional signaling pathways beyond ERK1/2 that also regulate the process under investigation. An ability to better quantify the effects of genetic manipulations on signaling, and identifying which other pathways may be affected, will be important for squaring these disparate observations.

Other Canonical MAPK Pathways

2b. P38—The p38 MAPK pathway is primarily known as a stress-activated protein kinase pathway. It is robustly activated downstream of environmental stress, such as ultraviolet light or hydrogen peroxide, and inflammatory cytokines, but can also be activated by various growth factors (Cuenda and Rousseau, 2007; Katz et al., 2007; Raingeaud et al., 1995; Rice et al., 2002; Tangkijvanich et al., 2002). The mechanism linking RTKs to p38 is unclear, but may proceed via RAC1 and CDC42 (Bagrodia et al., 1995; S. Zhang et al., 1995) (Figure 1B). There are four p38 isoforms in mammals: α, β, γ, and δ encoded by the *Mapk14, Mapk11, Mapk12,* and *Mapk13* genes, respectively. All four kinases have been knocked out, but only p38α has a developmental phenotype (Cuenda and Rousseau, 2007). Embryos lacking p38α die around E12.5 exhibiting anemia, liver, heart, and placental defects (Adams et al., 2000; Allen et al., 2000; Mudgett et al., 2000; Tamura et al., 2000). The placental failure is the cause of the other phenotypes, however, as p38α mutant embryos can be rescued by tetraploid complementation (Adams et al., 2000) or deletion of *Mapk14* specifically in the embryo proper using *Sox2-Cre* (Hui et al., 2007). Such embryos survive to term but subsequently succumb with apparent lung defects. Although p38β deficient mice are viable, $Mapk14^{flox/flox}$; $Mapk11^{-/-}$; *Sox2-Cre* mice die before E16.5 and exhibit exencephaly, a small liver, thin myocardium, and VSD (del Barco Barrantes et al., 2011).

While defects in neural crest development were not specifically assayed, craniofacial structures appeared to form normally in these embryos at E13.5. Defects in cardiac crest can lead to VSD, however these mice had numerous myocardial abnormalities, making myocardial dysfunction the most parsimonious explanation. *Mapk12*$^{-/-}$; *Mapk13*$^{-/-}$ double mutants are viable (Sabio et al., 2005).

These results argue that the p38 MAPK pathway is not essential for NC specification or early development. However, p38 has been linked to mechanisms that may be relevant at later stages. First, p38 regulates tracheal smooth muscle cell migration downstream of PDGF in tissue culture (Hedges et al., 1999). Because the neural crest is the source of pericytes and vascular smooth muscle in the head region of the embryo (Etchevers et al., 2001) and it is known that recruitment and stabilization of these cells around blood vessels is dependent on PDGF signaling (Levéen et al., 1994; Soriano, 1994), p38 may be an important effector in this process if both populations of smooth muscle function similarly. Second, p38 has been shown in fibroblasts to phosphorylate FGFR1 in order to regulate its transport to the nucleus and subsequent regulation of transcription (Sørensen et al., 2008). FGFR1 is important for neural crest development (Brewer et al., 2015; Trokovic et al., 2003; C. Wang et al., 2013), however it is currently unknown whether this particular mechanism is at play in NCCs or whether the signal driving p38 activity in this context is FGF itself or another pathway. p38 may regulate NC cell death, as PC12 cells overexpressing the RTK TRKA underwent apoptosis following NGF treatment and this was associated with a marked increase in p38 signaling (Yan et al., 2002). P38 can phosphorylate and activate the transcription factors DLX5 and SP7 (Osterix), promoting osteoblast differentiation in response to BMP2 (Ortuño et al., 2010; Ulsamer et al., 2008; X. Wang et al., 2007), and *Dlx5*$^{-/-}$ mice have craniofacial bone defects (Acampora et al., 1999). Finally, NCC deletion of the non-canonical TGF-β pathway effector *Tak1*, a p38 and JNK MAPKKK, leads to cleft palate (Song et al., 2013; Yumoto et al., 2013). These mice have strikingly reduced p38 signaling and additional defects in both JNK and SMAD activity. Conversely, hyperactive p38 signaling in *Tgfbr2*$^{flox/flox}$*; Wnt1-Cre* craniofacial mesenchyme is associated with reduced proliferation (Iwata et al., 2012). p38 is therefore likely to be an important pathway in NCCs, though it would be helpful to disrupt p38 directly in NC as the signaling changes and phenotypes discussed above are currently associated with mutations in more promiscuous upstream components. Furthermore, p38 is implicated primarily downstream of BMP and TGF-β rather than growth factor-mediated RTK signaling.

2c. JNK—The JNK pathway, like the p38 pathway, is a stress-associated MAPK pathway and can be activated by inflammatory cytokines, pathogens, UV radiation, oxidative stress, TGF-β signaling, as well as some GPCRs and WNTs (Zeke et al., 2016). JNK can also be activated downstream of RTKs through the CRK family of adapter proteins, which can engage JNK via the GEF C3G and small GTPase RAP1 (Larsson et al., 1999; Tanaka et al., 1997) (Figure 1C). CRK has also been reported to bind JNK, MKK4, and other signaling components in the JNK pathway, so the primary path(s) between CRK and JNK downstream of RTKs remains obscure (Girardin and Yaniv, 2001; Zeke et al., 2016). JNK is an important regulator of proliferation, apoptosis, and cell migration. Activated JNK regulates transcription, most famously by phosphorylating JUN, from which JNK derives its name,

and through other TFs such as MYC and TWIST1 (Zeke et al., 2016). It also has cytosolic targets, many of which are cytoskeletal proteins including PAXILLIN, β-CATENIN, and the microtubule associated proteins MAP1B, MAP2, and DCX, underlying its role in migration (Huang, 2004). There are three JNK isoforms in mammals, JNK1, JNK2, and JNK3 encoded by the *Mapk8*, *Mapk9*, and *Mapk10* genes, respectively. Mice deficient in any single JNK isoform, JNK1 and JNK3 together, or JNK2 and JNK3 together are viable (Kuan et al., 1999). Mice lacking JNK1 and JNK2 die by E12.5 and exhibit hindbrain exencephaly with reduced hindbrain apoptosis, but increased apoptosis in the forebrain (Kuan et al., 1999; Sabapathy et al., 1999). No difference in BrdU incorporation was noted in the neuroepithelium or cranial mesenchyme. The pharyngeal arches appear to form normally in these embryos and HNK-1/NCAM staining of neural crest cells in E10.5 embryos was indistinguishable from control littermates (Kuan et al., 1999). Additionally, a subset of $Mapk8^{-/-}; Mapk9^{+/-}$ embryos also display exencephaly, while surviving up to E15.5, but neural crest derivatives were not specifically examined (Sabapathy et al., 1999). JNKs have two upstream MAPK kinases, MKK4 and MKK7, that have also been mutated. $Mkk4^{-/-}$ mice die at E12.5 with liver defects (Di Yang et al., 1997; Ganiatsas et al., 1998; Nishina et al., 1999), while $Mkk7^{-/-}$ mice are viable (Schramek et al., 2011). The mice described to date argue against an essential role for JNK signaling in NC specification, growth, or survival, with the important caveat that *Jnk* triple mutants and *Mkk4/7* double mutants have not been analyzed either globally or specifically in NCCs using conditional mutagenesis. To the contrary, a recent study showed that morpholino-mediated depletion of mRNA encoding the tight junction protein MarvelD3 in *Xenopus* caused NC induction defects associated with elevated JNK signaling. Inhibiting JNK rescued the phenotype, suggesting suppression of JNK may be an important step in NC specification (Vacca et al., 2018).

