Modeling of the Mitogen-activated Protein Kinase Pathway

Diplomarbeit
zur Erlangung des akademischen Grades
“Magister rerum naturalium”

aus der Studienrichtung Mikrobiologie
durchgeführt unter der Leitung von
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Wien, im Juni 2005

Zusammenfassung

Die Mitogen-aktivierte Protein (MAP) Kinase Kaskade ist ein weit verbreitetes Modul in zellulären Signaltransduktions-Netzwerken von Eukaryoten. Sie besteht aus zwei Serin/Threonin Kinasen, gekoppelt mit einer dazwischengeschalteten Threonin/Tyrosin Kinase und hat evolutionär konservierte Funktionen in osmotischer und Zellzyklus- Regulation. Inhärente Eigenschaften der Kaskade — ihre verstärkende, ultrasensitive und bistabile Antwort auf aktivierende Signale — wurden mittels mathematischer Methoden aus der Enzymkinetik studiert. Diese Arbeit verfolg zwei komplementäre Ansätze, um solche theoretischen Modelle weiter auszuarbeiten und ihre Ergebnisse zu interpretieren:
(1) Ein *konzeptioneller Review* im Sinne von Blagosklonny und Pardee (2002) folgt der Integration der Raf/MEK/ERK Kaskade in globale Signaltransduktions Netzwerke. Eine graph-basierte Beschreibungs-Sprache (*bioLog*) wurde entwickelt, um experimentelle Beobachtungen aufzuzeichnen und um die Kluft von statischen und rein graphischen Interaktionsdiagrammen zu standardisierten Formaten zur Beschreibung biochemischer Reaktionsnetzwerke, z.B. der Systems Biology Markup Language (SBML), zu überbrücken. SBML Modelle werden eine eingehendere Analyse der komplexen dynamischen Eigenschaften erlauben, die in Reaktionsnetzkwerken mit Rückkopplung entstehen können: v.a. Oszillationen und Multistabilität.
(2) Die *SBML ODE Solver Library* ist eine Programmbibliothek, die Methoden der numerischen Analyse solcher Reaktionsnetzwerke implementiert. Sie soll Teil einer Serie solcher Bibliotheken werden, die wohlbegründete theoretische Methoden für eine Integration in komplexere Analyse Programme und systembiologische Applikationen anbieten.

Die Ras/Raf Schnittstelle bietet die meiste Information über die globale Vernetzung der Kaskade, während die Modulierung vorgeschalteter Komponenten durch ERK Rückkopplungsschleifen schließt, die die finale Reaktion einer individuellen Zelle definieren. Einige interessante Kandidaten solcher Rückkopplungsmodule konnten in dem konzeptionellen Review identifiziert und zu vier (Gruppen von) Hypothesen zusammengefasst werden. Parallele — oft oszillierende — Kalzium Signale und deren Rückkopplungsinteraktionen mit der Kaskade wurden als das vielversprechenste System für weitere theoretische Analysen

identifiziert.
Während solche Interaktionen meist stark von spezies– und zelltyp–spezifischen Expressionsmustern abhängen, lädt Kalzium als bivalentes Kation zu einem allgemeinen biophysikalischen Vergleich mit Protein Phosphorylierungen ein, wobei letztere als eine Immobilisierung negativer Ladungen von ATP auf makromolekulare Multi-Protein Komplexe — oft an bereits negativ geladenen Strukturen wie Membranen oder Actin Filamenten, interpretiert werden können. Diese biophysikalische Perspektive erlaubt, die duale Rolle von MAP Kinase Kaskaden in osmotischer und Zellzyklus-Regulation auf ein Prinzip zurückzufürhen, das auf einem Konzept lokaler osmotischer Regulation basieren könnte, die globale morphologische Übergänge während Zellmigration, –wachstum und –teilung integriert. In diesem Sinne, werden einige Szenarien des evolutionären Ursprungs der Kaskade in den Anfängen eukaryotischen Lebens vorgeschlagen. In Fortführung dieses Konzepts wird eine komplementäre Rolle von Kalzium und Protein Phosphorylierungskaskaden in integrativer Koordination von morphogenetischen Wachstumsprozessen am Beispiel des wirbeltier- spezifischen Prozesses der Somitogenese analysiert.

Solche biophysikalischen Überlegungen könnten im weiteren anhand der exisitierenden Konzepte der ‘elektrischen Dimension’ von Zellmembransystemen und der Gel-Sol Phasenübergänge von Polyelektrolyten zu einer *allgemeinen Theorie der zellulären Signaltransduktion* ausgearbeitet werden, die den Weg der Systembiologie zu einem integrierten Verständnis des Lebens auf molekularer Ebene wesentlich vereinfachen köennte.

Abstract

The Mitogen-activated protein (MAP) kinase cascade is a widely used module in cellular signaling networks of eukaryotes. It consists of two serine/threonine kinases coupled by an intermediate threonine/tyrosine kinase and has conserved roles in osmotic and cell cycle regulation. Inherent properties of this cascade — its amplifying, ultrasensitive and bistable response to activating signals — have been studied by mathematical methods of enzyme kinetics. Here, two complementary approaches have been followed to further elaborate such theoretical models and interpret their results:
(1) A *conceptual review* follows the integration of the Raf/MEK/ERK cascade into global cellular signaling networks. A graph-based description language (*bioLog*) was developed to keep track of experimental insights and to bridge from static and purely graphical interaction diagrams to standardized formats for describing basic biochemical reaction networks, such as the Systems Biology Markup Language (SBML). SBML models will allow further analysis of the complex dynamic properties that can arise in reaction networks with feedback, namely oscillations and multi-stability.
(2) The *SBML ODE Solver Library* is a tool for numerical analysis of such reaction networks. It is meant to become part of a series of tools that provide well-founded theoretical methods for easy integration into higher-level analysis tools and systems biological applications.

The Ras/Raf interface provides most information on the cascade's global wiring, while ERK modulation of upstream components closes feedback cycles that will ultimately define an individual cell's response. Several interesting candidates of such feedback modules have been identified in the conceptual review and could be condensed into 4 (sets of) distinct but interrelated hypotheses. The most promising candidate for further theoretical studies was found in the observed feedback interactions with parallel — often oscillatory — calcium signaling. While such detailed observations depend on species– and cell-type–specific expression patterns, calcium's nature as a bivalent cation inspires a general comparison with large-scale phosphorylations, interpreted as an immobilization of negative charges from ATP to large multi-protein complexes, often at already negatively charged structures such as membranes and actin filaments. From such a biophysical perspective, the

conserved dual role of MAP kinases in both osmotic and cell cycle regulation can be reconciled based on a concept of specific local osmotic regulation events, integrating global morphological transitions during migration, cell growth and cytokinesis. Some possible scenarios for the origin of the cascade at the roots of eukaryotic life are proposed. As a proof of concept, the putatively complementary roles of calcium and phosphorylation cascades in morphological coordination are carried forward to multi-cellular interactions and known signaling mechanisms in vertebrate somitogenesis.

Such biophysical considerations could be handled by existing concepts of the 'electrical dimension' of cellular membrane systems and of gel-sol phase-transitions of polyelectrolytes, and are proposed here as cornerstones of a yet to define *general theory of cellular signaling*. Such a theoretical framework could greatly support the systems biological goal of a system-level understanding of life on a molecular basis.

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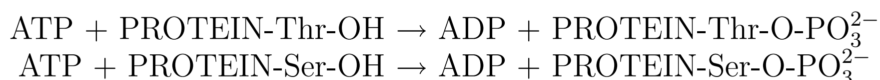
1 Introduction

1.1 The MAPK Pathway

Mitogen-activated protein kinases (MAPK[1]) are a large family of eukaryotic enzymes catalyzing the protein phosphorylation reactions:



$$\text{ATP} + \text{PROTEIN-Thr-OH} \rightarrow \text{ADP} + \text{PROTEIN-Thr-O-PO}_3^{2-}$$
$$\text{ATP} + \text{PROTEIN-Ser-OH} \rightarrow \text{ADP} + \text{PROTEIN-Ser-O-PO}_3^{2-}$$

i.e. transferring the γ-phosphate group (PO_4^{2-}) from adenosine-triphosphate (ATP) to the hydroxyl group of a serine (Ser, S) or a threonine (Thr, T) residue, embedded within specific structural motifs on the substrate protein. The enzymes thus belong to the family of serine/threonine protein kinases. Initially discovered in 1986 as an insulin stimulated serine/threonine kinase that phosphorylates the microtubuli-associated protein 2 (MAP-2) and related muscle proteins [454, 383], the kinase was soon found to be activated by a much broader range of mitogenic factors (e.g. [158]) and phosphorylates many other substrate proteins. The acronym MAPK could be kept, but it further stood for mitogen-activated protein kinase. The first identified MAPK is now known as the extracellular signal regulated kinase (ERK).

At this time protein phosphorylation has already been recognized as a widely used post-translational modification of enzyme activity [76]. The negative charge of the phosphate group, covalently attached to the hydroxyl of a serine, a threonine or a tyrosine (Tyr, Y), alters protein structure and function. This negative charge can often be mimicked by replacing the phosphorylated amino acid by aspartate (D) or glutamate (E), which are negatively charged themselves. And indeed, such mutations in both directions are common evolutionary and pathogenic transitions that render some protein's function sensitive (D/E → T/Y/S) or insensitive (T/Y/S → D/E) to regulation by a kinase.

It was soon found that the MAPK's activity itself is regulated by phosphorylation [384, 385]. The first recognized activating kinase was the MAPK/ERK kinase (MEK) [86]. A MAPK protein's catalytic activity is substantially increased upon dual phosphorylation at a Y and a T

[1]see section 7 for a list of used abbreviations and TLAs (three letter acronyms)

residue embedded in a TXY consensus motif, and subsequent dimerization. The MAPK kinases (herein called M2K) that catalyze these reactions are themselves activated by dual phosphorylation, this time again at a serine and a threonine residue. Enzymes that catalyze this latter step are called MAPK kinase kinases (M3K). This three-tiered cascade of dual protein phosphorylations is known as the 'MAP kinase cascade' or more general 'MAP kinase pathway'. See figure 1 for an abstracted illustration of the cascade. The figure omits however, that M2K's and MAPK's activities are counteracted by specific phosphatases, and the actual activation level highly depends on the concentration (expression level and activity) of the specific phosphatases.

The dually phosphorylated and dimerized MAPK phosphorylate various target proteins including other kinases - e.g. the ribosomal S6 kinases (Rsk) and other MAPK activated protein kinases (MAPKAP kinases or short MK) - thereby adding a fourth layer to the 'three-tiered kinase cascade'. Other important targets include cytoskeleton and metabolic proteins. Active MAPK is also translocated to the nucleus, where it specifically binds to and phosphorylates long-known MAP kinase targets like the transcription factors Fos, Jun, Myc, Elk-1, Egr-1, or chromatin -modifying integrators of diverse signals like the p300/CBP complex. The phosphorylated transcription factors induce the transcription of different sets of genes, among them the very same transcription factors that are substrates of ERK phosphorylation (Fos, Jun, etc.) [336, 335], feedback inhibitors of the pathway [31, 142, 485] or cell cycle genes [400, 375] (chapter 2.2.2). The observed modulation of gene expression by MAPK also includes phosphorylation of splicing factors [302], regulation of RNA transcript stability [533, 98, 349] and activation of DNA methyl-transferase expression [353], thus also reaching into the emerging world of RNA based regulation networks.

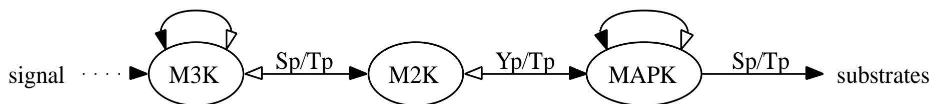


Fig. 1. The Mitogen-activated protein kinase cascade, in an abstracted semi-defined graph drawing, such as used throughout this work. Arrows usually relate to signal transmitting biochemical reactions. Section 3.1 '*bioLog* diagrams' of the methods chapter will give a short introduction, and the figures 31 and 32 introduce rough definitions of node and edge labels. The edge and node meanings in this figure are explained there in detail.

Networking and cross-talking: The term 'MAPK pathway' now subsumes a widely used module in eukaryotic signal sensing and cellular regulation that is embedded in various functional contexts. A 1999 review by Widmann reported 5 known MAPK cascades in yeast, 2 of which amplify 'stress signals', e.g. to counteract hyper- or hypo-osmolarity. The others are involved in regulation of invasive growth, cell wall remodeling and the haploid mating factor signal transduction [531]. The review listed 14 M3K, 7 M2K, and 12 MAPK identified in mammals, which can be further differentiated into 4 or 5 cascade families, culminating in ERK1/2, JNK and SAPK, p38MAPK or ERK5 activation, respectively. In mammals the p38 MAPK [301] and the JNK/SAPK (c-Jun N-terminal kinase / stress-activated protein kinase) are generally involved in responses to stress conditions (UV radiation, osmolarity, pH) and can induce apoptosis or cell cycle arrest. Raf/MEK/ERK signaling is often implicated in developmental and physiological contexts, mediating survival, mitogenic and chemotactic signals. Early interpretations of cell cycle regulation by external signals suggested such a clear distinction between proliferation (cell cycle), growth arrest (differentiation) and controlled cell death (apoptosis), with external signaling factors either acting mitogenic or anti-mitogenic through distinct linear pathways. While it is convenient to reduce the scenario to this conceptual classification for a rough interpretation of the many involved phenomena, experimental evidence now draws a much more complicated picture [34]:

Many signals have a clear specificity for one (or more) of the cascade families, but tend to also activate diverse other MAP kinases, although with often little efficiency [182]. The specificity of a certain MAPK signal is achieved through docking domains on substrates, and through scaffold proteins that sequester the kinases and substrates into larger 'signaling particles' (e.g. [326, 184, 286]), mediating transport and subcellular localization [60, 359]. The actual interpretation of a signal then highly depends on the cell's state, encoded in the expressed subset of signal mediating proteins, and activation of cross-talking signal transducing pathways, which mediate temporally and spatially diverse dynamics of signal relay [484, 297, 60, 34, 359, 51, 52]. Integration of distinct pathways occurs on many levels, from receptor activation, signal transducing protein complexes and second messengers, to complex multiple regulation of transcription factors [174, 530, 453, 449].

1.2 From Literature to Mathematical Models

It has recently often been stated, that our understanding of cellular signaling networks and their functional dynamics has grown too complex to be handled by purely conceptual (narrative) models but will require computational approaches. There is, however, a significant gap between theoretical and experimental methodology and knowledge. While experimental cell biology is interested in the very details of molecular interactions, mathematical approaches to model signaling pathways and networks are often interested in the most simple abstraction of the process that can account for observed dynamic behaviors. Here, two complementary approaches to the topic 'Modeling of the MAP kinase pathway' have been followed.

1.2.1 Conceptual Models

The first part of this thesis is dedicated to the vast experimental knowledge and conceptual models of the current understanding of the functional protein interactions of the vertebrate Raf/MEK/ERK cascade. *Conceptual Biology* has been proposed as a new job description. The conceptual biologist, according to Blagosklonny and Pardee 2002, is required to search existing literature for tested, known and suspected interactions, and deduce functional modules from the various experimental data [35]. Here, a pragmatic approach is adopted to catch such functional modules in a weakly defined diagrammatic notation, resembling the interaction sketches in cell biological and medical literature. As the graph notation used here employs the 'dot language' of the graphviz graph layout algorithm package [143], a definition of these diagrams towards better defined lower-level description standards, such as SBML (see below), is approached in a hands-on experimental adventure in conceptual biology, explained in section 3.1 *bioLog* diagrams.

The MAP kinase module and specifically its implementation in the vertebrate Raf/MEK/ERK cascade serve as a starting point to explore the current knowledge and to take a random and highly subjective walk on the graph of its published causal and correlative interactions, its spatio-temporal relationships and its biological functions. Several early and recent insights into the structure and function of the Raf/MEK/ERK pathway, as activated by receptor tyrosine kinases (RTK, section 2.1.1

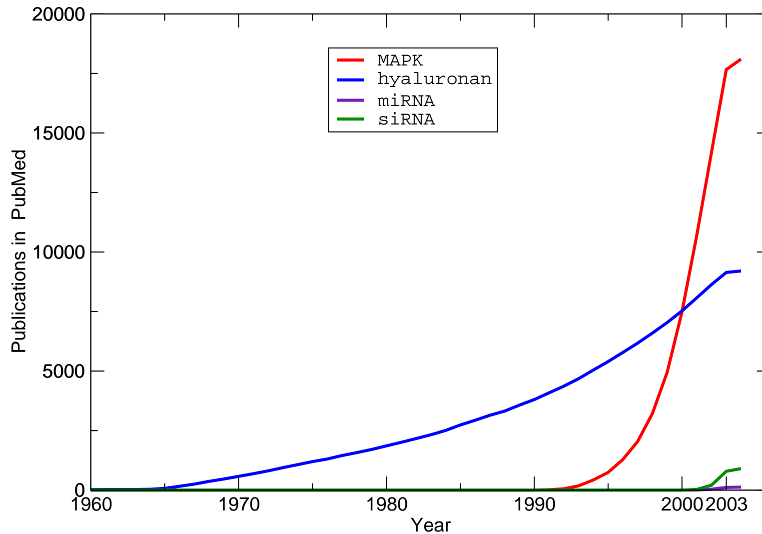


Fig. 2. Literature appearance of the MeSH terms for the 'Mitogen-activated protein kinase', compared to 'Hyaluronan' and 'siRNA' and 'miRNA', until February 2004

'Activation at RTK') and employed specifically in cell-cycle (chapter 2.2 'Spatio-temporal Regulation and Cell Cycle') and migratory (chapter 2.3 'Spatial Regulation and Cell Motility') signaling contexts, will be reviewed. The PC12 cell model is used to illustrate differential spatio-temporal control of the Raf/MEK/ERK cascade, as well as some experimental and molecular details.

While the chapters are written as mere reviews, they shall actually serve to outline questions and perspectives for further mathematical models. The *bioLog* diagrams follow a loose definition, and, while often also depicting molecular or experimental details and multi-level interactions, they are supposed to represent sketches for temporal logic descriptions that can later be converted to detailed quantitative models [469, 470]. The only (almost) strict convention of the diagrams is their consistency with regard to an abstract notion of a 'signal flow'.

Negative interactions can be counted along the path of ‘signal flow’: even numbers of negative interactions will cancel out and result in a positive action of the source of the path on the sink, while odd numbers indicate an overall negative influence (see figure 3).

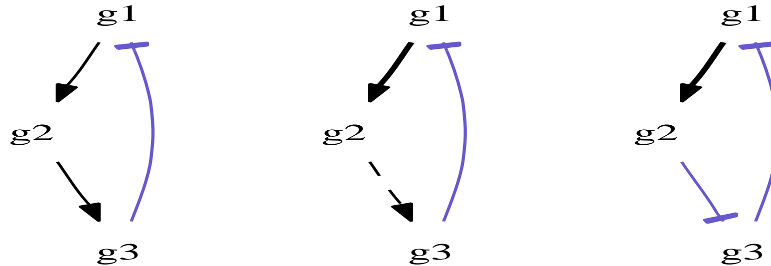


Fig. 3. Left: A negative feedback cycle can lead to adaptation or temporal confinement. Middle: a negative feedback cycle with delay (dashed arrow, e.g. transcription) can cause oscillations. Right: a double negative or any other positive feedback cycle can have multiple stable steady states, if at least one positive interaction is amplifying [470, 129, 490].