Like p38, there are several studies indicating a later role for JNK in NCCs, particularly regarding cell migration. Cardiac crest migration was inhibited following treatment with retinoic acid in a neural tube explant assay, an effect mediated by inhibition of JNK activity (J. Li et al., 2001). Intriguingly, *Jun* mutants lack NCCs in the cardiac outflow tract (Eferl et al., 1999). Mice harboring mutations in two JNK phosphorylation sites in c-JUN are viable, so JNK activity may not be necessary for c-JUN-dependent NCC migration (Behrens et al., 1999), though JNK also phosphorylates c-JUN at additional sites (Hibi et al., 1993). Mice homozygous for a S697A point mutation that disrupts a PKA phosphorylation site in the RTK RET have enteric NC migration defects and this mutation inhibits JNK phosphorylation, but not ERK1/2, p38, SRC, or AKT phosphorylation following stimulation with the RET ligand Glial Derived Neurotrophic Factor (GDNF) (Asai et al., 2006). Chemical inhibition of the JNK pathway negatively affects enteric NC migration, though this study did not find increased JNK activity following treatment with GDNF, so JNK may instead function as a permissive motility signal (Goto et al., 2013). Another substrate of JNK is TWIST1, a transcription factor that regulates epithelial to mesenchymal transition and is important in neural crest emergence and migration (Vincentz et al., 2008; 2013). JNK phosphorylation stabilizes TWIST1 in culture (Hong et al., 2011), however whether JNK affects TWIST1 in a meaningful way in NCCs is unknown. These data suggest JNK could be important for NCC migration in multiple contexts, but *in vivo* NC defects due to *Jnk*

mutation has not yet been demonstrated and whether JNK is required downstream of specific growth factors in NCCs is unclear.

2d. ERK5—ERK5, also known as Big MAPK (it is twice the size of the other canonical MAPKs due to an extended C-terminus), is encoded by the *Mapk7* gene. Like the other MAPKs, it can be activated by multiple stimuli such as cellular stress and growth factors, notably EGF (Kato et al., 1998) and NGF (Nishimoto and Nishida, 2006; Van T Hoang et al., 2017; Watson et al., 2001). Biochemical studies showed ERK5 can be activated by RAS in PC12 cells (Kamakura et al., 1999), although this view has been challenged (Fukuhara et al., 2000; Obara and Nakahata, 2010). ERK5 may instead be activated through the adapter LAD (Sun et al., 2003). It has a single upstream MAPKK, MEK5, which is in turn activated by the MAPKKKs MEKK2/3 (Figure 1D). Uniquely among MAPKs, the ERK5 C-terminus contains a MEF2-interacting domain and a transcriptional activation domain. Mice lacking either ERK5 or MEK5 succumb around E10.5 with cardiovascular defects (Regan et al., 2002; X. Wang et al., 2004). ERK5 mutants were also noted for having defects in cephalic mesenchyme and hypoplastic pharyngeal arches, possibly indicating a NC defect (Regan et al., 2002). and can regulate transcription from SREs by phosphorylating the ternary complex factor ELK4/SAP1 in HeLa cells. Newbern et al. deleted ERK5 in NC using the *Wnt1-Cre* driver and found this yields viable, fertile mice with reduced size, a shortened mandible, and truncated ear (Newbern et al., 2011). Previous studies had implicated ERK5 PNS development (Finegan et al., 2009), however the PNS was normal in these conditional mutants (Newbern et al., 2011). Thus, ERK5 supports cNCC development, though its impact on development is relatively minor and the underlying cellular defects have not been studied in NC.

3. PI3K Pathway

The PI3K pathway functions through the conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3) by the PI3K enzyme (Figure 1E). PIP3 recruits and activates the 3-phosphoinositide-dependent protein kinase-1 (PDPK1/PDK1) and recruits the PDK1 substrate protein kinase B/Ak thymoma viral proto-oncogene (PKB/AKT) (Fruman et al., 2017; Vanhaesebroeck et al., 2012). PDK1 phosphorylates AKT at T308 (Alessi et al., 1997; Stokoe et al., 1997), partially activating it and priming it for full activation by phosphorylation at S473 by the mammalian target of rapamycin complex 2 (mTorc2) (Sarbassov et al., 2005) or the DNA-dependent protein kinase (DNA-PK) (Bozulic et al., 2008; Manning and Toker, 2017). AKT is a Ser/Thr kinase which regulates cell survival, proliferation, migration, and differentiation via the mTorc1 pathway, p53 pathway, Forkhead box O (FOXO) transcription factors, Rho-family GTPases, and numerous other substrates (Manning and Toker, 2017). AKT activation is traditionally viewed as the principal output of growth factor-induced PI3K signaling, though more attention has recently been paid to AKT-independent roles of PI3K-PDK1 signaling in development (Grego-Bessa et al., 2016). PIP3 can also activate Rho-family GTPases through PIP3-binding GEFs (Campa et al., 2015; Goicoechea et al., 2014).

PI3K enzymes are divided into three functional classes, Class I, II, and III. Class I is further subdivided into Class IA enzymes, which mainly function downstream of RTKs and will be

discussed further, and Class IB enzymes, which primarily act downstream of GPCRs. For information on Classes IB, II and III we direct the reader to these recent reviews (Jean and Kiger, 2014; Thorpe et al., 2015). The PI3K enzyme is composed of a regulatory and a catalytic subunit. In the mouse, there are three Class IA regulatory genes, *Pik3r1*, *Pik3r2*, and *Pik3r3* encoding p85α/p55α/p50α, p85β, and p55γ, respectively. The *Pik3r1* gene encodes multiple subunits via alternative splicing. There are also three Class IA catalytic genes, *Pik3ca*, *Pik3cb*, and *Pik3cd* encoding p110α, p110β, and p110δ, respectively. Class IA regulatory subunits bind to and inhibit Class IA catalytic subunits in the absence of stimulation. Following stimulation with growth factors, regulatory subunits can interface with RTKs through their phospho-tyrosine binding SH2 domains by directly binding the phosphorylated receptor or a phosphorylated adapter protein. Binding has the dual purposes of relieving inhibition of the catalytic subunit and positioning it close to its substrate PIP2 at membranes (Fruman et al., 2017). Catalytic subunits can also be stimulated by the small G-proteins RAS (in the cases of p110α and p110δ) and RAC (in the case of p110β) (Fritsch et al., 2013). PI3K signaling is restricted both in space and time through the degradation of PIP3 by lipid phosphatases, most notably Phosphatase and Tensin homologue (PTEN), which removes the 3′ phosphate added by PI3K (Maehama and Dixon, 1998).