Eleven models will be proposed that are based in part on one or more *bioLog* graphs and could be condensed into four groups (I-IV, see below). Each can be studied by further theoretical modeling, and should then be tested and refined experimentally. Invidual models are hypotheses on their own, but their relations (see chapter 5) try to approach a ‘big picture’ that might emerge from ‘the sea of biological data’ [366] provided by the prolific last decades of molecular biology.

1.2.2 Mathematical Models

Interwoven feedback cycles can lead to complex dynamics, often called ‘emergent properties’ (e.g. [30], see [129, 490] for two excellent reviews), that are hard to grasp by qualitative, pure conceptual reasoning. Mathematical modeling is thus often employed to outline possible behaviors of a known process and identify crucial parameters for experimental measurement. Such models are not supposed to represent the process in molecular detail, using ‘real’ reaction parameters, but rather to abstract the process according to the experimental questions. The MAP kinase cascade with its curious dual phosphorylation mechanism soon proved as a grateful system to apply theories of enzyme kinet-

ics to phenomena of cellular signal transduction. From early response curves of MAP kinase activity to increasing concentrations of insulin signal (e.g. Figure 1 in Ray and Sturgill 1987 [383]), the activation could be suspected to show long-known phenomena such as ultrasensitive (all-or-none) response to a signal. Several models studied such properties arising within the cascade [202, 131, 132, 238, 295], while bigger models integrated the MAP kinase pathway into larger signaling networks and explored putative emerging properties of the cascade embedded in positive and negative feedback cycles that lead to bistability [30, 31, 129, 546, 295] or adaptation [45, 13, 31, 411] and — if involving a relatively slow propagation within a cycle — oscillations [238, 288] of the system. These models are discussed throughout the text, wherever appropriate, and in detail in subsection 2.4. Several of the published models have been converted to SBML for this work and are explained in the method subsection 3.2. The SBML files and example simulation runs produced by the SBML ODE Solver (see below) can be browsed at `www.tbi.univie.ac.at/~raim/mapk.html`.

The SBML ODE Solver Library For the majority of the published models of the MAP kinase pathway, a network of individual reactions has been converted into a system of ordinary differential equations (ODE), each describing the time development of the concentration of a participating chemical species (d[S]/dt). While pure analysis of the ODE system yields much insight, the system of ODEs must often be integrated numerically to study the exact dynamics. The SBML ODE Solver Library (SOSlib) is a simple command-line tool and programming library, that has been developed for exactly this purpose [287]. It is based on SBML (Systems Biology Markup Language), a standard format for exchange of reaction network data [203], the SBML library libSBML and CVODE, a powerful tool for numerical analysis of ODE systems [408].
Chapter 4 provides detailed documentation of program usage and architecture. Please consult the subsection 4.3.1 'Constructing ODEs from Reaction Networks' for an outline of the method, as implemented in the SBML ODE Solver. More detailed descriptions can be found in a variety of textbooks, e.g. in E. O. Voit's 'Computational Analysis of Biochemical Systems' [501]. Subsection 4.3.2 'Integrating ODEs Numerically' describes the implementation of the CVODE ODE solver.

The method is old and many implementations exist. The SOSlib is however designed as a 'library' and its functionality can be accessed by programmers of other tools. The SOSlib is thought to constitute a numerical back-end for high-level applications that open well-founded standards of mathematical biology for a non-expert use by biologists. Extensions of the solver library, including parallel tools at the same level, could provide a framework for dynamic systems analysis. Most interesting for developers are probably its straight-forward implementations of formula evaluation and symbolic differentiation, but also an interface function that allows to couple model simulation to external data, e.g. measured time-courses or input from multi-scale models.

The SOSlib is distributed under the free LGPL license, and users are invited to participate in further development. Amongst others, the CellDesigner project (`www.celldesigner.org`, [141]) as well as the private company Physiomics PLC (`www.physiomics-plc.com`) are currently implementing the library for their purposes and are actively contributing to its further development. At our working group the CelloS [14] and the MiniCellSim projects employ the library to study evolution of gene regulatory network dynamics. The library's analytical functionalities contribute to the implementation of sophisticated parameter identification methods, developed in collaboration with a group at the RICAM institute (`www.ricam.oeaw.ac.at`).

bioLog::SBML::SOSlib A common protocol for modeling the dynamics of biochemical and gene regulatory systems [150] would start off with an outline of the basic interactions of the system's components and then derive concrete reaction networks, including measured or estimated reaction parameters. The *bioLog* diagrams represent a hands-on 'standardization' to keep track of the most relevant among the diverse observed signaling modules that could later be translated into such detailed reaction network models. SBML introduces a standard description for such models [203, 133], while the SOSlib implements one of several possible second steps: the conversion of an SBML model into a system of ODEs. Numerical integration of the time-course of the system's components is often required for the third step, the identification of stable steady states of the system, while a fourth step would analyze the stability of stable steady states (therefore known as 'stability analysis'). Perturbations away from a stable state can ei-

ther drive the systems towards another stable steady state, or result in oscillations around a so-called instable steady state. A fifth step, 'bifurcation analysis', would then identify the domains in parameter space (the ranges of parameter values) that can result in the occurrence of multiple steady states or oscillations. Please see the recent reviews by Goldbeter ('Computational approaches to cellular rhythms', 2002 [150]), Ferrell ('Self-perpetuating states in signal transduction: positive feedback, double-negative feedback and bistability', 2002 [129]) and Tyson et.al. ('Sniffers, buzzers, toggles and blinkers: dynamics of regulatory and signaling pathways in the cell.', 2003 [490]) for short overviews of methods and applications of this process. The sixth step of this protocol closes the circle: refined experiments can ask whether the observed dynamics of the theoretical system can be approved or whether the theoretical model itself has to be refined or extended. The *conceptual review* is occupied with this last step of the protocol.

Computational models need not be detailed representations of reality, but rather resemble dynamic versions of the sketches and drawings of relevant interactions and signal flow, frequently found in literature. If cell-biological understanding of signal transduction now transgresses from linear pathway models to networks and from static models of cellular function to a more dynamic view of integrated cellular coordination, such dynamic models will certainly become indispensable not only for theoretical analysis, but also for **experiment design**. In a formalization of above protocol, the *bioLog* diagrams could represent specific multi-scale temporal logic descriptions. XML and RDF technologies of *semantic web* and data integration approaches could employ existing biological ontologies or controlled vocabularies to integrate weakly defined interaction diagrams or well-defined standards such as SBML with sequence/structure databases and with experimental data [512]. The *conceptual review* could be transferred to an online content managment system, similar to common *wiki* or *weblog* approaches, where both text and interaction models could be collaboratively developed by a scalable group of researchers and laboratories. Such an 'integrative biology' will enable the representation of our knowledge in searchable databases of dynamic models mapped to sequence data, which can also be considered as a **genotype/phenotype mapping** that might allow a comparative phylogenetics of pathway dynamics.

1.3 Summary of Proposed Models I-IV

Rhythms ... While theory clearly requires experimental observations, what can theory do for every-day experimental work, except for the currently hip narrations in mathematical and computer science speech ('systems biology')? One of the most fundamental manifestations of biological life are rhythmic phenomena. The cell cycle and circadian rhythms, cellular migration or intra- and intercellular calcium waves: many of the fundamental biological processes can be viewed as oscillations [150, 490], while cellular differentiation, or stable morphological and physiological states of an organism can be interpreted as (transitions between multiple) stable steady states of a given system [470, 129]. Several known oscillators are based on negative feedback by transcriptional repression in transcription factor systems [150, 90, 489, 85], but also some purely biochemical oscillators such as glycolysis [465], pseudopod formation and focal adhesion turnover during migration [497] or calcium oscillations [29] are now understood in fascinating but still quite incomplete molecular details. The MAP kinase pathways in general and specifically the Raf/MEK/ERK implementation, feed into and interact with many oscillatory processes, either driving them or freezing them into a stable state. Recently, it has e.g. been speculated that frequency and amplitude of calcium oscillations are optimized for efficient activation of the small G protein Ras and the Raf/MEK/ERK cascade [504, 261]. Sequential activation/inhibition cycles of Ras/Raf by calcium at the plasma membrane and at Golgi locations might also be involved in the PC12 cell model system for spatio-temporal coordination of ERK activity [33]. The vertebrate c-Raf protein is itself known to undergo complex cycles of activation, de-activation and re-direction to alternative sites of action [19]. Especially for such **rhythmic aspects** — most fundamental to biology — purely conceptual reasoning will not suffice to capture all possible dynamics.

(I, 1-4) A comprehensive model of NGF signaling in PC12 cells could integrate sustained ERK signaling via internalized and transported endosomes [559, 232] with morphological changes and metabolic requirements of neurogenesis. Sustained ERK activation depends on a cooperation of c-Raf and B-Raf and their differential activation by Ras and Rap1 [560, 451]. These two G proteins also relate directly to receptor internalization and endosomal trafficking (vesicle transport), to calcium

[33, 399] and to cAMP [453, 350], long known players in cytoskeletal remodeling [369, 420]. Vesicle transport along microtubuli, with its random dissociation and pathway changes [425], could be described by reaction kinetics in 'restricted dimensions' [27, 171]. Neurite outgrowth employs the cellular migration machinery, uncoupling protrusion of the leading edge from retraction of the uropod. The underlying interwoven and sequentially activated bistable lipid/G protein/kinase modules that define spreading [124], filo- and pseudopod formation [497], polarization [521], focal adhesion turnover [325], and uropod retraction are well studied independently and could be integrated to account for migration in detail. A membrane bound pair of an adenylate kinase (AK1β) and an AMP-sensitive protein kinase (AMPK) could sense sustained phosphorylations to drive local metabolic activity required for neurite outgrowth (subsection 2.4.3). This potentially old sensor module for metabolic state offers a nice perspective on general evolutionary aspects of intracellular signaling as an extension to basic metabolic, morphology and cell cycle coordination.

Multi-scale I: some evolutionary aspects Localized formation and dynamics of macromolecular multi-protein complexes — we will e.g. meet several proteins that function both as scaffold proteins and as protein kinases that modulate complex nucleation — is a fundamental mechanism of cellular signaling. These complexes depend on specific protein-protein interactions and are thus subject of evolutionary change, resulting in species– and cell-type–specificity and an impressive diversification of basic principles in complex organisms.

(II, 5) The vertebrate triplicates of a single invertebrate Raf protein [314] inspire speculations on evolutionary transitions by duplication of dimeric kinases, see subsections 2.1.2 and 2.3.3. B-Raf and c-Raf have been observed to cooperate, potentially by direct interactions as heterodimers [519, 326, 483]. c-Raf has in several cases been shown to inhibit other serine/threonine kinases by acting as a scaffold, independent of its kinase activity [19]. Here, such observations are elaborated into the second hypothesis about possible evolutionary origins of phosphorylation cascades, which could be suspected in an initial duplication of an osmotic pressure and growth regulating kinase/scaffold pair at the roots of eukaryotic life. A biophysical interpretation of ultrasensitively amplified phosphorylations further supports such scenarios:

... 'n Blues: some physico-chemical aspects While several important questions can not be captured within the frameworks of current theoretical approaches, they can give important clues for a more general interpretation of specific signaling modules. The vertebrate specific extracellular polysaccharide hyaluronan (HA) and its ancestry in the invertebrate chitin polymer of the ECM will serve to explore some biophysical aspects of developmental evolution and cellular function/structure relationships. Can phosphorylations and other widely used post-translational modifications, lipid and calcium signaling, be interpreted from a general biophysical perspective (subsection 2.3.3)?

RTK initiated phosphorylation events and complex formation can be considered as an immobilization of negative charges from soluble ATP to multi-protein complexes, often at already negatively charged macromolecular structures such as membranes and the actin cortex. Immobile negative charges are usually counteracted by positively charged ions — the so-called Gouy-Chapman cloud. The various potential differences at membrane systems have been described as an 'electrical dimension' of cells [95, 358, 304, 306]. While calcium oscillations, both intra- and intercellular, are a long-known phenomenon, the mechanics now become understood in molecular detail [29] and are accessible to theoretical studies [150]. Their occurrence over multiple spatial and temporal scales invites for interpretation as a globally integrative signal, e.g. in embryonic development [518]. The molecular read-out of frequency and amplitude is however only understood in fractions. As RTK usually activate both, ERK mediated large-scale protein phosphorylation waves and an often oscillatory increase in intracellular calcium concentration, they might have a complementary function with respect to modulation of the electrical dimension of cells.

(III, 6-9) Intracellular calcium oscillations have been observed to guide sequential activation and inhibition of Ras/Raf activity at subcellular locations [33] and ERK can feed back to local calcium release, at least in human platelets [403]. While these interactions depend on cell-type specific expression patterns, a biophysical perspective allows a very general interpretation. Protein phosphorylations can be imagined to cause additional inflow of solvent, and thus a kind of swelling of local structures. Oscillatory calcium signaling can in this sense be interpreted as a complementary mechanism, providing counter-ions that could neutralize charges and replace hydration layers, leading to contraction.

Such a general cooperativity might also be one function of an intracellular calcium gradient during migration, with a higher concentration at the rear end[170], where it is involved in myosin II based uropod detachment and retraction [120, 113] but also in focal adhesion turnover [145]. Such aspects can be handled by the concepts of the 'electrical dimension' of cellular membrane and cytoskeleton systems and of gel-sol phase transitions of negatively charged polymers [371]. The specific process of RTK and Raf activation by (Y) phosphorylations and complex formation and subsequent re-localization by additional feedback phosphorylations via ERK (at S/T) could serve as a first example to test such hypotheses theoretically. A specific experimental setup will be proposed in the discussion (chapter 5).

Multi-scale II: Development Experimental work often covers phenomena that span the range from atomic scales to organismic function in development and physiology (pathology) or even ecological interactions. While mathematical models exist on all levels – from multicellular developmental or physiological function of organs to the intracellular signaling networks that guide these processes, and finally to the tertiary structures of the involved proteins — so far only a hand-full of approaches exist, that integrate several scales (e.g. [342, 94, 315]).

(IV, 10-11) Vertebrate somitogenesis allows to interpret above hypotheses on general cellular biophysics within a specific developmental context. A posteriorly moving gradient of FGF8 defines a discrete 'determination front', that freezes the temporal pattern of the 'somitogenesis clock' into the spatial pattern of somites [83, 115]. This clock is defined by oscillatory expression of Hes transcription factors, coupled between neighboring cells by oscillatory activation of the Notch cell-cell signaling pathway [91]. The ultrasensitive and feedback modulated [131, 269, 485] signal response of the cascade could support the discrete front within the FGF8 gradient, while above proposed role of calcium could be repeated on a multi-cellular scale: long known embryonic calcium waves [147] can not only enforce cell-cell adhesion via calcium dependent cadherins [517, 459, 505], but in this case also tune the somitogenesis clock by its recently clarified role in Notch signaling [386]. A combination of the CelloS [14] and SOSlib tools for spatial representation of cell growth and movement and of intracellular coordination networks, respectively, could test these hypotheses theoretically.

As a conclusion, the electrical dimension of the cell and gel-sol phase transitions will be proposed as important cornerstones of a general theory of cellular signaling. Specific protein-protein interactions coordinate such phenomena with the oscillators of cell cycle, metabolism and migration. Cellular signaling may be based on a seizable set of biochemical and biophysical principles that can account for morphological and metabolic requirements, and are coordinated by sequence specific protein and nucleotide based reaction networks on which evolution can act to achieve the astonishing diversity of cell function seen in complex organisms such as mammals.

Such 'big pictures' can however only be drawn collaboratively. The SOSlib and maybe a formalization of the *bioLog* diagrams might hopefully contribute to integrative models and web-based tools, that would allow such a collective hypothesis generation and organized experiment design. It will require some sort of standard notation of cell-biological entities — recently compared to musical notations [135] — at higher levels than the reaction networks in SBML. Walter Kohn has first outlined possible diagrammatic conventions to depict protein-based cellular coordination networks [251, 252, 7] and this approach has been elaborated by Kitano et.al. [247] who work on an implementation of this notation in the CellDesigner [141]. Science's Signal Transduction Knowledge Environment (STKE) and the Nature Signaling Update — a collaboration of the Alliance for Cellular Signaling and Nature Publishing group — use abstract diagrams similar to the *bioLog* approach. Biocarta features several different pathways in specific cellular contexts, while the ROSPath database concentrates on ROS signaling [361]. The SigPath [55] and `www.biomodels.net` databases feature quantitative reaction network models, representable in SBML. The RTK consortium e.g. provides a large-scale interaction map of EGFR signaling, that has been created with the CellDesigner [351] and could thus be extended by the c-Raf cycle as presented in fig. 41. This work also approaches a complementary aspect, namely formalized and interlinked narrative text description (see fig. 45). Wiki or weblog like systems (see e.g.`http://en.wikipedia.org/wiki/MAPK`) could have a notion of biological categories and provide easy means for interlinking text descriptions with model and structure databases. One obvious use would be an educational application as dynamic online tutorials, such as approached for the Wnt pathway at `www.scivis.org/WNT`.

2 Models of the MAP Kinase Pathway (Results I)

2.1 The Raf/MEK/ERK Pathway

2.1.1 Activation at Receptor Tyrosine Kinases

The vertebrate Raf/MEK/ERK pathway is one of the best studied instance of the known MAP kinase pathways. The graph in fig. 4 depicts the classical and widespread understanding of typical receptor tyrosine kinase (RTK) mediated activation of this cascade, as known e.g. from epidermal growth factor (EGF) and its receptor EGFR, and many other RTK systems (PDGFR, FGFR, NGF:TrkA, VEGF:KDR, etc.). Extracellular ligands, often occurring as dimers and complexed with glucosaminoglycans of the extracellular matrix (e.g. heparin), bind to their receptors, and potential co-receptors, and lead to receptor dimerization and subsequent oligomerization, or clustering. The receptors carry a cytoplasmic kinase domain, and trans-phosphorylate multiple tyrosine residues on adjacent receptors. A range of adapter molecules bind to the phospho-tyrosines via a common Src-kinase homology domain 2 (SH2), many of which are themselves tyrosine-phosphorylated by the RTK. Some of these targets for RTK phosphorylation are involved in modification of membrane inositol phospholipids and cleavage of soluble second messengers. The phospho-inositide 3-kinases (PI3K), consisting of three subunits, bind to the RTK, and subsequently phosphorylate (mainly) phosphatidyl-inositol-4,5-bisphosphate (PI(4,5)P2, or shorter PIP2) at the 3' OH position of the inositol ring, to yield phosphatidyl-inositol-3,4,5-triphosphate (PI(3,4,5)P3), or shorter PIP3). Another family of protein domains, the pleckstrin-homology (PH) domain, binds to such phosphatidyl inositols, and specific PH containing proteins translocate to the membrane upon PI3K activity. The 3-phospho-inositide-dependent kinase 1 (PDK1) phosphorylates the protein kinase B (PKB or Akt, as it will be called herein), and this pathway is considered an anti-apoptotic (survival) branch of RTK signaling. The phospholipase Cγ (PLCγ) binds to and - again by RTK mediated tyrosine phosphorylation - is activated to compete with PI3K for PI(4,5)P2 as substrate, hydrolyzing it to produce the soluble second messenger inositol-1,4,5-triphosphate (IP3) and membrane-bound diacylglycerol (DAG). IP3 activates calcium release from endoplasmatic reticulum stores, and cytoplasmic calcium (intracellular calcium, iCa^{2+}) and DAG converge again on activation of some protein kinase C (PKC) isoforms. Many of these parallel pathways converge again at downstream nodes, and

also directly on activation of the Raf/MEK/ERK cascade. Several examples will appear in this work. However, tyrosine phosphorylation and membrane lipid modification cooperate in forming large (clusters of) macromolecular platforms for downstream signaling into the cell body.