PI3K and Neural Crest Developmental Defects—Like the ERK1/2 pathway, mouse mutants exist for most of the PI3K signaling cascade. An important point to remember is that disruption of Class IA PI3K genes particularly affects PI3K signaling downstream of RTKs, whereas mutation of downstream pathway components, effectors, or feedback enzymes affects non-RTK mediated PI3K signaling as well.

Mutant mice have been generated for all three Class IA catalytic genes. Global loss of p110α, or knock-in of a catalytically dead p110α, results in embryonic lethality around E10.5 (Bi et al., 1999; Graupera et al., 2008; Lelievre et al., 2005). Embryos develop to E9.5 with no organogenesis defects, but are smaller, have widespread hemorrhaging and occasionally exhibit blood-filled subepidermal blebs around the neural tube. Some of these defects are autonomous to endothelium as conditional deletion in endothelial cells results in lethality by E12.5 with angiogenesis defects (Graupera et al., 2008). Embryos null for p110β die around E3.5 and cells cultured from these blastocysts fail to proliferate (Bi et al., 2002). However, homozygous knock-in mice expressing catalytically inactivated p110β are recovered at birth at sub-Mendelian ratios indicating the early lethality is not due to loss of p110β enzymatic activity and PIP3 generation, but may instead be due to a non-enzymatic role for p110β in RTK and GPCR trafficking (Ciraolo et al., 2008). Further complicating matters, despite associating with Class IA regulatory subunits, p110β was initially found to be activated more strongly downstream of GPCRs (Guillermet-Guibert et al., 2008). It was then demonstrated that it could be stimulated synergistically by both RTKs and GPCRs, illustrating the potential for complex interactions among signaling pathways and receptors (Houslay et al., 2016). p110δ is primarily expressed in immune cells and accordingly p110δ null mice are viable with immune system defects (Jou et al., 2002; Okkenhaug et al., 2002).

Mutants in Class IA regulatory subunits more clearly demonstrate a role for growth factor-induced PI3K signaling in NC. Mutations that disrupt *Pik3r1* or *Pik3r2* alone are viable, but *Pik3r1*$^{-/-}$; *Pik3r2*$^{-/-}$ double mutants die between E10.5 and E12.5 (Brachmann et al., 2005).

Like p110α mutants, there is widespread hemorrhaging and subepidermal blebs. Some mice also exhibit a wavy neural tube and those that survive to E11.5 and E12.5 show a facial cleft. This is strikingly similar to the phenotype of *Pdgfra* mutant mice (Soriano, 1997). Indeed, point mutations in PDGFRα that disrupt its interaction with PI3K indicate PDGFRα signaling is primarily mediated through the PI3K pathway (He and Soriano, 2013; Klinghoffer et al., 2002). Furthermore, conditional mutagenesis indicates that the facial cleft and midfacial blebbing, but not the trunk blebbing or wavy neural tube, are autonomous to the NC (Tallquist and Soriano, 2003). PDGFRα can form heterodimers with PDGFRβ to support its activity in NC and this signaling is also primarily through the PI3K pathway (Fantauzzo and Soriano, 2016; Klinghoffer et al., 2002). Together these results indicate that *Pik3r1* and *Pik3r2* exhibit redundancy and are required downstream of PDGF in the NC to regulate craniofacial morphogenesis. Mice lacking *Pik3r3* have not been described.

Mutations in the downstream effectors PDK1 and the three mouse AKT isoforms exist but have not been especially informative regarding NC. PDK1 mutants die around E9.5 and lack pharyngeal arches and dorsal root ganglia at that time, though the embryos also lack somites and a heart and are developmentally delayed, so whether there is a specific defect in NC or the embryos simply experience growth retardation and/or toxicity is unclear (Lawlor et al., 2002). $Akt2^{-/-}$ and $Akt3^{-/-}$ single mutants and $Akt2^{-/-}; Akt3^{-/-}$ double mutants are viable (Dummler et al., 2006), $Akt1^{-/-}$ mutants are viable but exhibit growth retardation (W. S. Chen et al., 2001), $Akt1^{-/-}$; $Akt2^{-/-}$ mutants are born with reduced size, skeletal muscle atrophy, a delay in bone development, and die within a few hours of birth likely from respiratory failure (Peng et al., 2003), and $Akt1^{-/-}$; $Akt3^{-/-}$ mutants die between E11 and E12 with unclear defects, though they appear morphologically normal at E10.5 (Z. Z. Yang et al., 2005). None of the Class IA PI3K enzymes and few PI3K downstream effectors and regulators (PTEN being the exception, see below) have been studied conditionally in NC. It would be interesting to know if NC conditional compound AKT mutants phenocopy the facial cleft seen in $Pik3r1^{-/-}; Pik3r2^{-/-}$ mutants or if the phenotype is AKT-independent.

PI3K and Neural Crest Specification—Studying PI3K in mouse NC specification is hampered by the same drawbacks as those outlined above for ERK1/2. There is, however, some evidence from both mouse and other organisms that PI3K may be important in NC specification. A recent screen for chemicals that inhibit the NC reporter *Crestin:EGFP* in zebrafish identified a molecule, CAPE, which reduced reporter and NC gene expression as well as NCC migration. The study noted reduced AKT phosphorylation following CAPE treatment and the phenotype was rescued by constitutively active AKT expression whereas constitutively active PTEN caused similar defects to CAPE treatment (Ciarlo et al., 2017). The target of CAPE is unknown as is the signal driving PI3K/AKT activity. A recent study in *Xenopus* found that late stages of NC specification and subsequent NCC migration were disrupted following PFKFB4 depletion and these effects were due to reduced AKT activity. PFKFB4 was induced by the gene regulatory network governing NC specification, but whether the AKT activity it supports is dependent on a particular growth factor is unknown, as is the precise mechanism by which PFKFB4 promotes AKT activity (Figueiredo et al., 2017). In mouse, p110α mutants, various AKT compound mutants, $Pik3r1^{-/-}; Pik3r2^{-/-}$ double mutants, and $Pdgfra^{PI3K/PI3K}$; $Pdgfrb^{PI3K/PI3K}$ all form at least some NC, so RTK-

stimulated PI3K activity may not be strictly required to induce NC, though genetic redundancy precludes an unambiguous ruling on the matter. Furthermore, NC induction in these models has not been studied quantitatively or in great detail, so patterning could be negatively affected even if not absolutely inhibited. The missing pharyngeal arches and dorsal root ganglia in *Pdk1*$^{-/-}$ mice could reflect a NC induction defect, but this possibility has not been specifically investigated (Lawlor et al., 2002).