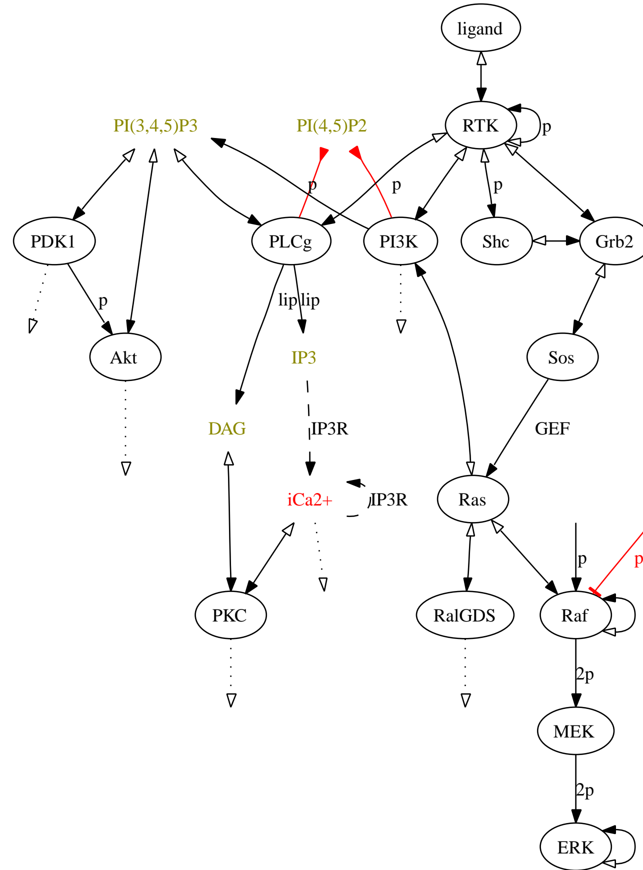


Fig. 4. A simplified picture of a typical receptor tyrosine kinase (RTK), activating the classical Raf/MEK/ERK cascade and parallel Phosphatidyl-inositol-3-kinase (PI3K) and Phospholipase Cγ (PLCγ) second messenger pathways (see text). IP3 is cleaved and binds to IP3 Receptor complexes, which release Calcium from internal stores in the endoplasmatic or the sarcoplasmatic reticulum. Calcium interferes with Ras signaling in several ways (see text), and has global effects on cell function. Lipid signaling interferes with Raf/MEK/ERK activation at several levels. DAG is a membrane lipid and Ras is also bound to the membrane. They are drawn below receptors and other lipids for layout reasons.The following chapters will feature several examples.

Membrane Lipids, Actin Cortex and Cytoskeleton The plasma membrane is organized into subcompartments, defined by their lipid or protein composition, such as cholesterol-rich lipid rafts, or specific protein (clathrin, caveolin) containing domains [364]. At the membrane, receptor complex formation and internalization are thought to be differentially modulated by dynamical (re-) organization of such subdomains. The space underlying the membrane is pervaded by actin filaments and forms the so-called cortical actin layer or actin cortex. Recently, above mentioned PIP2 has been identified as an important mediator of the membrane-cortex-cytoskeleton association, reviewed shortly in Nebl et. al. 2000 [339] and more comprehensively in Sechi and Wehland [428]. PLCγ activation by RTK markedly reduces the PIP2 mediated adhesion of the membrane to the (cortical actin) cytoskeleton [382]. Another branch of research on phosphatidyl inositol functions identifies a large number of diverse ion channels and pumps, involved in intracellular pH regulation and membrane potentials, that can be either positively or negatively regulated by PIP2 [6, 187]. PIP2, produced by a PIP-5-kinase, is e.g. also involved in Arf6 mediated endosomal recycling pathways [48], and such receptor internalization and endosomal transport systems will return later as an important regulator of spatio-temporal control of MAPK (ERK) signaling in the PC12 cell and other model systems. However, lipid signaling and membrane remodeling is certainly crucial for signal relay, and besides above mentioned phospholipases and lipid kinases, there emerges a whole cycle of regulated lipid modifying enzymes, see e.g. references [56, 279, 282], and such a cycle is likely to play evolutionary rather old roles in diverse cell morphological processes, as observed during cytokinesis and migration. Thus, RTK mediated signaling not only mediates specific protein interactions, but also involves local and/or global changes of the chemical environment, and induces e.g. a loosening of the submembrane compartment, to assure accessibility for complex formation and/or prepares for (receptor) internalization processes through endocytosis.

Small G proteins The RTKs start a cascade of several specific phosphorylation events, to initiate phospho-lipid, and phospho-tyrosine mediated formation of large adapter protein complexes. The latter mediate cell and ligand specificity. Figure 4 depicts the specific example of receptor complex formation involving the 'son of sevenless' (SOS) protein, which acts as GTP exchange factor (GEF) for the small guanine nucleotide binding protein (G protein or GTPase) Ras. Small G proteins like Ras act as molecular switches, that are in an 'on' state, when bound to GTP. They hydrolyze

their GTP to GDP, which the enzyme - now in its 'off' state - keeps bound until it can exchange its product by its substrate GTP, again. The latter step is induced by binding of a GEF, while the former step can be induced by GTPase activating proteins (GAP). A large superfamily of more than 170 members is named after Ras (see fig. 5), and their functional and evolutionary relations have recently been reviewed by Colicelli [77]. A broad range of the small G proteins, such as the Ras, Rap, Ral or the Rho and Arf subfamilies, are central parts of positive feedback switches with upstream lipids and downstream kinases, usually involved in remodeling of membrane composition and cytoskeleton during morphological changes of the cell during cytokinesis, cell migration or epithelialization. Notably, many proteins of the Ras (super)family interact with each other directly to cooperate in these processes, and several examples for such a cooperation will appear in this work, namely of Ras with Rap1, Ras with Ral and Ral with Rac/Cdc42. The Ran subfamily — implicated in shuttling of proteins and RNAs between nucleus and cytoplasm — and the Arf subfamily — involved in vesicle transport — will not appear further. However, all of these processes and the very same molecules are often directly or indirectly involved in direct or indirect regulation of the Raf/ERK activation.

Ras and PI3K Active Ras itself is of (oncogenic) fame as a major activating factor for the Raf family of M3K (c-Raf, B-Raf and A-Raf), but is also involved in above mentioned activation of PI3K, PLCϵ [443]. Several works report that Ras mediated activation of PI3K is also involved in Ras mediated Raf/ERK activation, depending on conditions and cell type [87, 178, 116, 245, 529]. Inhibitors of PI3K can also inhibit Ras activation [178, 529, 70] and constitutively active PI3K can activate Ras [201], which points to either a positive feedback interaction or side-effects of the inhibitors. Analyzing EGF effects on COS-7 fibroblast cells, Wennström and Downward speculate that PI3K plays a permissive role in Ras activation by low ligand and/or receptor concentrations (that are sufficient for mitogenesis, and more likely to resemble physiologic concentrations, though), while it might not be required at higher concentration of upstream signaling complexes. At intermediate EGF concentrations ERK activation is more sensitive to PI3K inhibition than Ras, indicating PI3K requirement upstream and downstream of Ras activation [529]. Chiloeches et. al. showed that even at high signal/receptor concentrations PI3K inhibitors can suppress Ras signaling, but only at later time points after ligand exposure (i.e. 10 - 60 minutes) [70]. The review of Parton and Hancock [364] on lipid rafts and

membrane micro-organization was cited above, and shall also be mentioned at this place, as therein Ras clustering and localization to such membrane subdomains is analyzed as the best known example for such processes. A complicated relation between PI3K lipid modifications and Ras mediated c-Raf activation is evident. How does this relate with above mentioned Ras clustering in membrane microdomains? Maybe the lack of clustering of low-density (ligand-bound) receptor complexes can be overcome by Ras oligomerizing itself, which again would depend on basal PI3K activity, responsible for appropriate membrane compositions.
The suspected direct pathway from PI3K to c-Raf/ERK activation leads via Rac/Cdc42 small G proteins and their effector PAK. RalGDS is a stimulator for Ral, a Ras like G protein involved in endo- and exocytic trafficking and vesicle fusion [39, 88]. Sequential activations of RalGDS, PI3K and Raf branches, coordinated via negative feedback cycles, might be involved in sequential formation of filo- and pseudopodia [149, 406, 475]. Both topics will be discussed in chapters 2.1.2 and 2.3. Here, a short discussion of recently dissected regulation cycles of Ras shall complete the picture and highlight some potentially important cross-connections.

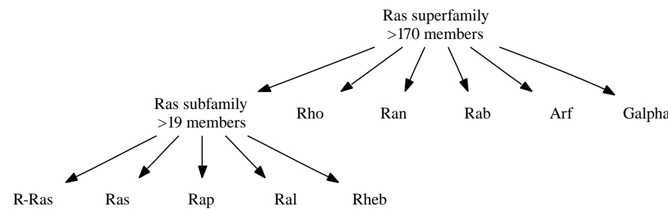


Fig. 5. Sketch of the Ras super- and subfamilies of small G proteins. See the reviews by Takai et.al. 2001 [461] or the more recent by Colicelli 2004 [77]

Ras Cycles As many vertebrate genes, the three Ras ORFs - H-Ras, K-Ras and N-Ras, are probably descendants of a single invertebrate protein, derived through two genome duplications during early vertebrate evolution [77]. While they share common effectors, major differences in sub-cellular localization have been outlined in several studies [71, 33, 105, 367, 396]. H-Ras reaches its peripheral membrane location via the endomembrane system, going through several modification steps. After farnesylation at the fourth C-terminal amino acid the first three are cleaved. Then the protein is methylated, and is finally targeted to the plasma membrane by palmitoy-

lation. The most recent experiments now exemplify, how reversible protein modifications — palmitoylations and farnesylations in this case — regulate differential sub-cellular location. H-Ras and N-Ras have been shown to constantly cycle between between plasma membrane, Golgi and endoplasmatic reticulum systems, while K-Ras is found to be exclusively localized at the plasma membrane [396]. The translocating Ras can bring along its activation status or bind e.g. to a Golgi-located Ras effector Rain [324], and induce locally scaffolded Raf/MEK/ERK signaling cascades (see chapter 2.3) or other effectors at the respective locations [71, 396].

Ras and Calcium As depicted in fig. 6, a differential activation of plasma membrane vs. Golgi located H-Ras can be mediated through Src dependent PLCγ1 activation leading to an increase in intracellular calcium (see fig. 4), which activates the (inhibitory) Ras GAP CAPRI at the plasma membrane, thus terminating initial Ras activation at this site, and subsequently the (activating) Ras GEF RasGRP1 at the Golgi. This process was again shown to be involved in differentiation of the PC12 cells, which reappear frequently in this work, as well as in mechanistically similar but functionally distinct phenomena in T cells and fibroblasts [33]. Recent experimental observations indicate several relations of Ras with receptor/PLC induced calcium oscillations. Cullen and coworkers suppose that above cited CAPRI senses amplitude, while RASAL - another calcium activated Ras GAP - oscillates between membrane and cytosolic sites in synchrony with calcium oscillations, and thus their frequency [503, 504]. They moreover propose that the 'frequencies of calcium oscillations are' even *'optimized for efficient calcium-mediated activation of Ras and the ERK/MAPK cascade'* [261]. The experimental treatment of cells underlying this hypothesis seem somewhat crude. Purely biochemical oscillations are generally hard to detect in cell culture, when no means of synchronization are available. The 2002 review by Cullen [88] outlines the observed details of calcium oscillations' relation with Ras signaling. Intracellular calcium oscillations occur both locally and as global (cell-wide) waves, encoding information both in their frequency and amplitude, which are sensed by many other signaling modules [29], now putatively joined by Ras cycles of Raf/MEK/ERK activation. Moreover, calcium oscillations can be coupled between adjacent cells and produce intercellular waves across a whole embryo in early development [517]. While the mechanisms underlying such oscillations are comparatively well understood, the functions and the mechanistics of readout, i.e. their influence on global signaling and regulatory networks, seem to be diverse

and no general picture has yet emerged. Please consult the review by M.J. Berridge et.al. 2003 [29] for mechanisms of calcium oscillators. Developmental calcium oscillations and waves have been recently discussed by S.E. Webb and A.L. Miller 2000 [517], while A. Goldbeter 2002 [150] provides a great introduction to rhythmic biological phenomena from a theoretical perspective.

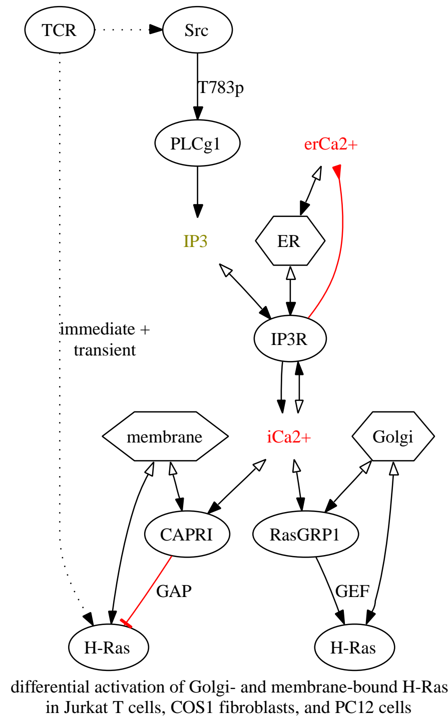


Fig. 6. Bivona et.al 2003 observed that calcium activated Ras GAP CAPRI inactivates H-Ras at the membrane, resulting in an immediate but transient activation, while H-Ras activation at the Golgi by RasGRP1 is responsible for a later phase of H-Ras activity. This has been shown for TCR signaling in Jurkat T cells (shown above), for EGF signaling in COS-1 cells, and for phorbol esther and NGF induced differentiation in PC12 cells [33]. The IP3 receptor (IP3R) is a channel at the ER, that is activated by IP3 to pump calcium from the endoplasmatic reticulum (ER, $erCa^{2+}$), into the cytosol, resulting in a local increase in intracellular calcium (iCa^{2+}) that further activates IP3R (calcium induced calcium release (CICR)), which can account for autonomous iCa^{2+} oscillations [29])).

The short detour into some of the upstream processes in activation of the Raf/MEK/ERK cascade was taken, because several of the mentioned processes will be relevant for the following. First, both the calcium, and the

lipid / cytoskeleton connections are central to the understanding of integration of survival, mitogenic, and migratory actions of the Raf/MEK/ERK cascade. The lipid/cytoskeleton connections relate to more biomechanical aspects of cell function and morphology. Calcium oscillations can by themselves show very complex dynamics [260, 424, 150], and are coupled to a wealth of other oscillatory phenomena. Just naming the cell cycle, oscillatory processes can be considered to be a quite fundamental manifestation of biological life [150], and the Raf/MEK/ERK cascade feeds into several of these systems. Both the mechanical membrane/cytoskeleton connections, as well as (calcium) oscillations are likely to resemble old phenomena, common to or similar in all (eukaryotic) cells. In contrast, sequence specific protein/protein interactions are object to evolutionary change and 'innovation'. Second, the PC12 cell line was shortly introduced, a defined and well studied model system for spatio-temporal control of cellular signaling networks. Complex spatio-temporal dynamics of signaling networks, eventually integrated into fundamental oscillations, are probably the most interesting aspect for quantitative analysis. While at the moment the separate knowledge of signaling modules seems too diverse and fragmentary, an integration to comprehensive models of intracellular functions, integrated into intercellular networks, can be spotted on the horizon. Computational models for mathematical analysis and numerical simulation of time-courses will be invaluable to outline the required *basic architectures* in theory as well as to understand their evolutionary diverse *real life implementations* in experiments. Such models will discussed at several points of this work. But let's follow Ras' triggering of the cascade, first.

2.1.2 c-Raf

Activation of the c-Raf protein (648 amino acids, 72-74 kDa) is a quite complicated mechanism by itself, and besides active Ras several other molecular interactions are required to start the Raf/MEK/ERK cascade via c-Raf. Both, Ras and Raf protein families have been discovered as viral oncogenes and cellular proto-oncogenes, frequently mutated in diverse kinds of cancer. As the pathway is involved in mitogenic and anti-apoptotic signaling, activating mutations can lead to uncontrolled proliferative activity. Thus, a tight control of this step makes sense. As we will see below, B-Raf — due an evolutionary older and simpler activation mechanism — is the main target of oncogenic mutation [314], while both A-Raf and c-Raf have acquired additional other functions [19].

The c-Raf protein cycles between different states of activity and subcellular localizations, that are defined by its phosphorylation state and distinct multi-protein complexes, mediating differential interactions of the three conserved domains of Raf proteins: the N-terminal regulatory domains (CR1 and CR2, conserved regions) and the C-terminal kinase domain (CR3). Figure 7 depicts a rough and unscaled outline of c-Raf's protein domain structure, highlighting specific amino acid residues, and some of the interactions and modifications that are involved in its complex activation and inactivation cycle. Several excellent recent reviews by Kolch [254], Baccarini [18, 19], Dhillon and Kolch [103, 104], Mercer and Pritchard [314], O'Neill and Kolch [359] and by Wellbrock, Karasarides and Marais [528] were helpful guides to primary literature, and the reader is referred to these works, where this work cuts down on potentially crucial molecular details, and (the varying reliability of) experimental methods involved, as well as on cell type and species specificity of results.