PI3K in Neural Crest Proliferation—PI3K is known to promote growth through AKT and mTorc1. Indeed, activating mutations in many PI3K components and inactivating mutations in *Pten* are common in many cancers (Fruman et al., 2017). This connection between PI3K and the cell cycle is unsurprisingly borne out in NCCs during development. *Pdgfra*$^{flox/flox}$; *Wnt1-Cre* mice show reduced proliferation in the medionasal prominence (MNP) (He and Soriano, 2013). At least some of this activity is PI3K dependent because *Pdgfra*$^{PI3K/PI3K}$ mice also show reduced proliferation in this region (He and Soriano, 2013). Expression of a constitutively active allele of *Pdgfra* in the *Meox2*-Cre (epiblast), *Wnt1-Cre2* (NC), or *Col2a1-Cre* (chondrocyte) lineages led to cranial suture malformations and ectopic cartilage in this region (He and Soriano, 2017). The opposite phenotype was seen in *Pdgfra*$^{PI3K/PI3K}$ embryos. Indeed, PDGFRα activation drove increased AKT and PLCγ activity. BrdU assays indicated an increase in proliferation in this region, though additional effects on migration and differentiation cannot be ruled out (He and Soriano, 2017). Furthermore, loss of *Pten* in NCCs caused enhanced PI3K-AKT signaling and subsequent overgrowth of tissue (T. Yang et al., 2017). GDNF-stimulated proliferation of enteric neuroblasts is blocked by a PI3K inhibitor (Focke et al., 2001). PI3K is therefore an important driver of NC proliferation.

PI3K and Neural Crest Survival—PI3K signaling can promote cell survival through two well-characterized mechanisms. One is the phosphorylation and subsequent inhibition of the pro-apoptotic FOXO transcription factors. In enteric NC, a GDNF-RET-PI3K-AKT signaling pathway was shown to promote survival by antagonizing FOXO1 and FOXO3A (Srinivasan et al., 2005). FOXO4 and FOXO6 are expressed in cranial NC in *Xenopus* where they are antagonized by AKT, and depletion of FOXO4 causes craniofacial abnormalities (Schuff et al., 2010). *Foxo4*$^{-/-}$ mice have no detectable phenotype, although this could be due to redundancy of *Foxo* genes (Hosaka et al., 2004). AKT can also promote survival through antagonizing the p53 pathway. A proteomics study from our own laboratory assayed for PDGFRα-induced AKT substrates in MEPMs and identified several negative regulators of p53 (Fantauzzo and Soriano, 2014). Consistent with this observation, *p53* mRNA levels are reduced following PDGF-AA treatment, loss of one copy of *Tp53* rescues a subset of *Pdgfra*$^{PI3K/PI3K}$ skeletal phenotypes including a strong reduction in the incidence of cleft palate and significant rescue of vertebral malformations (Fantauzzo and Soriano, 2014), and *Pdgfra*$^{-/-}$ mice have increased apoptosis in cranial mesenchyme (Soriano, 1997).

PI3K and Neural Crest Migration—PI3K activity is critically important for NC migration and numerous studies support a role for growth factor-induced PI3K signaling in this process. Motility of cardiac crest towards Fgf8 (Sato et al., 2011) and enteric neurons towards GDNF (Goto et al., 2013) is sensitive to PI3K inhibition. Supporting this, mouse

embryos expressing a *Ret* Y1062F mutation that disrupts a binding site for multiple adapter proteins had a reduced or missing ENS (Jijiwa et al., 2004; Wong et al., 2005). This mutation affected both ERK1/2 and PI3K signaling, but a later study partially rescued ERK1/2 activity by deleting the negative regulator *Sprouty2*, while PI3K activity, as assessed by pAKT levels, remained suppressed. The ENS in these double mutant $Sprouty2^{-/-;}\ Ret^{Y1062F/Y1062F}$ embryos better colonized the stomach compared to $Ret^{Y1062F/Y1062F}$ single mutants, but failed to rescue along the length of the intestine, suggesting the phenotype is primarily PI3K dependent (Miyamoto et al., 2011). It remains to be determined whether the primary defect in these embryos is migration, as suggested by the inhibitor study (Goto et al., 2013), or proliferation.

In *Xenopus*, NC cells migrate toward PDGF-AA and PDGFRα was shown to mediate cell dispersion and contact inhibition of locomotion through regulation of N-Cadherin. This effect was sensitive to inhibition of PDGFRα, PI3K, and AKT (Bahm et al., 2017). In mouse, $Pdgfra^{flox/flox}$; *Wnt1-Cre* conditional mutants have lineage tracing defects in the cranial crest, with fewer cells reaching the frontonasal prominence and pharyngeal arches (He and Soriano, 2013). Neural crest explant assays of *Pdgfra* conditional mutants showed reduced migration out of the explant compared to controls. Cells that did emerge had a smaller size, fewer focal adhesions, and disorganized lamellipodia (He and Soriano, 2013). PDGF signaling is associated with robust PI3K activity, however lineage tracing in $Pdgfra^{PI3K/PI3K;}\ Rosa26R^{LacZ/+;}\ Wnt1\text{-}Cre$ embryos did not reveal a migration defect and MEPMs cultured from these embryos responded normally to PDGF-AA in transwell assays (He and Soriano, 2013). Perhaps PDGFRA regulates migration via a different signaling pathway than PI3K *in vivo*. He et al. further implicated RAC1 downstream of PDGFRA (He and Soriano, 2013) and RAC1 can be activated by RTKs independent of PI3K (Feng et al., 2011; Schiller, 2006). An alternative explanation is that $Pdgfra^{PI3K/PI3K}$ NCCs can still activate PI3K through heterodimer formation with PDGFRB (Fantauzzo and Soriano, 2016). Supporting the involvement of Rho-family GTPases in NC development, conditional NC mutants of *Rac1* (Fuchs et al., 2009; Thomas et al., 2010), *Cdc42* (Fuchs et al., 2009; Y. Liu et al., 2013), or mice with NC-specific expression of a dominant negative Rho Kinase (ROCK) (Phillips et al., 2012) all display pronounced facial clefts. RTK-driven PI3K signaling can activate these GTPases, but so can PI3K independent mechanisms also active in NC (Fort and Theveneau, 2014; Schiller, 2006). It is currently unknown to what extent these GTPases are dependent on PDGFRα, other RTKs, PI3K, and other stimuli altogether for their overall activity. Inhibitor studies clearly demonstrate the importance of PI3K in NCC migration and the necessity of PI3K activity to induce motility following treatment with growth factors, but genetic studies have yet to conclusively show the source of this PI3K activity originates at a specific RTK. Directly visualizing migration in $Pik3r1/2^{-/-}$ (Brachmann et al., 2005) or $Pdgfra^{PI3K/PI3K}$; $Pdgfrb^{flox/flox}$; *Wnt1-Cre* (Fantauzzo and Soriano, 2016) mutant embryos and conducting genetic interaction studies between RTKs, small GTPases and/or PI3K components would be enlightening.