Complex formation at the membrane 'Quiescent' c-Raf is phosphorylated (at least) at serines 259 and 621 which both lie within a conserved RSXSXP motif. An early report of Raf regulation by phosphorylation reported 2-fold activation of a S259 mutant, while the S621 mutation resulted in kinase inactivation [331]. At the same time, the 14-3-3 proteins were found to be required for Raf activation [136, 213, 140]. The RSXSXP motif was then revealed to constitute the 14-3-3 binding sites [337, 549]. S621 phosphorylation was proposed to mediate kinase inhibition by cAMP dependent PKA [168], while also being required for kinase activation [321]. This opposing functions is now attributed to the activity of dimeric 14-3-3 proteins [474, 492, 558, 184]. Dimeric 14-3-3 proteins binds phospho-serines at those two, and possibly a third site around serine 233, holding c-Raf in an inactive (quiescent) conformation in the cytoplasm, where the phosphorylated N-terminus inhibits the C-terminal kinase domain. As outlined in subsections 2.1.1 and 2.2.1, RTK induce formation of a tyrosine-phosphorylated adaptor platform that provides GEFs to activate membrane-bound Ras. c-Raf then binds to Ras via its Ras-binding domain (RBD), and to the membrane via a cysteine-rich domain (CRD). It is unclear however, if c-Raf membrane translocation occurs by pure diffusion and sequestration by Ras, or other processes. Recently it was postulated, that an inactive fraction of c-Raf might reside in the plasma membrane and that RTK signaling induces initial phosphorylation at S259 succeeded by S621 phosphorylation and 14-3-3 binding [183, 184]. However, di- or oligomeric Ras induces di- or oligomer-

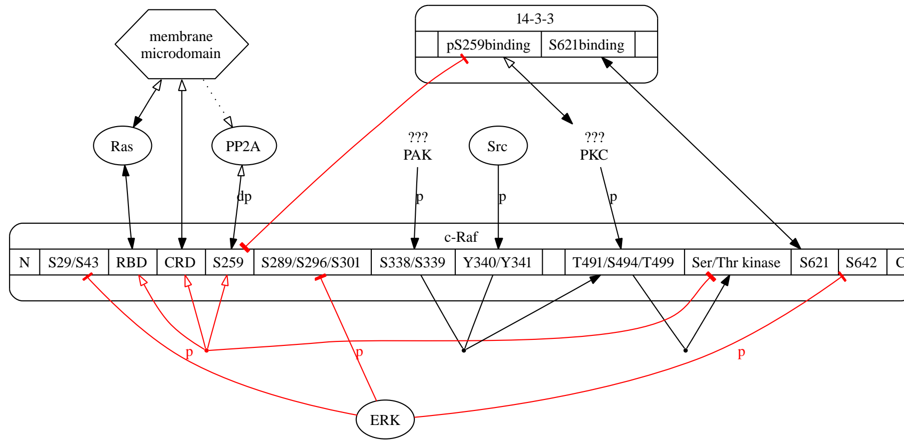


Fig. 7. Simplified sketch of c-Raf activation/inactivation cycles: In resting cells, c-Raf is held in an inactive conformation by 14-3-3 binding to phospho-S621 and phospho-S259. Upon ligand stimulation, c-Raf binds to activated Ras (Ras.GTP) at the membrane, via its Ras binding domain (RBD) and to the membrane directly via a cysteine rich domain (CRD). Ras displaces 14-3-3 from phospho-serine 259 [398], enabling dephosphorylation by PP2A [257], but also inducing rephosphorylation of activated c-Raf [101]. 14-3-3 are dimeric proteins, and c-Raf dimerization is also thought to be crucial for its activation [283, 128]. The free 14-3-3 site might be involved in recruiting S/T and Y kinases. Phosphorylations in the C-terminal region of the kinase domain at S338/S339 by PAK1/3 [244, 63, 456, 564, 482, 8] or unknown other kinases [70] or not at all [352], at Y340/Y341 [127] by Src family kinases [293, 300, 482] and finally in the 'kinase activation loop' at S497/S499 by PKC isoforms [255, 59, 421] and at T491/S494 by unknown kinases or possibly by autophosphorylation [72] cooperate to induce full MEK kinase activity [300, 72]. Cascade activation depends on scaffold proteins, like the kinase suppressor of Ras (KSR), which are not depicted. Feedback phosphorylation by ERK leads to inhibiting hyperphosphoylation of serines and threonines throughout the protein, and such inactivated c-Raf is insensitive to further stimulation by growth factors. Importantly, the total cellular pool of c-Raf appears to shift to hyperphosphorylated form, indicating that the mechanism is independent of c-Raf activation, and the possibility of cross-regulation by other ERK activators. Desensitized c-Raf is reactivated - after several hours - by a cooperation of PP2A and the Pin1 prolyl isomerase [112], which is also not depicted above. Inhibitory phosphorylations, can be mediated through cAMP activated protein kinase A (PKA) (S259, S43, S233) [102, 118], or via PKB/Akt by the lipid signaling branch (S259) [567, 397].

ization of c-Raf at the membrane. 14-3-3 are constitutive dimers and they are suspected to play an essential role in Raf dimerization [283, 128]. Active Ras induces a conformational change of Raf, displacing 14-3-3 from the pS259 binding site [398]. The S259 site can then be de-phosphorylated by the PP2A phosphatase [2, 257, 102].

The function of 14-3-3 in dimerization and possibly in recruitment of activating kinases, explains the conflicting early results on the function of S621 phosphorylation, required both for kinase activity and for inactivation [331]. In contrast, S259 is a true inhibitory site, and it was observed to be phosphorylated by PKA [102], together with S43 and S233 whose role in inhibition are again less clear [539, 82, 433, 117, 118], but also by PIP3 induced Akt/PKB serine kinase activity [567, 397]. S259 has been dubbed a 'gate keeper' for c-Raf activation, and the S233 14-3-3 binding site a 'secondary site' for inhibition by PKA [118]. Two distinct binding sites with different functions seem to be a common theme for 14-3-3 regulated proteins, as such a scenario is also suspected to underlie 14-3-3 interactions with Bad, Cdc25, Cbl and KSR [491]. S43 phosphorylation by PKA has been observed to inhibit interaction with Ras [539] and was suspected to constitute a release mechanism in c-Raf inactivation [103]. It might also to induce interaction with Rheb, another member of the Ras family [557], maybe only sequestering and inhibiting c-Raf[75]. Little work has been done on this interaction, but it is possible that it constitutes a cAMP/PKA controlled alternative pathway of c-Raf activity [254].

S338-Y341 Phosphorylations As a last step in activation of the kinase domain, all Raf kinases share the requirement for phosphorylation in the 'activation loop' of the kinase domain, at S491 and S494 in c-Raf. These residues have no identified kinase and might be phosphorylated by auto- or trans-phosphorylation in dimers [313]. However, in the case of c-Raf (and A-Raf) this last activation step requires prior phosphorylations that mediate the release of the inhibitory N-terminal domain from the catalytic domain. After dephosphorylation of S259, 14-3-3 dimers stay bound to the pS621 site and might be involved in recruiting one or more kinases, that contribute to full activation of c-Raf's MEK kinase activity. Members of the Src family of tyrosine kinases are the best established c-Raf kinases, phosphorylating at Y340 and Y341 [127, 293] and this step requires the serine-phosphate at position 621 and its binding to 14-3-3 [558]. Activating c-Raf tyrosine phosphorylation has also been observed to be catalyzed by Abl [437, 527] and JAK kinases [541, 448], but their target sites have not been mapped. Y338 and Y339 lie 10 amino acids N-terminal of the glycine-rich loop of the ATP binding domain of the kinase, and their phosphorylation has been observed to be succeeded by phosphorylation of the preceding serine residues S338 [300], a step that is more controversial, however (see below). Mutation of this site and adjacent S339 to alanine blocked activation by oncogenic v-Src

[107]. Phosphorylations at both sites are often speculated to cooperate in full kinase activation in an synergistic or additive manner by mediating the release of the inhibitory N-terminal CR1 domain from the CR3 catalytic domain [300, 558, 482], but might also regulate the specific interaction between c-Raf and MEK1 [542]. The latter study supported evidence for the tryptophan at position 342 being required for these phosphorylations [308, 542]. However, activating double mutants of these sites (S338D,Y340D) didn't show increased basal kinase activity compared to wild-type and single mutants, and thus the cooperation is probably not simply additive [72]. Recently, Hekman et. al. [184] have dissected the c-Raf the time course of S621, S259, S338 and Y340 phosphorylation and de-phosphorylation cycles and the role of 14-3-3 scaffolding in PC12 cells. They present the latest in a series of models of the cycle, that also accounts for a possible cooperation of c-Raf with its paralogue B-Raf, and this connection will be outlined below in more detail.

Kinases of the PAK family, PAK1 and PAK3, have been the only suspects for S338/S339 phosphorylation. They are effectors of Rac and Cdc42, members of the Rho family of small G proteins. This would constitute the link between above mentioned requirement for Ras and RTK mediated PI3K lipid signaling at the membrane in subsequent c-Raf activation [87, 178, 116, 245, 529]. Rac and Cdc42 are activated by PIP3 and their direct effector PAK was observed to mediate this requirement by S338 phosphorylation [244, 63, 456]. However, the requirement for PI3K mediated signaling, as well as PAK3 activity has been disputed [70]. The phosphorylation of S338 should happen at the plasma membrane, while PAK phosphorylation has been observed to happen in the cytoplasm [244, 63, 70]. The relation to PI3K/lipid signaling is likely to be quite complex. As mentioned above PIP3 effector Akt/PKA kinase can inhibit c-Raf by S259 phosphorylations [567], but in a differentiation-state specific manner [397]. Lipid signaling is tightly connected to cytoskeleton regulation, e.g. via the Cdc42/Rac and PAK modules, while RTK signaling itself is thought to be tightly integrated with integrin signaling [137]. Recently PAK1 has been proposed to constitute both a scaffold and activating kinase for the whole Raf/MEK/ERK pathway at integrin adhesion sites [199, 119, 457]. The intricate relation of the cascade with cytoskeleton remodeling will be discussed in more detail in chapter 2.3). Another fascinating perspective has been unclosed by the observation of Alavi et.al., that PAK1 mediated phosphorylation at S338 targets c-Raf to mitochondrial locations, while Src phosphorylation at Y340 keeps c-Raf at the membrane for cascade activation [8]. This will be discussed in a moment.

PKC Mediated Activation PKC proteins are divided into the three classes of conventional, novel and atypical forms, depending on their dependence on DAG and calcium binding. They are thus considered to be direct effectors of phospholipase products IP3 (via calcium release) and DAG, where different phospholipases are activated by RTK (PLCγ), trimeric G protein coupled receptors (GPCR, PLCβ), by Ras (PLCϵ) or inflow of external calcium [374, 363]. Diverse kinds of PKC isoforms have been shown to catalyze phosphorylations at adjacent S497/S499 residues as a step in c-Raf activation [255, 59, 493] and PKC mediated c-Raf activation was one of the earliest observed inputs to this pathway, via direct PKC activation by phorbol ester treatment [271, 200, 97, 535, 471, 345, 255, 166, 320, 179, 496, 493]. The only observed phosphorylation sites at S497/S499 lie within the kinase activation loop [255, 59, 493], but their mutation to alanine does not interfere with MEK activation [421]. However, PKC activation has been speculated to be independent of Ras [296, 493]. It was later shown that Ras is required, but not blocked for this activity by the dominant negative mutant RasASN17 [292], that was used in many of the preceding studies that showed Ras independence [271, 97, 166, 320, 179, 496, 460]. Thus PKC activation of the c-Raf/MEK/ERK cascade might involve a different mechanism of Ras activity, and constitute an alternative pathway of c-Raf activation [292, 460]. However, PKC isoforms are activated by DAG and calcium, and these second messengers will appear at several points in this work. Moreover a positive feedback between PKC and ERK signaling was proposed to underlie a bistable behavior of these pathways [30, 31], which will be discussed in section 2.4.2. The mathematical models employed for these works are shortly discussed in section 3.2.6 (see fig. 33).

Cascade Scaffolds As outlined below, c-Raf's first vital functions in development and physiology could be independent of its kinase activity. It acts itself as a scaffold that inhibits other S/T kinases in anti-apoptotic [360, 18] and migratory contexts [122]. However, activation of the MAPK pathways is itself highly dependent on scaffolding by a variety of proteins, connecting various components of the cascade. Several chaperones have been identified that stabilize c-Raf's tertiary structure and are also involved in its regulation. The complex relation of the Raf scaffold 14-3-3 with Raf activation cycles has been outlined above. Please see the review by Walter Kolch [254] for details on the many scaffolds and chaperones involved. It has been observed that Ras induces large macromolecular signaling complexes containing both c-Raf and B-Raf [326]. The actual activation of the

cascade depends on scaffold proteins, that sequester Raf, MEK and ERK at specific sub-cellular localizations. Scaffolds mediate localization to and interaction with different signaling complexes, and thus impose specificity on this ubiquitous signaling molecule [254]. Please consult this and other recent reviews by Park et.al. 2003 [362] or Ptashne and Gann 2003 [379] for general discussions of MAPK scaffolding.

... at RTKs: The KSR (Kinase Suppressor of Ras) was initially identified as a suppressor of c-Raf/MEK/ERK signaling in over-expression experiments. While KSR has a kinase activity and was shown to phosphorylate c-Raf at T269 [552, 545], a site that is also an in vitro autophosphorylation target [331], its central function now is considered to act as a scaffold for the whole cascade. At normal expression levels, it supports the pathway, by bridging the components and increasing their local concentration at the plasma membrane. Such differential function of scaffolds, depending on their expression levels, was also studied in a theoretical model by Levchenko et.al.[274]. Together with the C-TAK1 kinase [332] or the connector-enhancer of KSR (CNK) [265], KSR facilitates MEK/ERK activation at the plasma membrane. Likewise SUR-8 forms a complex with Ras and c-Raf and enhances signaling to the cascade [277].

... at integrin adhesions: One of the best studied cascade scaffolds is the yeast Ste20 protein, which is also known as an activating kinase of yeast M3Ks [92]. While yeast has no homolog of the Raf proteins, Ste20 is a homolog of the vertebrate PAK kinases. Their controversial role in c-Raf phosphorylation has been outlined above, and their role as both a kinase and a scaffold for c-Raf/MEK/ERK activation at integrin adhesion sites will be discussed in more detail in chapter 2.3.

... at endosomes: MEK partner 1 (MP1) was identified as an enhancer of ERK signaling [416] and is now thought be bound to late endosomes via p14 and MP1 to sequester c-Raf at this location [262]. Endosomal Raf/MEK/ERK signaling will return below in the context of spatio-temporal control of ERK signaling by a putative cooperation of c-Raf and B-Raf in PC12 cells, although in this case internalized receptor complexes are made responsible and MP1 has not been shown to be involved.

... at the Golgi: Sef and Sprouty proteins are known as feedback inhibitors of developmental FGF signaling via ERK [485], which act via several paths, e.g. at FGFR level, but also just between MEK and ERK (see left graph in fig. 8). Sef was recently also found to direct Ras signaling via MEK and ERK at the Golgi apparatus, from where it activates cytoplasmic ERK activity, but inhibits nuclear translocation [481, 370]. In contrast, in

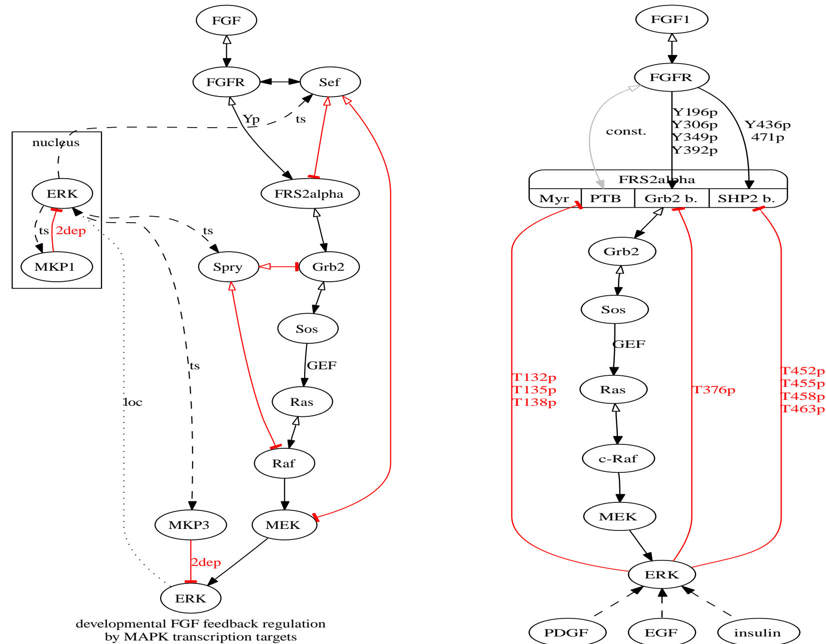


Fig. 8. Genetic vs. biochemical negative feedback of FGF signaling via the Raf/MEK/ERK cascade. Left: Sef, Sprouty and MKP proteins are expressed upon ERK activity and interfere with ERK activation at diverse levels [485]. Right: FRS2α is an adaptor protein in FGF receptor signaling, that can be inhibited by ERK, either as a negative feedback to FGF signaling or as a trans-inactivation via other ERK activating factors. The latter could be of relevance in developmental signaling, where one ERK signal could render the cell insensitive to an FGF signal from another source [269]. NOTE, that the left graph shows feedback that depends on gene expression, while the right graph depicts direct biochemical feedback. These two different kinds of feedback can exhibit very different kinds of dynamics of ERK activation.

a short comment to the insights of above mentioned H-Ras and N-Ras cycling between plasma membrane and Golgi apparatus, Meder and Simons speculate that Golgi activation could support nuclear ERK translocation by activation in close proximity to the nucleus [309].

De- and Re-sensitization Finally, active ERK phosphorylates a range of negative regulatory sites in c-Raf. c-Raf becomes de-sensitized for further activation, and rests in an inactive cytoplasmic conformation. PP2A seems to be a key player in c-Raf cycles, dephosphorylating inhibitory phosphoresidues at several points of the cycle, and possibly recruiting activating

kinases [2, 257]. Re-sensitization of c-Raf is catalyzed by a cooperation of Pin-1 with PP2A and takes several hours [112]. Thus, not only phosphorylations by cAMP/PKA and PIP3/Akt signaling branches, but also feedback phosphorylations by ERK inhibit c-Raf for further cascade activation. While there exist several lines of evidence, that the cAMP and lipid mediated inhibition actually might redirect c-Raf activity to other targets, the ERK mediated inhibition at several sites fits into a general emerging picture of tight feedback control of c-Raf activation in different contexts, both biochemically by posttranslational modifications [269, 50, 457], and genetically by induced expression of inhibitors [485]. Importantly, the latter process can be expected to happen on a much slower time-scale than the former. Fig. 8 comprehends several examples for both levels of feedback inhibition.