PI3K and Neural Crest Differentiation—There are also several reports pointing to PI3K as a regulator of NCC differentiation. One recent study ablated the negative regulator *Pten* in NC, resulting in enhanced proliferation as well as osteoblast differentiation in

cephalic NCCs (T. Yang et al., 2017). This result is consistent with previous work in our laboratory that PDGF-AA treatment of MEPMs promotes osteoblast differentiation in a PI3K-dependent manner (Vasudevan et al., 2015). It is also likely that PI3K plays a role in smooth muscle differentiation through the positive regulation of SRF-MRTF complexes, which will be discussed in more detail below. Overexpression of FGF8 in the anterior palate caused increased cartilage formation at the expense of bone as well as increased proliferation (J. Xu et al., 2018). Surprisingly, ppERK1/2 levels were unchanged but p85α protein levels were reduced, suggesting FGF-signaling may antagonize PI3K signaling to prevent bone differentiation (J. Xu et al., 2018).

4. Serum Response Factor: A shared MAPK and PI3K Effector

An interesting example of how MAPK and PI3K signaling can elicit some of their effects in NC through a common effector is found in Serum Response Factor (SRF). This transcription factor cooperates with MAPK-activated Ternary Complex Factors (TCFs) such as ELK1 to induce expression of genes regulated by Serum Response Elements (SREs). Alternatively, it can form a complex with Myocardin or the two Myocardin Related Transcription Factors (MRTFs), MRTF-A and MRTF-B (D. Wang et al., 2001; Z. Wang et al., 2004; Zaromytidou et al., 2006). SRF-MRTF complexes are regulated by PI3K, RHO-actin, and ERK1/2 signaling, and are important for regulating genes involved in the cytoskeleton, migration, and muscle differentiation (Panayiotou et al., 2016; Posern and Treisman, 2006).

Newbern et al., investigated NC-specific conditional mutants of *Srf* in conjunction with their study of the ERK1/2 pathway in NC. Interestingly, these mice partially phenocopied ERK1/2 conditional mutants. They displayed mandibular hypoplasia, outflow tract defects, a midline cleft, and defects of the thymus and thyroid gland, but had less severe maxillary defects and retained their ear and tongue, arguing that the effects of ERK1/2 may be partially mediated through SRF (Newbern et al., 2008). In DRG neurons, SRF was required for axon growth, branching, and target innervation but not survival (Wickramasinghe et al., 2008). Restoring expression in NCCs rescues the phenotype, indicating the defect is autonomous to NC (J. Li et al., 2005). SRF is also linked more directly to RTKs. The craniofacial phenotype of $Srf^{flox/flox}$; *Wnt1-Cre* mice is strikingly similar to $Pdgfra^{flox/flox}$; *Wnt1-Cre* mice and both genes interact genetically (Vasudevan and Soriano, 2014).

Unsurprisingly, given their similar phenotypes, SRF has been shown to regulate many of the same cellular behaviors as the upstream signaling pathways that regulate it. Deletion of *Srf* in NC causes a reduction in proliferation in the frontonasal region (Vasudevan and Soriano, 2014). Cultured primary frontonasal prominence cells from *Srf* conditional mutants failed to migrate towards a source of PDGF-AA, unlike their control littermate counterparts (Vasudevan and Soriano, 2014). This is consistent with SRF's well-characterized role in cell migration and cytoskeletal gene expression (Franco et al., 2013; Schwartz et al., 2014). $Mkl2^{-/-}$ embryos, which lack SRF's partner MRTF-B, underwent initial NC specification and migration, but cardiac NC then failed to differentiate into smooth muscle, causing cardiac outflow tract defects.

SRF seems to be deployed differentially via distinct signaling inputs. Our lab recently found that whereas both FGF and PDGF could cause SRF-TCF complex formation in MEPMs in

an ERK1/2-dependent fashion, only PDGF stimulated SRF-MRTF complex formation, in a PI3K-dependent fashion (Vasudevan and Soriano, 2014). A detailed study of MRTF-A activation further showed that both ERK1/2 and actin signaling were in fact required for full MRTF activation in NIH3T3 cells, illustrating how multiple pathways can combine to differentially regulate outcomes through a single protein (Panayiotou et al., 2016).

5. The Agony and Ecstasy of Crosstalk

The importance of achieving the proper level and duration of a particular signaling pathway is attested by the striking example of the PC12 cells (Marshall, 1995), the fact that GoF and LoF mutants in the same pathway both generate severe defects, and the importance of feedback genes in development and disease (Neben et al., 2017). In addition to feedback of one pathway onto itself, however, many pathways also cross regulate each other. Indeed, the point has been made that activation of signaling *pathways* by RTKs might better be described as activation of signaling *networks* (Jordan et al., 2000; Manning and Toker, 2017; Raju et al., 2014) (Figure 2). This is especially important to keep in mind and test for, as it can fill in crucial gaps connecting pathway perturbations with phenotypes. A defect that seems due to loss of ERK1/2 activity could be more directly attributable to a consequent hyperactivation of PI3K/AKT signaling, or a combination of both. Indeed, cross-regulation between the ERK1/2 and PI3K pathways has long been recognized (Mendoza et al., 2011). In MEPMs, we see increased AKT activation in response to MEK1/2 inhibition and increased ERK1/2 phosphorylation in response to PI3K inhibition (Vasudevan et al., 2015). Consistent with this, FGF1 induces differentiation of MEPMs, normally a PDGFAA-PI3K driven process, when MEK1/2 is also inhibited. A similar phenomenon was noted in zebrafish, where FGF did not normally stimulate AKT activity, but did in the presence of a MEK1/2 inhibitor (Ciarlo et al., 2017). There is also crosstalk between MAPK pathways, likely mediated partially by dual specificity phosphatases (DUSPs), negative feedback genes that are specific to particular MAPKs (Owens and Keyse, 2007). MEK1/2 inhibition leads to an ectopic induction of JNK activity in response to either FGF1 or PDGFAA in MEPMs (Vasudevan et al., 2015). It is therefore possible that a phenotype such as apoptosis thought to be due to reduced ERK1/2 or PI3K activity may instead be caused by non-physiological JNK or p38 signaling. These possibilities will be exciting to explore as our understanding of growth factor signaling in NC advances.

6. Future Questions and Approaches

Considerable headway has been made towards understanding where and when growth factors and the signaling networks that they activate are deployed during NC development. Genetic studies have made clear the importance of RTK signaling for NC development and generated a long list of RTK families and signaling proteins known to be involved. Nevertheless, detailed knowledge of how specific growth factors modulate signaling *in vivo* and how these signaling changes are decoded by NCCs to generate specific behaviors remains lacking. Indeed, as simple on-off signaling models are discarded in favor of network-oriented, dynamic, and quantitative ones, the problems of studying this *in vivo* only grow. Thankfully, several new approaches are allowing developmental biologists to interrogate signaling with methods once strictly in the domain of cell biologists.

To characterize pathway activity, fluorescent signaling sensors for MAPK and PI3K signaling represent an exciting opportunity to assay pathway activity in living cells and tissues (Gross and Rotwein, 2015; Regot et al., 2014; Vandame et al., 2014). Combined with time-lapse imaging and existing mouse mutants, they could illuminate unappreciated *in vivo* signaling domains, dynamics, and crosstalk. To date they have already been successfully utilized in cells, worms, flies, zebrafish, and mice (Aoki et al., 2017; Goto et al., 2013; Kamioka et al., 2012; Kumagai et al., 2015; la Cova et al., 2017; Yoo et al., 2010; Q. Zhang et al., 2018). They require a careful approach, as sensors first characterized in cells may not behave the same way in embryos, can be difficult to visualize and quantify. Moreover, depending on the sensor's own dynamic range, sensitivity, timescale, and localization in the cell, they may report incompletely on a particular molecule's relevant activity.

It is clear that RTKs can drive divergent cellular responses, depending on the cell type, the identity of the RTK itself, as well as ligand identity, concentration, and duration of exposure. A more complete understanding of how downstream signaling and transcriptional responses vary according to these factors will be key to untangling the complexities of growth factor signaling in NC development. The increasing practicality of mass spectrometry and RNA sequencing, including at the single-cell level, will help to address these questions. These approaches are already generating considerable insight into how NCCs are specified, migrate, and respond to signals (Lignell et al., 2017; J. A. Morrison et al., 2017; Rada-Iglesias et al., 2012).

In addition to techniques that enhance our ability to read out how NCCs are responding, there are several tools facilitating perturbation of signaling systems in more sophisticated ways. Genetic knock-in of point mutations in RTKs to disrupt particular downstream effector molecules has been an effective approach (Asai et al., 2006; Brewer et al., 2015; Hoch and Soriano, 2006; Jijiwa et al., 2004; Kimura et al., 2004; Klinghoffer et al., 2002), but one largely limited to the mouse and time consuming to undertake. CRISPR-Cas9 should extend this technique, and genetic disruption generally, to other systems (Gandhi et al., 2017; Lai et al., 2017; Z. Liu et al., 2016; Willems et al., 2015) and increase throughput where it already exists.

Last, optogenetic control of growth factor signaling is an exciting technique to probe signaling dynamics because of the exquisite spatial and temporal control it allows (Toettcher et al., 2013). It has already been used to great effect to study the ERK1/2 pathway in cells and flies (Johnson et al., 2017; Toettcher et al., 2013; Wilson et al., 2017), and a similar system for studying RAC signaling was recently described (Graziano et al., 2017). The system could be difficult to deploy in mice as samples must be cultured under microscopic observation and is mostly applicable to relatively transparent or superficial cells, but the approach is likely to be useful in culture or smaller embryos.

Out of 150 years of NC research, well over 100 have seen the use of RTK mutants (Dunn, 1937; Little, 1915) and this line of research shows no signs of slowing. Indeed, the past history heralds even more exciting things to come.

Acknowledgments

We thank Stuart Aaronson, Katherine Fantauzzo, Robert Krauss, Sergei Sokol, and our laboratory colleagues for discussions and critical comments on the manuscript. We apologize to authors whose work we have not cited due to space limitations. Work from the author's laboratory is supported by NIH/NIDCR grants R01 DE022363 and R01 DE022778 to P.S. C.J.D. is supported by NIH/NIDCR fellowship F32 DE026678.

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Highlights

- ERK1/2 and PI3K signaling play critical roles in neural crest development.
- Recent publications have shed light on the in vivo roles of these pathways in multiple developmental models.

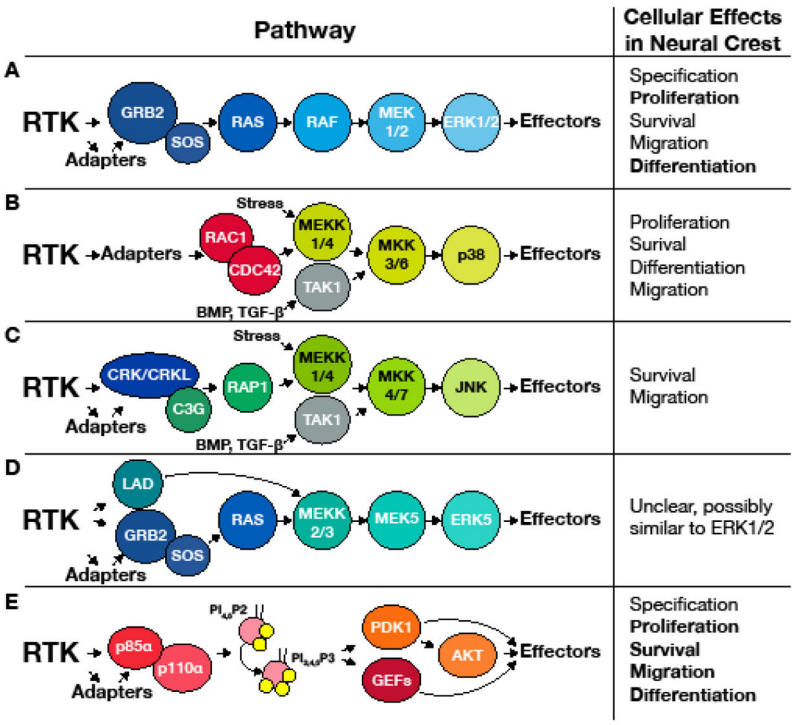

Figure 1. RTK signaling pathways in isolation

The ERK1/2 (A), p38 (B), JNK (C) ERK5 (D), and PI3K (E) pathways and their known or likely means of activation downstream of RTKs are shown at left. For simplicity only crucial adapter proteins are shown and specific effector proteins and crosstalk between pathways have been omitted. See the text for further clarification. To the right are demonstrated (bold) and suggested (regular) cellular downstream behaviors in NC.