Redirecting c-Raf The most surprising insight of a c-Raf knockout experiment in mice was, that the protein's first vital functions during development - and also its transforming potential - are independent of its kinase activity [207, 318]. Instead, c-Raf was lethally missing from a mitochondrial apoptosis pathway, crucial for development. These knockout mice die around midgestation (E11.5 to E13.5) from accelerated erythropoiesis and consequent depletion of erythropoietic stem cells from the fetal liver, the source of early embryonic hematopoiesis [318, 253]. Generation of erythrocytes involves parts of the apoptotic caspase cascade to degenerate the nucleus. Several potential paths link c-Raf to apoptotic protection (reviewed in[18, 19]). Recently fascinating mechanisms have been indicated: c-Raf translocates to mitochondria [510, 409] upon PAK1 phosphorylation at S338/S339 [8], where it can suppress activation of the pro-apoptotic kinases Ask1 [68, 550], independent of its kinase activity and rather acting as a scaffold. Mitochondrial c-Raf has long been known to bind anti-apoptotic Bcl-2, where it might facilitate or directly catalyze inhibitory phosphorylations of the pro-apoptotic Bcl-2 antagonist BAD at S112 [510, 509]. Recently, PAK1 phosphorylation at S338/S339 has been observed to induce also this latter function [225] and the pro-apoptotic kinase MST2 (mammalian sterile 20-like kinase) could be identified as one essential target of anti-apoptotic kinase-independent scaffold function of mitochondrial c-Raf [360]. The graphs in fig. 9 comprehend some of the last cited works. However, another recent study knocked out c-Raf and A-Raf in mice couldn't find any (significant) influence on neither apoptosis nor ERK activation of the single knockouts. The differences to other knock-out studies were attributed to the genetic background of the mice [313].

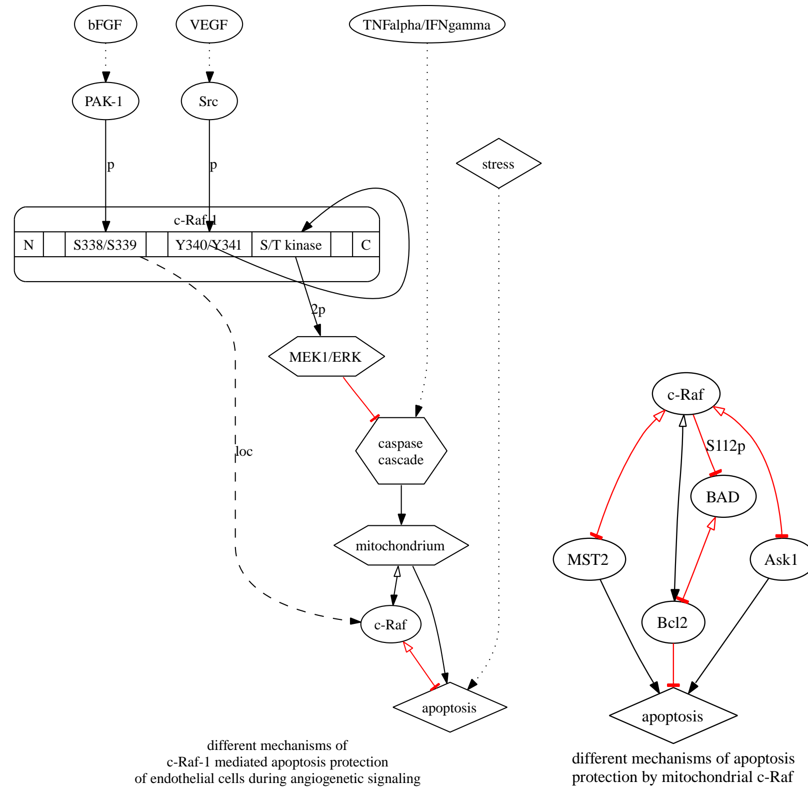


Fig. 9. Left: In endothelial cells VEGF induces PAK1 mediated c-Raf phosphorylation at S338/S339 and translocation of c-Raf to a mitochondrial location, where it protects against stress induced apoptosis. In contrast bFGF (FGF2) induces Src mediated phosphorylation of Y340/Y341, subsequent activation of the MEK/ERK cascade, and protection against external - inflammatory - signal induced apoptosis (TNFα/IFNγ) [8]. Right: Major anti-apoptotic functions of mitochondrial c-Raf has been delineated to the inhibition of the Ask1 and the MST2 kinases, where it acts as a scaffold rather than a kinase [68, 360], but also by activating complex formation with Bcl-2 and displacement and inhibitory phosphorylation of pro-apoptotic BAD [225].

The most recent experiments with (conditional) mouse knock-outs identified yet another function of c-Raf in the scaffolding of a Rho effector, the kinase Rok-α, which is involved in microtubuli assembly and actomyosin-based contractility at the trailing edge of migrating cells; again this function is independent of c-Raf's kinase activity. Keratinocyte-specific disruption of c-Raf leads to a significant decrease in an experimentally defined wound healing rate, due to impaired migration of keratinocytes towards the wound

edge [122]. The connection to adhesion and migration — and the connections of the former with apoptotic signaling — will be discussed in chapter 2.3.

Depending on its phosphorylation status, c-Raf can fulfill diverse functions at several sub-cellular locations, where scaffolds ensure specific cascade activation. It has several kinase independent functions, which are controlled by its subcellular localization (reviewed in [19]). Phosphorylations at S43 by cAMP/PKA [557, 75] and at S338/S339 by PAK1 [8, 225] have been observed to redirect c-Raf interactions. Thus, phosphorylations might not only activate or inactivate kinase activity, but induce relocation of c-Raf to fulfill these alternative functions, possibly after having contributed to early MEK/ERK signaling. The many involved and suspected c-Raf kinases that cooperate in full activation, modulation of activity, inactivation and localization indicate that c-Raf is a hub for multiple signaling pathways. However, it is difficult to conclude about the 'connectivity' of certain proteins, as this may be an artefact of research interest [194]. So let's stay with the cascade and investigate potential roles of the other two Raf kinases:

2.1.3 B-Raf (and A-Raf)

While long being considered the specific MEK/ERK activator of only neuronal or hematopoietic cells, B-Raf is now known to be expressed more widely, although at often much lower levels than the ubiquitous c-Raf and in tissue-specific isoforms [23]. Phylogenetic analysis by protein alignments reveals B-Raf as the closest homolog of the only known invertebrate Raf gene [314, 52] (see fig. 20). B-Raf is subject to alternative splicing, giving rise to a range of proteins between 75 and 100 kDa, while A-Raf is expressed as a 68 kDa polypeptide. It carries phospho-mimetic aspartates D447 and D448 instead of Y340 and Y341. Phosphorylation of B-Raf's S445, the site corresponding to S338 in c-Raf, elevates its basal activity, but is found to be constitutive [300]. While in c-Raf and A-Raf the respective phosphorylations at these sites cooperate in full activation, B-Raf only requires Ras (or e.g. Rap1 - see below), respectively [294, 300]. Thus, B-Raf activation can be said to 'short-circuit several events, required for c-Raf activation' [359]. Consequently, B-Raf, but not c-Raf or A-Raf, is a major target of oncogenic mutations [314, 52]. The deactivation process for B-Raf, involving conserved as well as B-Raf specific phosphorylation sites [163], is slower than for c-Raf and A-Raf [313]. Of the three inhibitory PKA targets in c-Raf, S43, S233 and S259, only the latter is conserved in B-Raf (at S364) and A-Raf, which is also a target of Akt phosphorylation. B-Raf carries specific additional

Akt target sites S364, S428, T439 [163], which - until further investigation - allows two opposing interpretations: B-Raf is either more sensitive, inhibited even by weak Akt, as it has several possible targets of which only one suffices for inhibition, or less sensitive, if all three targets need to be phosphorylated for inhibition [163, 313].

Sustained vs. Transient ERK Activation In c-Raf knockouts, B-Raf and A-Raf are thought to fully cover for MEK/ERK activation alone. As O'Neill and Kolch put it: 'B-Raf emerges as the main regulator of the MEK-ERK pathway, while both c-Raf and A-Raf seem to provide ERK-independent apoptosis protection in different tissues' [359]. The above cited recent double knockout mice (araf$^{-/-}$ and craf$^{-/-}$) indicate, however, that these proteins are required for an initial phase of ERK activation but not for a later sustained phase, that would then depend on B-Raf. Mouse embryonic fibroblasts (MEFs) from these mice are delayed in the G_1/S transition of the cell cycle, and — apparently as a consequence — the newborn mice are smaller than wild-type controls [313]. This fits into an emerging picture of cooperation of vertebrate Raf proteins in spatio-temporal control of ERK signaling.

Differential activation profiles of the Raf/MEK/ERK cascade are correlated with differential responses to signals, and ERK activation can lead not only to proliferation but also to growth arrest (and differentiation) or merely mediate survival (apoptosis protection) signals. Biphasic ERK activation in fibroblasts, with an initial peak and a sustained phase for 5-6 hours hours in response to growth factors and adhesion mediated integrin activity, is required for cyclin D1 expression and progression through the G_1 into the S phase of cell cycle [520]. A transient activation of ERK, that can in this case persist for more than one hour when activated by growth factors in non-adherent (suspended) fibroblasts [401], or only 10 minutes by serotonin [312], inhibits cell cycle progression. Please see [400] or [375] for reviews on this issue. Cell cycle inhibition by expression of cyclin-dependent kinase inhibitor (CKI) proteins p21Cip-1/WAF1 can also be achieved by manipulating Raf to induce stronger ERK signaling [537, 236], and thus the intensity of the signal also seems important. In a primary culture of rat hepatocytes an 'acute/phasic' MAPK activation can stimulate, while a 'chronic' activation inhibits DNA synthesis [476], depending on its stimulation of expression of p21Cip-1/WAF1 [15]. In Schwann cells a transient mitogenic activation of ERK and ribosomal S6 kinase (Rsk) depends on low concentration of cAMP, while a sustained signal, inducing differentiation, requires high

cAMP concentrations [338], and a crosstalk with cAMP signaling, mediated via differential activation of the Raf isoforms c-Raf and B-Raf, has long been implicated in the differential response in PC12 cells (e.g. [125]). An intricate interplay between upstream activators Ras and Rap1, and M3Ks c-Raf and B-Raf has e.g. also been shown for thrombopoietin response of megakaryoblastic cell line UT7, that also requires sustained ERK activity for differentiation [144].

Several interesting observations - somewhat different than above cited - have recently been made in an elegant study of the activation of the B cell receptor (BCR): B-Raf and c-Raf cooperate in temporary distinct activation phases of the MEK/ERK pathway. While B-Raf is responsible for an early and a sustained phase of ERK activation, c-Raf contributes to the intermediate phase [51]. B-Raf is also regulated by negative feedback phosphorylation at an ERK target site, in this B cell system as well as in PC12 cells [50]. These experiments carried out by Brummer and colleagues are comprehended in the two graphs of fig. 10.

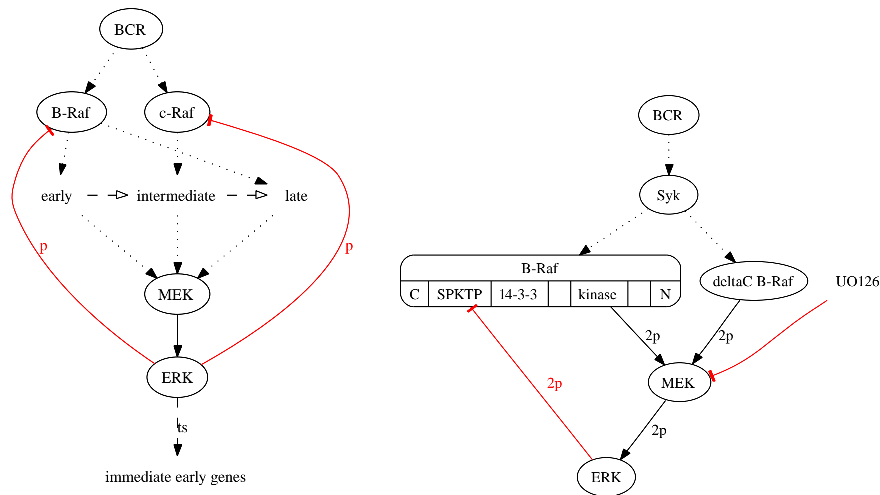


Fig. 10. Brummer T. and colleagues have investigated the cooperation of B-Raf and C-Raf in early, intermediate and late phases of ERK activation [51], and in a later study identified a negative feedback inactivation of B-Raf by ERK. A deletion mutant, lacking the C-terminal domain, that contains a potential ERK target site, cannot be deactivated by ERK, and the MEK inhibitor U0126 inhibits phosphorylation of this site [50].

Hetero-dimerization? Dimerization of c-Raf has been found to be crucial for its activation [283, 128], and dimerized Ras has been suspected to be involved in this process [211]. A fascinating perspective for Raf cooperativity is opened by the finding that c-Raf and B-Raf can hetero-dimerize upon Ras activity [519, 507], that c-Raf's isolated auto-inhibitory domain can directly inhibit B-Raf's catalytic domain [483] and that kinase-impaired but still oncogenic B-Raf mutants can trans-activate c-Raf [507]. B-Raf has been detected in Ras-induced large (400 kDa) macromolecular complexes that activate c-Raf for ERK signaling [326]. Dimerization is a wide-spread phenomenon in protein function. Hetero-dimerization of two diverged descendants of a single gene offers a great mechanism for evolution to fine-tune protein function. Together with the increase in alternative spliced variants, such a scenario might be central to the 'success' of vertebrate evolution.

The recent insights into Raf biology indicate that there is much more to learn before a clear evolutionary and mechanistic scenarios can be outlined. The PC12 cells have become a model system for differential cellular responses to differential time-courses of ERK activity. Several of the involved processes have already been mentioned above, and the PC12 cells will stay in focus of the upcoming discussions below. While the detailed mechanistics of Raf cycles await further clarification, we can 'zoom out' and investigate the already known embedding in cellular signaling networks on the specific example of cAMP signaling, which will allow to connect to the PC12 cell system as well as to integration of the cascade with cytoskeletal regulation in cell adhesion and migration.

2.1.4 Cross-talk: cAMP and Ca^{2+}, lipids and cytoskeleton

Experimental evidences for cross-talking can be found between virtually all signaling 'pathways' and at all levels - from extracellular to nuclear interactions, as well as through modulation of protein expression. Here, only some of the immediate and intensively studied network connections will be sketched, staying in context with above and following topics:
BCR signaling to the cascade - as mentioned above - is mediated by the protein tyrosine kinases Syk, Lyn and BTK, members of the Src family of kinases ([51, 50], see fig. 10). Src kinases have appeared above, as activating kinases for PLCγ1 and c-Raf, and will appear again in chapter 2.3 as a partner of the focal adhesion kinase (FAK) in integrin signaling. The members of this family often have (partially) overlapping functions, and often multiple knockout cells, such as $(Src, Yes, Fyn)^{-/-}$ cell lines are em-

ployed to study their function. Src kinases are involved in RTK and many other signal pathways. A complex of Src with the Focal Adhesion Kinase is central in adhesion and migratory signaling, and this topic will be outlined in chapter 2.3. Here, Src merely serves to hit the road from above example of c-Raf/B-Raf cooperation towards another enigmatic crosstalk with the cAMP/PKA pathways (adenosine 3', 5'- cyclic monophosphate, protein kinase A) — the first-known eukaryotic signaling system. cAMP is produced by a (large) variety of adenylate cyclases (AC), often activated by trimeric G protein coupled receptors (GPCR), but also by many other stimuli, e.g. Wnt/Frizzled signaling [66]. Among several cAMP responsive proteins, the PKA family is the most prominent. A cytoplasmic tetramer of two inhibitory and two catalytic subunits dissociates upon cAMP binding, freeing the catalytic subunits for phosphorylation of substrate proteins. Importantly cAMP - via PKA-dependent and -independent tracks - can both inhibit and activate ERK activation as well as growth factor induced mitogenesis, depending on cell type, signaling history and culture conditions. A 2002 review by Stork and Schmitt summarizes cAMP and MAPK interferences [453], while Stork in 2003 [451] focuses on Rap1's functions in this cross-talk. The right graph in fig. 11 comprehends various of these paths from cAMP to activation of B-Raf and inhibition of c-Raf. Signaling via the cAMP pathway has often been considered to serve as a positive or negative gate for a number of other signaling pathways [216], and so has the state of the cytoskeleton and adhesion of cells to extracellular matrix or other cells [137]. Recent observations indicates how these gating functions might be integrated [199, 137], and the following discussion will analyze some of this evidence from the perspective of Raf/MEK/ERK gating.

Rap1 Rap1 is a closely related member of the Ras subfamily of small G proteins. It is now understood to be an important relay between cAMP and Raf/ERK pathways as well as a partner of Ras in spatio-temporal fine-tuning of c-Raf/B-Raf activation of the cascade. Often, a spatio-temporal cooperation of Ras and Rap1 is found to be required for 'proper' modulation of ERK activity (e.g. [356, 42, 354]). Rap1 is very similar to Ras in its effector binding regions. It binds to c-Raf, but doesn't activate it. It could thus be able to sequester inactive c-Raf away from Ras activation [451], and thereby also redirect Ras signaling to other targets, e.g. PI3K [74] or merely to B-Raf. (However, just as a note, phosphorylated c-Raf has been found to also be activated by Rap1 at specific membrane subdomains [57].) In contrast, B-Raf is found to be bound as well as activated efficiently

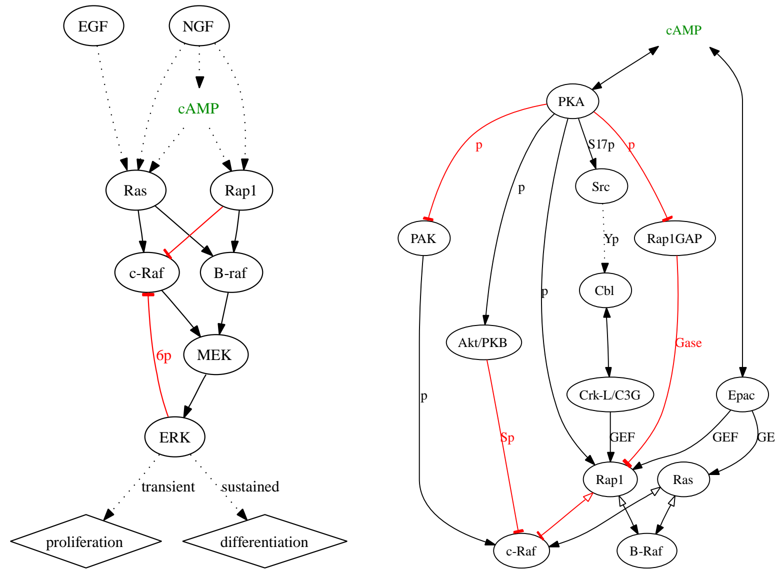


Fig. 11. Left: a cooperation of c-Raf and B-Raf signaling in PC12 proliferation or differentiation, modified from O'Neill and Kolch [359]. Right: a comprehension of observed pathways of modulation of c-Raf and B-Raf mediated MEK/ERK activation by the cAMP signaling system, modified from the review by Stork and Schmitt 2002 [453].

by Rap1. This differential activity of Rap1 towards c-Raf and B-Raf is now often hypothesized to constitute a core mechanism of Raf kinase family cooperation in spatio-temporal modulation of ERK signaling [254, 359]. cAMP can activate Rap1 via several ways, both dependent on and independent of PKA. PKA catalyzes e.g. an activating phosphorylation of the Src tyrosine kinase at serine 17 [350] (see the right graph in fig. 13 for the experimental model and setup). Independently of PKA, the cAMP sensitive Epac proteins (exchange protein directly activated by cAMP) [96, 123] comprise GEFs for both Ras and Rap1. cAMP and Epac signaling is (of course;) also regulated by compartmentalization, as analyzed in [109]. Just alike, many routes seem to lead from cAMP to ERK activation, both via Ras and Rap1, and via c-Raf and B-Raf [102, 101, 418, 453, 359].