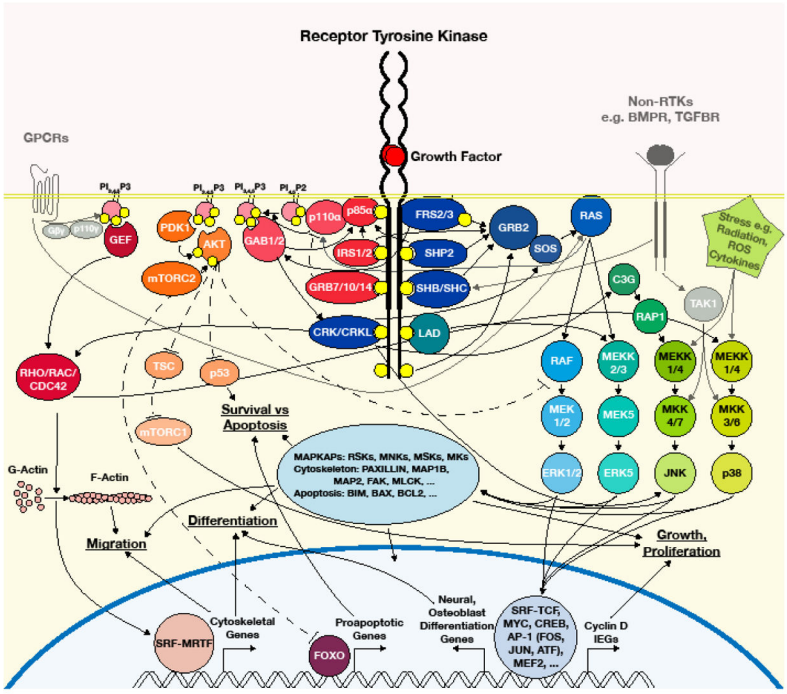

Figure 2. RTK signaling pathways acting in concert

A generic RTK is shown at center in an activated state. Signaling proteins that activate the MAPK pathways (e.g. GRB2-SOS) and PI3K pathway (e.g. p85α) can be recruited directly to the RTK or via signaling effectors that act as intermediates (e.g. FRS2/3, SHB/SHC, CRK/CRKL, SHP2, GRB7/10/14, IRS1/2, etc.). Many of these proteins can recruit one another and receptor activation thereby creates multimeric signaling complexes. The exact composition of effectors and activated pathways will depend on the RTK, ligand, and cell type involved. MAPK signaling regulates cytoplasmic targets such as MAPKAPs, cytoskeletal proteins, and apoptosis/survival proteins, and nuclear targets, mainly transcription factors. Some MAPKAPS will translocate to the nucleus and regulate TFs, potentiating the response. PI3K activity can activate Rho-family GTPases that regulate the cytoskeleton and cytoskeleton-regulated TFs (e.g. MKL1/2), and activate AKT signaling, which in turn regulates p53, FOXO, and mTORC1 activity. MAPK and PI3K can regulate each other and some of this crosstalk is shown (e.g. AKT inhibition of RAF). MAPK and PI3K can also be activated by other receptors or cellular stress and these sources are shown in a lighter tone. Last, RTKs can activate additional signaling pathways not covered in this review (PLCγ, PKC, Src Family Kinases, and STATs). While many of these pathways can interact with MAPK and PI3K pathways, they are omitted for clarity. Effectors and pathways more associated with MAPK signaling are shown in blue tones and green tones, while those more tightly associated with PI3K signaling are shown in red and orange themes, though the high degree of interconnectedness makes such distinctions somewhat contrived.

Table 1

Summary of MAPK and PI3K mutant phenotypes discussed in the text. For simplicity, growth factor receptor mutations have been omitted.

Genotype	NC Defects	Lethality/Other Defects	Reference
ERK1/2 Pathway LoF			
Mapk1$^{-/-}$ (*Erk2*)	NA	Peri-implantation lethal. Extraembryonic tissue defects.	(Hatano et al., 2003; Saba-El-Leil et al., 2003)
Mapk3$^{-/-}$ (*Erk1*)	NA	Viable and fertile. Background-dependent hyperactivity.	(Mazzucchelli et al., 2002; Pages et al., 1999)
Map2k1$^{-/-}$ (*Mek1*)	NA	Mid-gestation lethal. Placental defects.	(Giroux et al., 1999)
Map2k2$^{-/-}$ (*Mek2*)	NA	Viable and fertile.	(Belanger et al., 2003)
Mapk1$^{flox/flox}$; *Wnt1-Cre*	Micrognathia, hypoplastic maxilla, cleft palate, DORV, PTA, VSD, osteoblast differentiation defects.	Perinatal lethal.	(Newbern et al., 2008; Parada et al., 2015)
Mapk1$^{flox/flox}$; *Mapk3*$^{-/-}$; *Wnt1-Cre*	Above + aglossia, missing external ear and thymus, misplaced thyroid. Hypoplastic PAs at E10.5. No NCC- derived Schwann cells. DRG degenerate. Lacrimal aplasia.	Lethal E18-E19.	(Garg et al., 2017; Newbern et al., 2008; 2011)
Mek1$^{flox/flox}$; *Mek2*$^{-/-}$; *Wnt1-Cre*	Similar to above.	Similar to above.	(Garg et al., 2017; Newbern et al., 2008; 2011)
Braf$^{flox/flox}$; *Raf1*$^{flox/flox}$; *Wnt1-Cre*	Similar craniofacial and cardiovascular phenotypes as above.	Similar to above.	(Newbern et al., 2008)
Mapk1$^{flox/flox}$; *Mapk3*$^{-/-}$; *Dhh-Cre*	PNS not analyzed. Hypo- and unmyelinated PNS axons. Tremor, hindlimb paresis.	Lethal by P28.	(Newbern et al., 2011)
Mapk1$^{\Delta/flox}$; *Sox2-Cre*	Agnathia, aglossia, hypoplastic maxilla, VSD, PSA.	Lethal by E16.5. Short forelimbs and missing hindlimbs.	(Frémin et al., 2015)
Mapk1$^{\Delta/flox}$; *Mapk3*$^{+/-}$; *Sox2-Cre*	NA	Small size, lethal E10.5.	(Frémin et al., 2015)
Ptpn11$^{flox/flox}$ (*Shp2*); *Wnt1-Cre*	Reduced or absent NCC-derived craniofacial bones and cartilage, PTA, variable VSD. Osteoblast differentiation defects. Lacrimal aplasia.	Lethal between E15.5 and birth.	(Garg et al., 2017; Nakamura et al., 2009a)
ERK1/2 Pathway GoF			
Tg(CAG-cat,- Ptpn11^{Q79R}*); Wnt1-Cre*	Variable cleft lip/palate. Domed skull, hypertelorism, small size. Osteoblast differentiation defects.	Lethal P1–P5 if cleft lip/palate present.	(Nakamura et al., 2009b)
ROSA26-Map2k1^{DD}*; Dhh-Cre* and *ROSA26- Map2k1*^{DD}*; Egr2-Cre*	Continuous myelin growth in PNS Schwann cells.	NA	(Sheean et al., 2014)
Tg(P0-Raf1/ER); Activated in adult Schwann cells	Schwann cell dedifferentiation, increased proliferation, muscle weakening.	NA	(Napoli et al., 2012)
ERK1/2 Pathway Effectors			