Calcium NGF:TrkA induced differentiation of PC12 cells depends on PLCγ activity and is thought to involve CalDAG-GEFs for both Ras and Rap1 [399] (see middle graph in fig. 13). Neuronal calcium signaling is observed to activate the Rap1/B-Raf/ERK branch, potentially via adenylate cyclase ac-

tivation and cAMP signaling [165, 161], while cAMP has also been observed to induce calcium signaling [563], lending some evidence to a potential positive feedback between these systems (see left and middle graphs in figure 12, respectively). In PACAP/GPCR mediated neuronal differentiation, a sustained activation of ERK via Rap1 requires Ras, PKC (and thereby calcium), and cAMP/PKA [498, 42] [2]. The various proposed feedback systems - involving various combinations of cAMP, calcium, and lipid signals, responsive GEFs like Epac, CalDAG-GEF proteins, and feedback from Ras, Rap1, or Rap2b to PLCϵ [226, 442, 235], would only resemble several known similar situations where several small G proteins interact with each other, with lipid and with calcium signaling, forming positive feedback switches that mediate local formation of macromolecular complexes, that further nucleate actin polymerization processes. Several examples will be shortly discussed in chapter 2.3. Calcium appeared above in its cooperation with localized Ras activity and will be met at dispersed points throughout the following.

The cytoskeleton connection There exists serious doubt, however, whether Rap1 is directly involved in cAMP mediated B-Raf activation [568, 123, 248]. Again, the evidence for functional hetero-dimerization of c-Raf and B-Raf (see above) provides a nice playground for speculation. Maybe Rap1's activation of B-Raf is achieved by mere sequestering c-Raf away from both Ras and from an inhibitory hetero-dimerization with B-Raf. Active Ras would be free to activate free B-Raf. While above speculations all refer to vertebrates and their three Raf genes, just recently, a direct Rap1/B-Raf connection got strong support from a study in Drosophila, which possesses a protein called D-Rap1, and the one invertebrate Raf, called D-Raf in Drosophila. Signaling via the Torso RTK can activate D-Raf/ERK directly via D-Rap1 but independently of D-Ras1. Combined knockout embryos of D-Ras1 and D-Rap1, mimicked the D-Raf knockout [322]. The Rap homolog in Dictyostelium and yeast are again mainly known for their involvement in cytoskeletal organization, a role which could constitute another or a complementary branch to B-Raf activation. Rap1's involvement in cytoskeletal changes was reviewed extensively by J.L. Bos [40]. It is - to give only some examples, central to the process of cell spreading; Rap1 stimulates integrin mediated adhesion [380, 124], or localizes Rac [11] to nucleate actin polymerization. As a more detailed example, EpacI has been observed to mediate calcium stimulated calcium release and exocytosis [40, 231].

[2] Please see the review by Wetzker and Bohmer [530] for the important, but here totally neglected cross-connections of GPCR, RTK and Raf/ERK signaling!

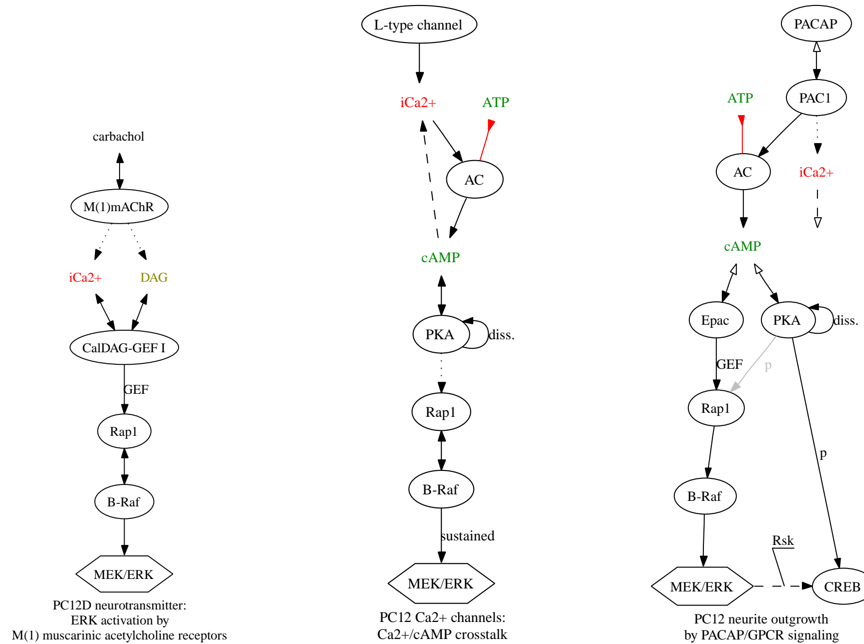


Fig. 12. PC12 neurogenic signaling via Rap1 and B-Raf: diverse activators of Rap1 signaling are involved in sustained ERK activation, neurite outgrowth and cell differentiation. Many pathways employ both calcium and cAMP signals. Left: Calcium and DAG signals activate CalDAG GEFs for Rap1 [165]. Middle: Calcium inflow activates Adenylate Cyclase [161], while cAMP/PKA signaling can lead to intracellular calcium release and ERK activation [563], pointing to a putative positive feedback between calcium and cAMP signaling. Right: PACAP signaling via cAMP activates Rap1, but also signals through intracellular calcium. All pathways converge again at the level of transcription factor modification. Here only the dual influence on the cAMP responsive element binding protein (CREB) is sketched. Comprehensive model modified from Fig. 1 in [498].

In Dictyostelium the reorganization of cortical actin, membrane ruffling, the extension of lamellipodia, and phagocytosis are the studied domains of Rap function. In yeast, the homolog is called Bud1, because of its central involvement in a Cdc24/Cdc42 (again 2 small G proteins, members of the Rho family) induced actin polymerizations at the future site of cell budding [40]. Interestingly, yeast has no Raf homolog, and the yeast homolog of Ras is involved in nutrient-sensing pathways regulating adenylate cyclase [254]. Cdc42 is also known as the main activator of the Ste20/PAK which has conserved roles both as an upstream activator of MAP kinases [92] and cytoskeletal signaling (see below).

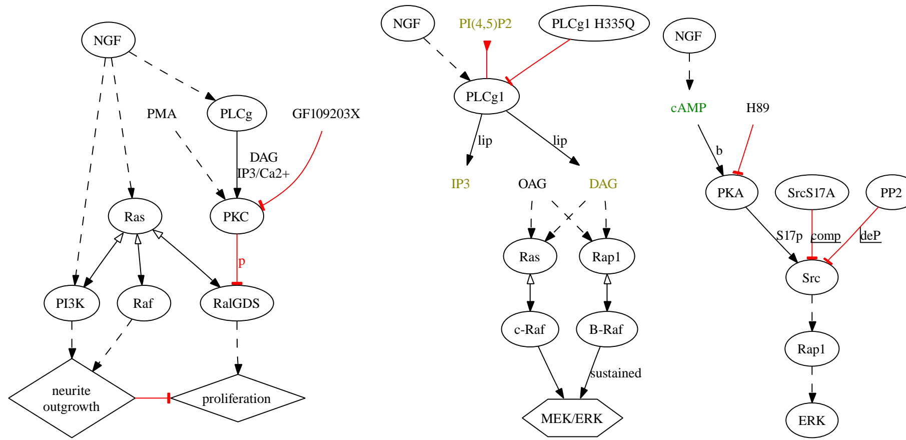


Fig. 13. PC12 NGF signaling: neurite outgrowth and differentiation. Left: PKC signaling can redirect Ras signaling to the Raf and PI3K branches by inhibiting the RalGDS branch. Enforcing the RalGDS branch via activators of PKC leads to cell proliferation, while PKC inhibitors enhance neurite outgrowth [149, 406]. A follow-up work in COS-7 fibroblasts EGFR signaling showed that PI3K mediated activation of PDK1 and phosphorylation of RalGDS is the second stimulus for its activation besides Ras binding [475] - see fig. 24. Middle: dominant negative PLCγ1 protein inhibits NGF induced Rap1 activation, sustained ERK activation and differentiation and neurite outgrowth, while the DAG analog 1-oleyl-2-acetylglycerol (OAG) induces these processes [399]. Right: PKA inhibitor H89 abolishes Src serine 17 phosphorylation and inhibits Rap1 and sustained ERK activation, and so do expression of a Src S17A mutant or of PP2 phosphatase [350].

The left graph in fig. 11 collapses diverse experimental knowledge about differential ERK activity in the PC12 cell line by NGF and EGF signals, and the differential consequences for cell function, while the right graph in fig. 12 comprehends some of the interactions involved in PACAP/GPCR mediated neurogenic signals. NGF and PACAP induce a sustained activation of ERK and start a neuronal differentiation program, whereas EGF induces only a transient activation of ERK and start of the cell cycle, i.e. inducing proliferation of PC12 cells. It is clear that cAMP is involved in PACAP signaling via activation of Rap1/B-Raf [498]. Artificial cAMP signaling via forskolin was observed to convert EGF into a neuronal differentiation signal in PC12 [553]. cAMP has however rarely been directly implicated in NGF signaling, which is thought to induce sustained ERK signals also via a direct activation of Rap1 through receptor complex associated GEF C3G [298, 498]. However, Src kinases are involved in NGF::TrkA receptor complex formation [559, 232, 540], and Src activation and sustained ERK activity can

be blocked by the H89 inhibitor of PKA [350]. (see figures 15 and 16 in next chapter). While Ras is known to be essential for ERK activation by EGF and NGF, it is not required by PACAP signaling, which on the other hand is independent of PKA [498], and likely depends on direct activation of Epac proteins by cAMP. As we will see below, sustained activation of ERK highly depends on receptor internalization dynamics, and thus on remodeling of cytoskeletal structures. Furthermore neurite outgrowth itself is of course a cytoskeletal remodeling process, and cAMP is known to be tightly integrated with cytoskeletal integrity [420, 137]. Thus, the generation of a cAMP signal in NGF signaling might be a secondary process and depend on prior induction of neurite outgrowth.
While it is not clear whether and how 'sustained' and 'transient' activities in different cell lines correspond to each other [400], the cited evidence clearly demonstrates that spatio-temporal control of ERK activity is crucial for the cell's response, challenging classical models of cell cycle regulation [36]. However, the knowledge and narrations about NGF (and other neurogenic) signals in PC12 cells offer a nice system to delineate the complex integration of cellular signaling networks and cytoskeletal dynamics with cell survival, cell cycle arrest and developmental differentiation programs.

2.2 Spatio-temporal Coordination and Cell Cycle

2.2.1 From the Membrane ...

PC12 Receptor Dynamics The PC12 cell culture is derived from rat pheochromocytoma cells - a tumor cell line arising from chromaffin cells, neural crest descendants in the adrenal medulla - that often serves as a model system for neuronal differentiation [160, 297]. Soon after their establishment in 1976 [160] the differential responses to EGF and NGF, and also a possible involvement of cAMP [186], also made PC12 cells a popular model for spatio-temporal modulation of ERK signaling. The addition of Neuronal Growth Factor (NGF) to culture medium causes PC12 cells to initiate a neuronal differentiation process, involving a cell cycle arrest, expression of neuronal genes and outgrowth of neurites [484] - similar to other neurogenic signaling pathways [498]. While in an initial peak of ERK activation both c-Raf and B-Raf are involved, a second sustained phase (1-2 hours) of neurogenic signaling via B-Raf/ERK is crucial to differentiation. As for PC12 cells, the differential kinetics of receptor internalization were already mentioned in Marshall's oft-cited review as a crucial mediator of differential ERK sig-

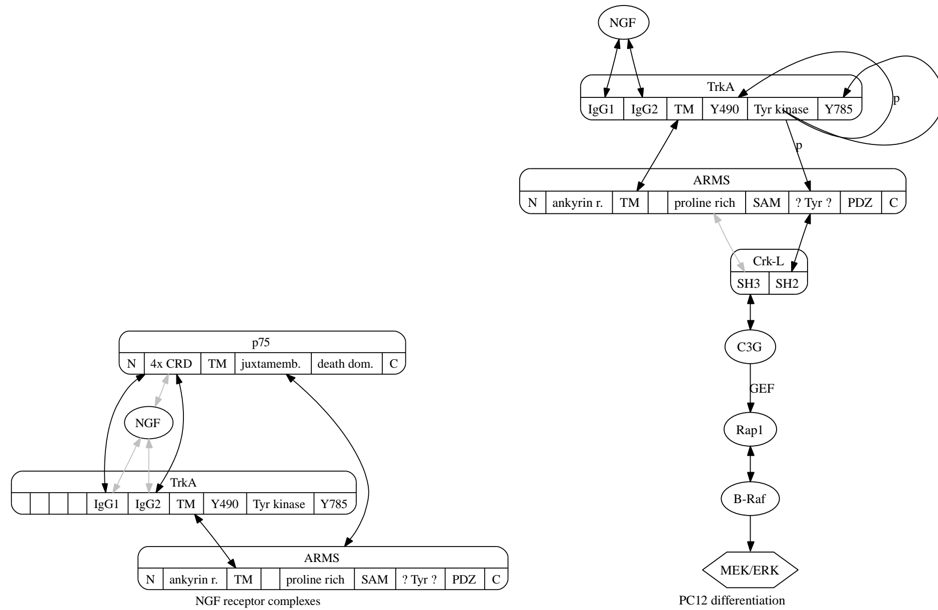


Fig. 14. Left: NGF:TrkA receptor complexes with co-receptor p75 and membrane-bound ARMS proteins [61]. Right: ARMS acts as an adapter for C3G containing complexes, directing activation towards a Rap1/B-Raf/ERK branch [9]

naling [297]. The sustained phase is now attributed to Rap1 on endosomal vesicles, containing internalized NGF:TrkA complexes [560, 559, 232, 538]. RTK are activated by dimerization, oligomerization and potential clustering, putatively organized within membrane subcompartments. Usually receptors are associated with co-receptors or receptor complex scaffolds and transmembrane adapter molecules. In the case of PC12 cells, p75 acts as a TrkA co-receptor for NGF [3], enhances NGF affinity and associates with the membrane bound ARMS protein [61], that scaffolds the Crk/C3G complex for Rap1 activation [9] (see fig. 14). However, retrograde transport vesicles containing the TrkA receptor complexes are speculated to fuse with Rap1 containing vesicles, thereby keeping TrkA signaling active via the B-Raf/ERK branch [239]. In contrast EGFR complexes are not only subject of quick negative feedback phosphorylation (of e.g. SOS), but are internalized much faster, and targeted for proteasomal degradation through ubiquitinylation, mediated by (E3-ligase) Cbl containing complexes [232, 538]. EGFR complexes thus induce only a transient peak of ERK activity, that triggers

[3] p75 also mediates TrkA independent NGF survival signals via the PI3K/Akt branch

the cell cycle [484, 297]. The graphs in figures 15 and 16 depict experiments and models from the cited works [560, 559, 232, 538].

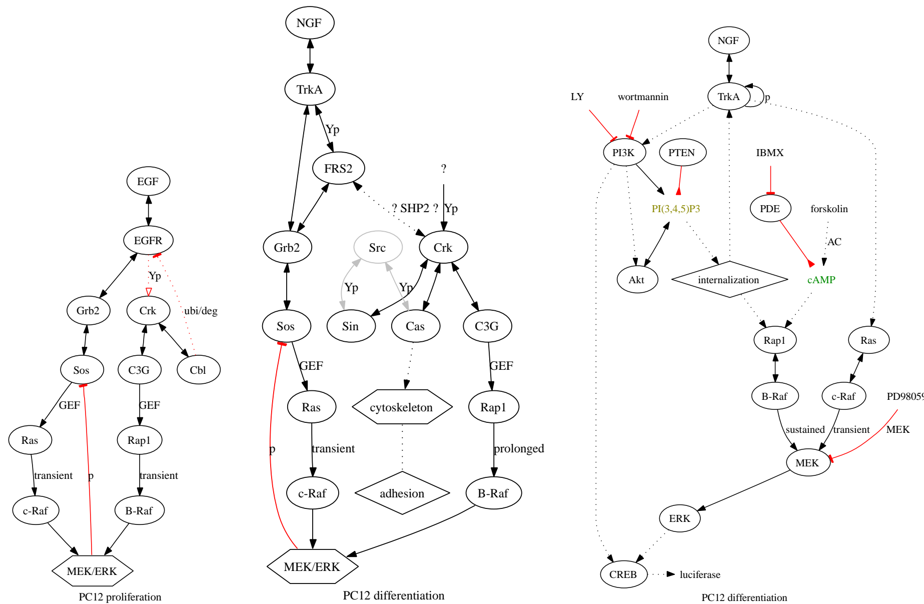


Fig. 15. Differential receptor complexes in PC12 signaling. Left and middle: Based on experiments by Kao et.al. 2001. Both, EGFR and TrkA receptor complexes are associated with C3G, a GEF for Rap1. The C3G complex at the EGFR contains Cbl, and E3-ligase, that mediates ubiquitinylation and subsequent proteasomal degradation of the EGFR. Negative feedback to SOS terminates and initial Raf activation in both cases [232]. Right: based on experiments by York et.al. 2000: internalization and transport of TrkA requires PI3K lipid signaling, and leads to endosomal TrkA/Rap1/B-Raf signaling. Downstream of PI3K, the Rap1/B-Raf branch can be activated by cAMP increasing agents. A transient Ras/c-Raf mediated signal is essential for transient ERK activation [559].

Recent experiments allowed to assign survival signaling to the TrkA complex at membrane via prolonged Akt signaling, while internalized TrkA mediates cell differentiation signaling to the nucleus [566]. An artificial fast internalization of TrkA - resembling the EGFR situation - mediates trophic (growth and proliferative) but not neurogenic signals [410]. This fits to some evidence that Ras is more responsible for initiation of cell differentiation, while Rap1 is required for neurite outgrowth [560, 298].