Genotype	NC Defects	Lethality/Other Defects	Reference
Rps6ka$^{-/-}$ (*Rsk2*)	Delayed mineralization of craniofacial bones. Wide cranial sutures at birth. Dental defects.	Viable. Reduced long bone length and decreased mineral density.	(Laugel-Haushalter et al., 2014; X. Yang et al., 2004)
Ets1$^{-/-}$	Ectopic NCC-derived cartilage in heart, VSD. Rare surviving mouse had white belly spot. Increased apoptosis of melanoblasts.	Perinatal lethal on C57Bl/6 background.	(Gao et al., 2010; Saldana-Caboverde et al., 2015)
Srf$^{flox/flox}$; *Wnt1-Cre*	Facial cleft, micrognathia, thymic and thyroid aplasia, PTA, VSD, DRG innervation defects.	Background dependent lethality between E13.5 and late gestation.	(Newbern et al., 2008; Vasudevan and Soriano, 2014; Wickramasinghe et al., 2008)
P38 LoF			
Mapk14$^{-/-}$ (*p38α*)	NA	Lethal by E12.5. Anemia, liver, heart, and placenta defects.	(Adams et al., 2000; Allen et al., 2000; Mudgett et al., 2000; Tamura et al., 2000)
Mapk14$^{flox/flox}$; *Mapk11*$^{-/-}$; *Sox2-Cre* (*p38α/β*)	NA	Lethal by E16.5. Exencephaly, small liver, thin myocardium, VSD.	(del Barco Barrantes et al., 2011)
Mapk12/13$^{-/-}$ (*p38γ/δ*)	NA	Viable and fertile.	(Sabio et al., 2005)
Tak1$^{flox/flox}$; *Wnt1-Cre*	Cleft palate.	Perinatal lethal.	(Song et al., 2013; Yumoto et al., 2013)
JNK LoF			
Mapk8$^{-/-}$ (*Jnk1*), *Mapk9*$^{-/-}$ (*Jnk2*), *Mapk10*$^{-/-}$ (*Jnk3*), *Mapk8/10*$^{-/-}$, *Mapk9/10*$^{-/-}$	NA	Viable and fertile.	(Kuan et al., 1999)
Mapk8/9$^{-/-}$	Normal.	Lethal by E12.5. Exencephaly and neural apoptosis defects.	(Kuan et al., 1999; Sabapathy et al., 1999)
Mkk4$^{-/-}$	NA	Lethal by E14.	(Di Yang et al., 1997; Ganiatsas et al., 1998; Nishina et al., 1999)
Mkk7$^{-/-}$	NA	Viable and fertile.	(Schramek et al., 2011)
JNK Effectors			
Jun$^{-/-}$	Lack of NCCs in cardiac OFT.	Lethal E13. Apoptosis of hepatoblasts and hematopoietic cells.	(Eferl et al., 1999)
Jun$^{AA/AA}$	NA	Viable and fertile. Small size, resistant to epileptic seizures and neuronal apoptosis.	(Behrens et al., 1999)
ERK5 LoF			
Mapk7$^{-/-}$ (*Erk5*)	Disorganized cranial mesenchyme, small PAs	Lethal at E10.5. Cardiovascular defects.	(Regan et al., 2002)
Mek5$^{-/-}$	NA	Similar to above.	(Wang et al., 2004)
Mapk7$^{flox/flox}$; *Wnt1-Cre*	Micrognathia, truncated ear, reduced size.	Viable and fertile.	(Newbern et al., 2011)
PI3K LoF			
Pik3ca$^{-/-}$ (*p110α*) or *Pik3ca*$^{D933A/D933A}$	NA	Lethal at E10.5. Hemorrhaging, subepidermal blebs, angiogenesis defects.	(Bi et al., 1999; Graupera et al., 2008)
Pik3cb$^{-/-}$ (*p110β*)	NA	Lethal at E3.5.	(Bi et al., 2002)
Pik3cb$^{K805R/K805R}$	NA	Subviable.	(Ciraolo et al., 2008)
Pik3cd$^{-/-}$ (*p110δ*)	NA	Viable with immune defects.	(Jou et al., 2002; Okkenhaug et al., 2002)

Genotype	NC Defects	Lethality/Other Defects	Reference
Pik3r1$^{-/-}$ or *Pik3r2*$^{-/-}$	NA	Viable and fertile.	(Brachmann et al., 2005)
Pik3r1/2$^{-/-}$	Facial cleft.	Lethal between E10.5-E12.5. Hemorrhaging, subepidermal blebs, wavy neural tube.	(Brachmann et al., 2005)
PI3K GoF			
Pten$^{flox/flox}$*; Wnt1-Cre*	Overgrowth of craniofacial structures. Increased proliferation, enhanced osteoblast differentiation.	Perinatal lethal.	(T. Yang et al., 2017)
Pten$^{flox/flox}$*; Dct-Cre* (melanocytes)	Temporary overgrowth of dermal melanocytes. Resistance to graying.	Most mice die within two months of life due to neurologic defects. Small size.	(Inoue-Narita et al., 2008)
PI3K Effectors			
Pdk1$^{-/-}$	Absent PAs and DRG.	Lethal E9.5. No somites or heart, generally delayed.	(Lawlor et al., 2002)
Akt1$^{-/-}$	NA	Viable and fertile. Growth retardation.	(Chen et al., 2001)
Akt1$^{-/-}$, *Akt1/2*$^{-/-}$	NA	Perinatal lethal. Dwarfism, skin, muscle, and bone defects.	(Peng et al., 2003)
Akt2$^{-/-}$, *Akt3*$^{-/-}$, *Akt2/3*$^{-/-}$	NA	Viable and fertile.	(Dummler et al., 2006)
Akt1/3$^{-/-}$	NA	Lethal between E11-E12. Morphologically normal at E10.5.	(Z. Z. Yang et al., 2005)
Mkl2$^{-/-}$ (also regulated by other pathways)	Cardiac OFT smooth muscle defect.	Perinatal lethal.	(Li et al., 2005)
Rac1$^{flox/flox}$; *Wnt1-Cre*	Cleft lip and palate, PTA, ectodermal detachment, reduced proliferation. Possible differentiation defects. Reduced DRG.	Lethal between E12-E13.5.	(Fuchs et al., 2009; Thomas et al., 2010)
Cdc42$^{flox/flox}$; *Wnt1-Cre*	Cleft lip and palate, shortened snout, PTA, VSD, possible migration defects, reduced proliferation, small DRG.	Lethal between E14.5- birth.	(Fuchs et al., 2009; Liu et al., 2013)
Tg(Rock^{DN}*); Wnt1-Cre*	Facial cleft, hypoplastic PAs, shortened snout, OFT defects, cytoskeletal disorganization, increased apoptosis.	Lethality between midgestation and E18.5.	(Phillips et al., 2012)