Many members of the small G protein family are known to be involved in endo- and exocytosis, as well as in vesicular trafficking, all of which also involve remodeling of lipid composition of membranes. Here, only a direct

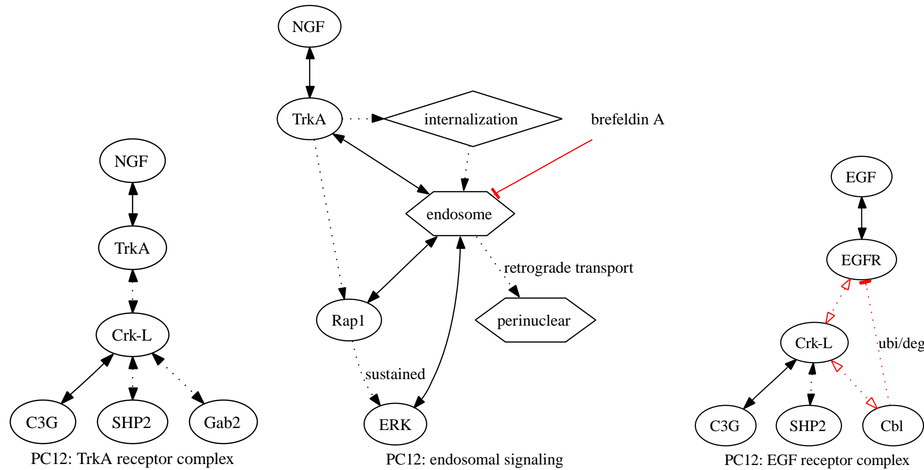


Fig. 16. Differential receptor complexes in PC12 signaling. Similar to the experiments shown in fig. 15, Wu et.al. 2001 dissected EGFR and TrkA receptor complexes in PC12 cells, and requirement of internalization and retrograde transport of intact endosomes for sustained signaling [538].

connection to NGF induced ERK activation and neurite outgrowth shall serve as a further example of this integrated processes. PKC has been implicated in NGF signaling already in 1988 [172]. PKC's influence might involve the sequential activation of Ras branches of RalGDS, PI3K and Raf activation. RalGDS is an activator of the small G protein Ral, that is implicated in the formation of filopodia. In PC12 cells PKC was shown to phosphorylate RalGDS, which does not inhibit its interaction with Ras but its activation by Ras. Moreover, this PKC mediated inhibition seems to redirect towards the other branches of Ras, namely activation of PI3K and Raf and inhibition of PKC renders the NGF signal mitogenic instead of neurogenic [149, 406]. RalGDS requires a second input for activation, besides binding to Ras, and in EGF signaling in COS-7 cells the same group identified PDK1 mediated phosphorylation as a sufficient second input. PDK1 is again activated by PI3K's product PIP3 [475], pointing to a complicated relation of all these processes. This module of EGFR and TrkA signaling might thus likely be involved in differential ERK signaling in PC12 cells. As neurite outgrowth can be considered to employ parts of the cellular machinery for directed migration and Ral is known to interact with exactly this machinery, in fractions depicted above in the cross-talk section 2.1.4 (fig. 13), as well as in the adhesion/migration chapter 2.3 (fig. 24).

Membrane Networks Diverse processes are involved in receptor dynamics. To approach a global picture, we will have to quickly let some of the so-far uncited experimental indications pass by:

... Integrins: Cells connect to the outside world - via integrins they are associated tightly with the extracellular matrix. Similarly - with involvement of overlapping protein machinery, cadherins mediate cell-cell contacts. Some of the RTK / Raf / ERK connections to cell adhesion and integrin signaling will be outlined in the chapter 2.3 on migration and adhesion.
... Ion Channels and Transporters: Besides their tight integration with the cytoskeleton, both RTKs and integrins activate ion channels and pumps e.g. via inositol-phosphates, such as the ubiquitous Na+/H+ exchangers (NHE), or calcium channels [358, 187]. More downstream components, such as the 14-3-3 scaffold protein and ERK target p90Rsk also interact with NHE [272] and so do cAMP as well as ATP [156, 53, 100, 180, 5, 111]. Of course also ERK has been observed to activate e.g. NHE1 [181]. NHE proteins control the intracellular pH values. The cascade is also tightly directly linked to Cx43 gap junctions, mediating 'pH gating', the closing of these cell-cell connections upon pathogenic intracellular acidification [266]. While a general function of growth factor induced decrease or increase of intracellular pH [329, 556, 106] are not known, they were frequently observed to be linked with the calcium signaling branch [215, 185, 422, 289, 463, 93].
... ROS signaling: A recent branch of research identifies, yet another group of small metabolites, the reactive oxygen species (ROS) as important mediators of receptor clustering and lateral signal propagation [4], and stable and strong signaling to the ERK cascade. ROS generation - putatively via RTK mediated activation of a REDOX pathway involving NADPH oxidase, mediates inhibition of protein tyrosine phosphatases responsible for RTK inactivation, by reversible (!) oxidation of cysteine residues [477]. This double-negative, and thus positive feedback is required for EGFR clustering in fibroblasts [391]. The work by Reynolds et. al. includes a nice mathematical reasoning with a small reaction network model, that outlined central questions for experimentation (see left graph in figure 17). ROS induced receptor clustering has recently also been observed to be obligatory for neurogenic TrkA signaling in PC12 cells [229] (right graph in fig. 17). Just to draw a cross-connection to cytoskeletal processes again: ROS has also been found adhesion signaling, where it e.g. inhibits a tyrosine phosphatase responsible for FAK deactivation [69].

[4] i.e. the trans-activation of receptors that are not bound by extracellular ligands

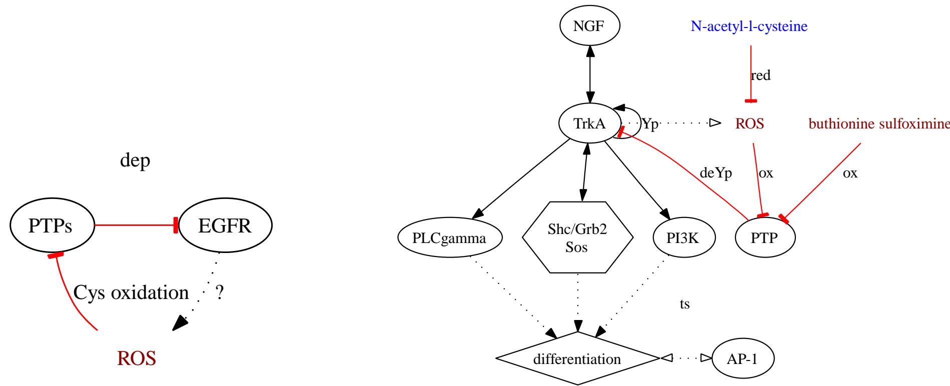


Fig. 17. Lateral receptor activation is mediated by reactive oxygen species, as shown in detail for fibroblast EGFR [391], and for the NGF:TrkA system in PC12 cells [229]

A biophysical Perspective : Protein Charge So we have met massive multiple phosphorylations in large proteineous complexes, but also ion flows, adenosine-phosphate signaling, lipids and lipid products, and even reactive oxygen species involved in receptor mediated signaling into the cell's body. The signals don't just enter the nucleus and 'reprogram' the cell, the cell 'itself' responds, including morphological changes, involving localized reorganizations of the cytoskeleton. Some questions shall shortly be discussed, that in the author's view might get too little attention in cell biological research as well as in recent 'systems biological' approaches. What are (macromolecular) biochemical and -physical implications of all of these processes? The actin cortex - its protein polymers, but also the phospho-lipid membrane - are known to carry a negative net charge, and so does the extracellular matrix (ECM).

From a biophysical perspective, a more general discussion of the phenomena of complex formation at the membrane could be encouraged. Receptor clustering is mediated by ROS signaling [391, 229] and potentially directed by differential membrane compartments, where also active Ras proteins cluster [364]. Cholesterol, a defining compound of membrane rafts, alters the dipole moment of the membrane (i.e. the membrane potential), and this might affect receptor signaling [12]. And finally Raf activation is tightly linked to lipid modification, especially such cholesterol containing rafts [183], where active Raf appeared as dot-like structures in fluorescence microscopic imaging [184]. Intracellular calcium oscillations are known to be induced by the lipid signaling branches of RTKs, GPCRs, Ras itself,

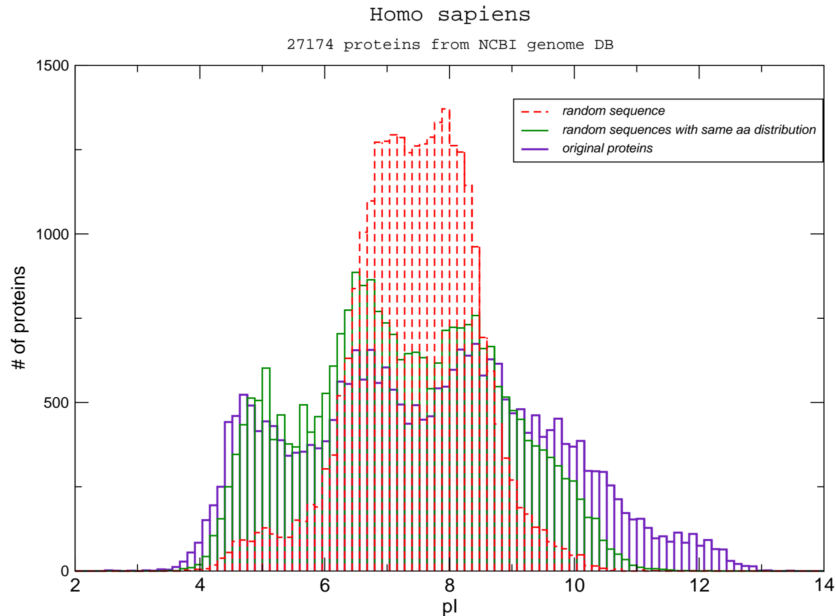


Fig. 18. Predicted isoelectric point (iP) distribution of the human proteome. Three peaks arise through properties of individual amino acids, but are supported by global natural and further enhanced by individual proteins' amino acid composition.

but also by extracellular calcium waves, often implicated in multicellular integration during development [148, 517, 459, 506]. How does a massive local increase in concentration of the bivalent cation calcium interact with this negatively charged matrix? Can the intense multiple phosphorylation events at RTK complexes be re-interpreted from this viewpoint? An ATP consuming trans-phosphorylation immobilizes a negative charge from the small (thus relatively fast diffusing) ATP metabolites to large-scale macromolecular complexes. Does such a charge immobilization (functionally) influence the constitution of the membrane and cortex compartments by itself, independent of the (sequence-) specific events of protein interactions?

Interesting preliminary evidence of in silico proteomic analysis points to a general and old role of protein charge in general. Unfortunately, annotation of protein databases with information of subcellular localization is still sparse, but allows first approaches for global analysis. Figure 18 shows the distribution of automatically calculated isoelectric points of the human proteome, compared with random sequences of the same amino acid distri-

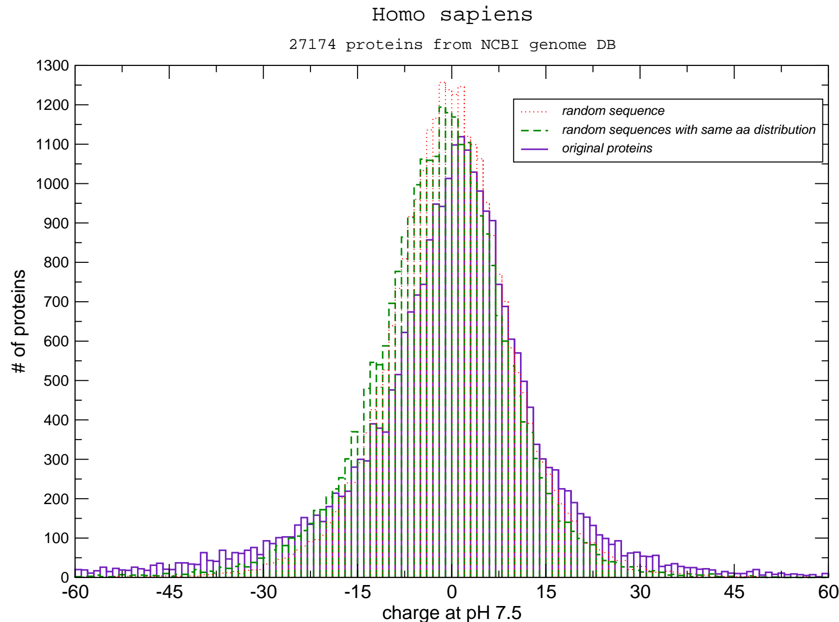


Fig. 19. Predicted charge distribution of the human proteome at pH 7.5. Highly charged protein can not be explained by amino acid composition and might represent a functional role of protein net charge. There is a general deviation towards positive charges and positively charged proteins tend to have a nuclear localization and are often involved in nucleic acid binding.

bution and with completely random sequences. It appears that the natural protein composition leads to tri-modal distribution of isoelectric points (pI, see figures 18 and 19), while in bacteria the distribution is in all analyzed cases only bimodal [426, 524] (R.M. and Stefan Washietl, unpublished data, some preliminary test runs can be browsed at `http://www.tbi.univie.ac.at/~raim/proteincharge/`). While the three peaks arise through properties of the amino acids themselves rather than through sequence evolution [524], an analysis of proteome annotation pointed to a function in subcellular localization. E.g integral membrane proteins clustered at the pI 5.5 peak, and cytoplasmic proteins accumulate at pI 9. The third eukaryote-specific peak at pI 7 is thought to represent nuclear proteins [426], which can be further subdivided according to their pI values [32]. While calculated pI values don't account for protein structure — e.g. completely neglecting disulfide bridges of cysteine residues, they inspire speculations of a general function of protein charge in subcellular distribution and point to a po-

tential biophysical function of hyper-phosphorylation of proteins. It is for example well known, that proteins are less soluble at pH values near their pI [32], i.e. when they carry a neutral net charge. c-Raf in its 'desensitized' cytosolic state can carry 13 or more phosphate residues (see figures 7 in chapter 2.1.2 'The three Raf kinases' and 41 in the discussion, chapter 5). The mouse c-Raf amino acid sequence (Swiss-Prot entry Q99N57) obtains a theoretical pI of 9.4 when using the online calculation tool at `http://bioweb.pasteur.fr/seqanal/interfaces/iep.html`. It is thus probably positively charged, calculated to + 24.11 at pH 7. The sequential phosphorylation cycles will gradually decrease the pI towards cellular pH values. Phosphorylations on e.g. 13 positions will introduce 26 negative charges instead the neutral S, T and Y residues and thus will result in a pI near cellular pH values. Thus, it is allowed to ask whether pure electro-static forces are involved in Raf's cytoplasmic localization in inactive particles, in clustering of active Raf complexes at the membrane, or in transport to mitochondria or trailing edges of migrating cells. Without collecting the relevant interaction data and numbers (of net charge distributions, membrane potentials, protein and metabolite concentrations, actual phosphorylation reactions. etc.), not much more can be said about such hypotheses. However, they open a fascinatingly simple perspective for a global picture of the complex intracellular signaling networks and the function of charge altering post-translational modifications.

An Evolutionary Perspective : Raf Gene Duplication The Cdc42-PAK1 module, which was in part outlined above, is central to neutrophil chemotaxis [278, 548, 310] and will be discussed in this context in chapter 2.3. The slime mold Dictyostelium is evolutionary far away from vertebrates, but uses a similar machinery for directed migration. Cdc42 has not yet been identified in Dictyostelium but interestingly, its PAK homolog seems to have a different role at the tail rather than at the leading edge of the cell [497].

The three vertebrate Raf genes have been derived from a single invertebrate protein by gene duplication. Alignment of manually chosen common core sequences of the Raf genes places B-Raf closer to the invertebrate Raf gene [52] (see fig. 20). Several scenarios of their functional radiation after duplication events can be imagined. E.g. different functions of the old Raf protein could have been split between the new proteins, or the new proteins could have acquired completely new, slightly changed or even opposing functions by mutation and incorporation of additional domains, often involved in differential localization signals. Does the single Raf protein have e.g. c-Raf's

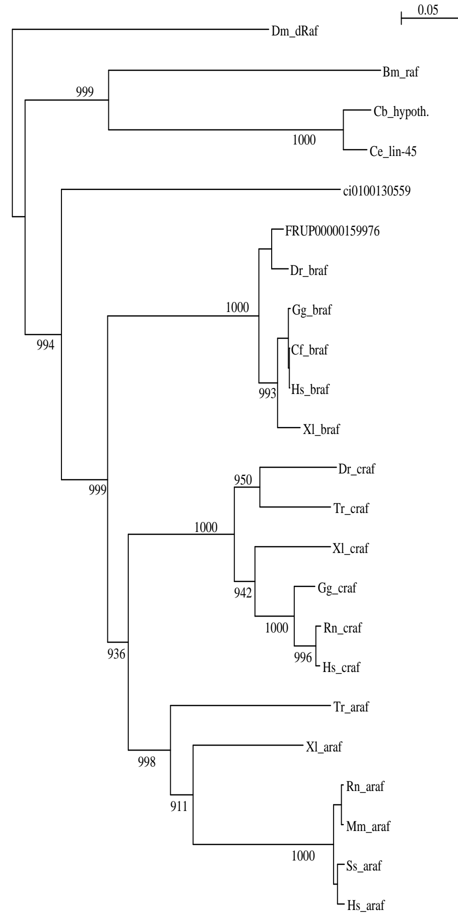


Fig. 20. Alignment of metazoan Raf proteins, and predicted proteins from Ciona intestinalis and Takifugu rubripes (Fugu rubripes) using ClustalW's implementation [472] of pairwise alignment algorithms [157]. Ciona and Fugu genes have been obtained by online Blast search at the Joint Genome Institute's website at http://www.jgi.doe.gov/. Numbers at the branches are bootstrap values.

functionality in migrational scaffolding of RhoA/Rokα signaling at the trailing edge [122] or inhibiting apoptosis mediators at mitochondrial pathways [360], or are these new 'inventions' of c-Raf? A possible hetero-dimerization of c-Raf and B-Raf, [519, 507] (reviewed in [104, 19]) offers probably the most fascinating evolutionary scenario. Is such a direct cross-regulation involved in the cooperation in spatio-temporal modulation of ERK activity? A general outline for a detailed computational and conceptual analysis of

evolution of di- or oligomeric S/T protein kinases and derived scaffolds follows in the discussion, chapter 5. It will require both experimental and computational analysis to resolve such questions. In the meantime, let's return to the cascade and follow ERK ...

2.2.2 ... to the Nucleus

Many questions about cytoplasmic signaling mechanisms lie still in the dark, only fractions of specific protein-protein interactions and their integration with second messenger and ion signaling are understood. However, let's follow ERK to to an even darker place [5], the nucleus.

Nuclear Signaling Complexes Things are - as usual - quite complicated when looking at the details. Not only ERK and possibly MEK are translocated to the nucleus. Many and functionally diverse proteins are found to cycle between nuclear and cytoplasmic locations. Just to note one interesting example, recently a whole nuclear lipid signaling mechanism has been identified, that relies on the same - translocated - components as membrane lipid signaling: PI3K, PLCγ1 [555, 554] or PKC isoforms and e.g. the DAG kinase (DGK) [480] (see e.g. the review by Neri et.al. [340]). Other examples of observed nuclear translocation upon growth factor signaling include e.g. PAK1 [436] and fascinatingly even FGFR isoforms [377, 378, 446].

However, as stated above, ERK is thought to dimerize upon dual phosphorylation. Mutation of dimerization motifs hinders active transport to the nucleus [3]. This and related studies have however established that ERK can also enter the nucleus as an inactive monomer. In fact a picture emerges, that there is a continuous shuttling of ERK between cytoplasmic and nuclear compartments. ERK has however neither a nuclear import sequence (NLS, nuclear location sequence) nor a nuclear export sequence (NES). In contrast MEK carry a highly efficient NES and have been speculated to also been shuttled and constitute the 'driving exporting-force' for inactive ERK, which unlike active, is associated with MEK [4] in the cytoplasm of resting cells. Long mitogenic signaling and sustained ERK activation, lead to nuclear accumulation of inactive ERK. Nuclear MKP1 and MKP2 inactivate and sequester inactive ERK in the nucleus forming a nuclear 'inactivating

[5]The emerging research topic of small non-coding RNAs has led researches to call such functional(ly structured) RNA molecules 'the dark matter' of biology, due to there notorious resistance to experimental or computational detection

center' as opposed to a cytoplasmic 'activating center', a MEK-ERK containing complex. Please consult the review by Pouyssegur and Lenomard for details about the latter interpretation and its experimental evidences [375]. However, apart from fragmentary details, e.g. even a possible involvement of endocytic trafficking [239], the exact mechanism of ERK nuclear export and import are largely unknown. As shown for the STAT proteins — indicated by a theoretical model and validated by experiment — the dynamics of nuclear-cytoplasmic shuffling can be crucial for signal outcome [458].

'Immediate Early Genes' as Feedforward Sensors Mitogenic and other signals induce, apart from direct changes in metabolism and cell structure, the activation or repression of the expression of so-called 'target genes'. In the case of MAPK (ERK, JNK, p38MAPK) activation, target gene expression can be differentiated into immediate early, early and late response genes. The 'immediate early genes' (IEG) consist largely of transcription factors (e.g. Fos, Jun, Myc, Egr-1), that guide subsequent expression of late response genes, but also include the above mentioned nuclear MAP kinase phosphatases (MKP-1 and 2), which thus act as negative feedback modulators of ERK activation [62, 455, 191, 46, 407] (see chapter 2.4). Transient and sustained activation of ERK activate different sets of target genes, when e.g. induced by different doses of LPA in Rat-1 fibroblasts [81].

Studies by Murphy and colleagues have identified several important characteristics of IEGs, that mediate interpretation of signal strength and duration [336, 335]. As previously known, c-Fos and other ERK targets are unstable proteins with half-lives of less than an hour, due to immediate ubiquitinylation and proteasome-mediated degradation. They can be stabilized by an initial phosphorylation by ERK or its downstream effectors of the p90 Rsk family (90K-ribosomal S6 kinase) (see e.g. [357, 138]). Murphy and coworkers identified a so-called DEF domain (**d**ocking site for **E**RK, **F**XFP, see [220]) in the analyzed IEG products. Upon initial phosphorylation of the IEG proteins, the DEF domain (by e.g. ERK or Rsk1) is exposed and can bind activated ERK, at least in vitro, while in vivo the corresponding DEF amino acid residues are required for transcription factor activity. In this model, ERK would bind and then phosphorylate further residues in its own IEG products, and moreover active ERK would get locally concentrated at the transcription site and mediate unknown further effects on gene expression. Due to the delay between initial expression, translation and translocation to the nucleus, these transcription factors thus act as **feedforward sensors** for sustained ERK activity. In the absence of further activity, the

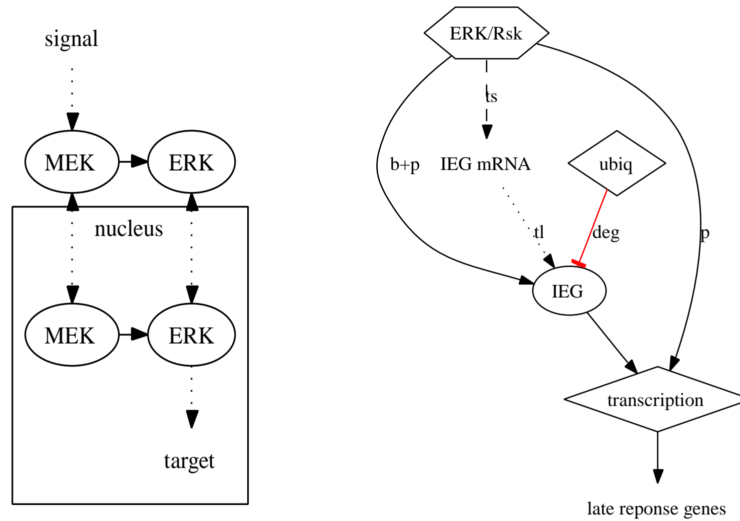


Fig. 21. Nuclear Targets of ERK; left: ERK and possibly MEK are translocated to the nucleus upon activation; Immediate Early genes (IEG) can act as 'feedforward sensors' for sustained nuclear ERK and Rsk activation: their transcription is initiated upon early ERK and Rsk activity, but the proteins are quickly degraded unless active ERK/Rsk stabilize them by specific binding and phosphorylation. Some IEGs then work as transcription factors for late response genes, and this transcription again depends on additional downstream targets of ERK/Rsk [336, 335].

IEG products will just be quickly degraded, while they require sustained ERK activity to exert their effects as transcription factors. An interesting recent contribution to the PC12 cell problem (differentiation vs. proliferation), shows that constitutively active Rsk1 mutants were completely sufficient for PC12 differentiation [435]. Rsk activation requires sequential ERK mediated phosphorylation, autophosphorylation and PDK1 mediated phosphorylation, and thus RTK mediated lipid and Raf/MEK/ERK signals again converge on this crucial mediator [224, 138] in differentation signaling.

A similar 'feedforward' scenario, initial induction and later stabilization, can be speculated for negative feedback regulators MKP, as they are also unstable and require further ERK mediated phosphorylations to become stabilized [47, 441]. In this case the feedforward sensor would mediate negative feedback (see left graph in fig. 8).

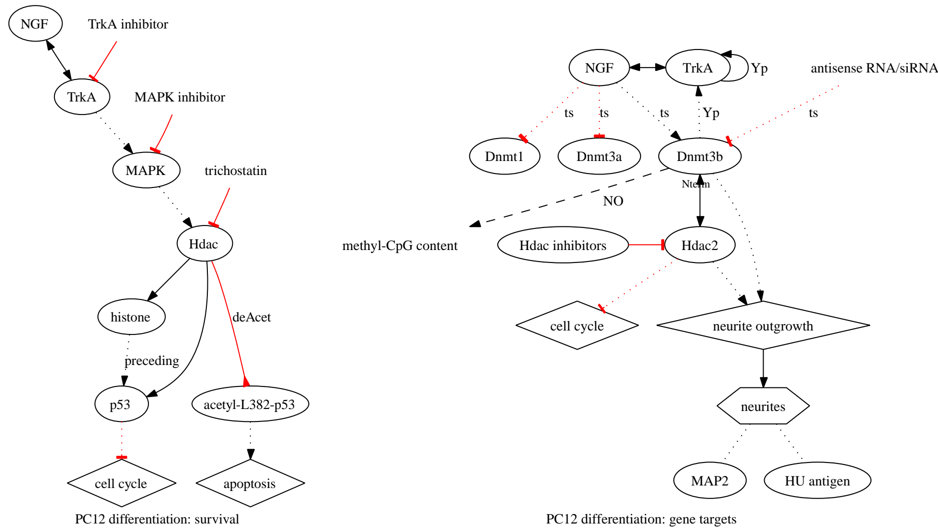


Fig. 22. Chromatin remodeling involved in NGF mediated cell cycle arrest and neurite outgrowth in PC12 cells. After [494, 20].

2.2.3 Feeding into the Cell Cycle

The basic regulatory network of the cell cycle can be conceptualized as a step-wise oscillator, with stationary states in during each of the $G_{0/1}$, S, G_2 and M phases [490, 373, 546]. The MAPK modules feed into this complex 'step-cycle' and trigger or block transition between the multiple stationary states. As stated above MAPK activation is often necessary, and for some cell types sufficient, to enter the cell cycle. In HeLa cells and NIH 3T3 fibroblasts, cell cycle progression has been observed to require cyclic ERK and PI3K activities, but each branch during different phases [394]. After inducing the G_1/S transition through activating expression of Cyclin D1/D3 and other cell cycle regulators, ERK is further involved e.g. in nucleotide synthesis during S phase [400, 375].

Intermingled feedback cycles determine complex responses of the protein-protein interactions in regulation of cellular functions. Positive cycles can lead to multiple stationary states (the phases) and negative feedback cycles can lead to adaptation (to a signal) or - if a delay (e.g. a transcription process) is involved - cause oscillatory behavior. It is clear, at least since the ideas and experiments of Jacob and Monod on the E.coli lac operon [219, 218], that feedback is crucial to biological regulation[328]. Several

models address feedback properties within and around the MAP kinase cascade, and simple models of adaptor protein or enzyme targeted feedback have been analyzed for their capability to explain above temporal regulation of ERK activity [45, 13]. A recent work tries to incorporate the knowledge of differential Ras/Rap1 and c-Raf/B-Raf interactions in PC12 cell EGF and NGF signaling [411]. The experimental evidence indicates however, that large scale processes, such as receptor internalization and its kinetics will have to be accounted for in theoretical models. Birgit Schoeberl et.al. [419] have presented a more detailed and experimentally backed model of differential ERK activation by membrane and internalized receptors, and this model can serve as a well-defined starting point for further extension to the PC12 cell problem. The models by U. Bhalla and R. Iyengar finally incorporate the ERK cascade into large-scale networks with multiple feedback in neuronal regulation [30, 31]. They speculate on the bistability - shown by positive feedback of the ERK cascade with lipid/calcium and PKC signaling - as a potential biochemical memory mechanism. A short pulse of signal can switch on the positive feedback cycle, and it keeps itself on long after the signal has disappeared [30]. An additional negative feedback, involving transcription of an MKP phosphatase can then render the system mono-stable for subsequent activating pulses. When the MKP is expressed, the system reacts with short and lower ERK activity, without being able to switch to the independent stable activity [31].
However, comparing these models with experimental knowledge on protein interactions, reveals their vast simplification of the processes and restricts their general applicability for modeling 'real life' large-scale signaling networks. The models are based on crude parameter estimations, fitting the simplified reaction network to experimental data, which however is only measurable for some cornerstones of the modeled reaction system. Spatio-temporal interactions usually involve huge macromolecular protein complexes and fine spatial organization of pathways. Modeling in detail would exponentially increase the sizes of current models. An alternative approach to employ such modeling techniques to questions of the cascade, thus asks more fundamental questions and analyze the cascades' basic architecture. The kinases organization into a cascade of several dual phosphorylation events could lead to 'ultrasensitive' behavior. A broad range of low signal concentration will not effect the cascade, but upon increase over a threshold, the cascade gets fully activated [202, 131] (see chapter 2.4). On the other hand, the cell cycle itself has been a great subject for theoretical studies, by e.g. the groups of J.J. Tyson or J.E. Ferrell. The cell cycle phases can be explained in terms of stability and oscillatory behavior, arising from inter-

mingled feedback cycles on both, biochemical and transcriptional regulation levels [129, 490]. One of the recent important contributions in this regard examines the role of bistability of Cdc2 activity in establishing the mitotic oscillator [373]. The MAP kinase cascade can trigger the transition or support the stability (and function) of these phases [400, 375]. A coupling of the bistable MAP kinase cascade with the bistable Cdc2 system cooperate in the irreversible triggering of the cell cycle in Xenopus oocytes [546].

SBML versions of some of the cited models are available at `http://www.tbi.univie.ac.at/~raim/mapk.html`. Chapter 2.4 will introduce the concepts of ultrasensitivity and above outlined bistability and irreversability as applied to the MAP kinase cascade, as prepared by Goldbeter and Koshland and elaborated by Ferrell, Kholodenko and many others. But let's first elaborate on the complex cell-biology of ERK regulation from one more perspective, that will be relevant to interpret and evaluate signal transduction and its cellular functions from the perspective of computational modeling.

2.3 Spatial Coordination and Cell Motility

Obviously spatial coordination is of immediate relevance for global cellular functions, such as adhesion to extracellular matrix or to neighboring cells, cytokinesis and directed migration. The subcellular organization of the modules controlling these processes is thus a direct *ligne de fuit* (*Fluchtlinie* as interpreted by post-modern philosophers Deleuze and Guattari [99]) to organization on a cellular and multi-cellular level. Here only a tiny fraction of these processes is discussed, again with a special focus on the integration of RTK and Raf/MEK/ERK signaling pathways.

2.3.1 Adhesion ...

PAK1 and the Integrin connection To stay at the plasma membrane and with upstream kinases for c-Raf in MEK/ERK activation, the PAK family of kinases, especially PAK1, shall be discussed shortly, as the recent insights in its regulation of c-Raf allow to point to one important function of c-Raf, the integration of mitogenic and survival with adhesion and migratory signal responses in vertebrates, as well as to putatively older cross-connections, the above mentioned lipid, calcium and cAMP pathways.

Several members of the Ras superfamily of small G proteins are central players in adhesion and migration signaling. First, RhoA is mostly respon-

sible for generating acto-myosin based contractile forces, responsible for the formation of integrin-based focal adhesions, that link extracellular matrix proteins such as fibronectin to the cytoskeleton via so-called stress fibers consisting of actin bundles. cAMP has long been known to induce disassembly of cytoskeletal organization, mainly by opposing the functions of the RhoA and its effectors. These processes have been reviewed by Schoenwaelder and Burridge [420] and are sketched in fig. 25. Second, Cdc42 and Rac are close relatives of RhoA, but — quite similar to the Ras and Rap1 pair — serve opposing functions. The p21-activated protein kinases (PAK) are effectors of Cdc42 and Rac and modulate adhesion and cytoskeletal signaling. PAK are homologs of the yeast Ste20 MAPK scaffold, and thus apparently old regulators of this pathway. PAK1 and PAK3 have long been suspected to act as S338 kinases of c-Raf [244, 63, 456, 482]. Although conflicting experiments cast doubt on a role of PAK3 in S338 phosphorylation [70] and even the necessity of S338 phosphorylation in general [352], recent evidence indicates that PAK1 not only phosphorylates c-Raf, but also MEK and moreover can act as a scaffold protein for all three kinases of the cascade at integrin adhesion sites. This complex mediates communication between growth-factor signaling via RTKs and adhesion of the cell to collagen fibrils of the ECM (extracellular matrix) via integrins [199, 119, 457].

Howe and Juliano showed, how the well-known phenomena of anchorage-dependence might be mediated via PAK, and via another well-known phenomenon, the inhibition of c-Raf/MEK/ERK signaling by cAMP inducing stimuli [199, 121]. Two different kinds of anchorage-dependence are known: upon loss of ECM adhesion a failure in activation of c-Raf/MEK/ERK by growth factor signaling induces fibroblasts to acquire a state of growth-arrest, while epithelial and endothelial cells even undergo 'anoikis', a special form of apoptosis [137]. The left graph in fig. 23 comprehends the model, which Howe and Juliano deduced from their experiments. In short: adhesion to the ECM via integrin complexes, and general 'cytoskeletal integrity', especially that of the cortical actin layer, keep PAK active and available for c-Raf activation. Loss of adhesion seems to activate cAMP/PKA signaling, that has long been known to disrupt the cellular cytoskeleton and especially the cortical actin layer ([420], see fig. 25), within which receptor complexes reside. PKA mediated phosphorylation then inhibits PAK for c-Raf activation. Thus an inhibition of the cAMP/PKA systems allows transient, anchorage-independent c-Raf activation by PDGF in these cells [199] (see left graph in fig. 23). Liisa Sundberg-Smith and coworkers recently observed how PAK1 can act as a scaffold protein, sequestering the c-Raf/MEK/ERK

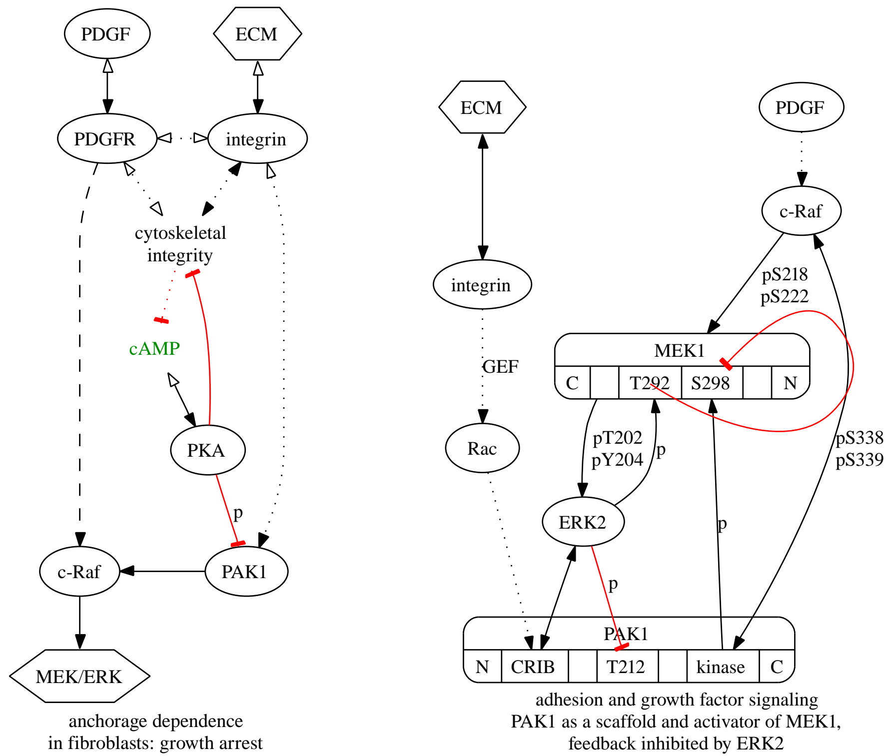


Fig. 23. Cell-cell adhesion via integrins interacts at multiple points with Raf/MEK/ERK activation, and PAK has been observed to be an important mediator, by scaffolding the cascade at integrin adhesion sites [199, 119, 457]

module at adhesion sites, and as a modulating kinase at the same time. In this complex, PAK1 activates MEK1 by phosphorylation at S298, enhancing Raf binding to MEK and thus increasing transmission to ERK, as previously shown [139]. ERK2 then quickly inhibits PAK1 in a direct negative feedback phosphorylation [457], and by direct phosphorylation of MEK1. The latter inhibits PAK1 phosphorylation of MEK1. [119]. The right graph in fig. 23 depicts the observations of Sundberg-Smith et.al. and Eblen et.al.

2.3.2 ... or Migration

Together, these and much herein uncited data point to a tight (spatial) integration of the integrin and the RTK mediated signaling complexes [420, 137].

This integration is not only relevant for adhering but also for migrating cells. Especially the quick feedback control of PAK/ERK activation could play an important role in directional migration [457]. Let's elaborate on migration shortly, as it allows to take a step out and broaden our perspective to outline the functional integration of signaling 'pathways' with each other and with their biochemical and biophysical environment, to draw the connection to organismic function in vertebrate biology, and to finally get an impression of possible evolutionary scenarios of Raf functions.

Lamellipodia Cycles & Polarization Migration of a resting cell usually starts with a process called spreading. The cell forms so-called lamellipodial extensions at its periphery in a cyclic process every 30 to 60 seconds. During directed migration, the usual case in the organismic (vertebrate) context, lamellipodial and pseudopodial extensions are preferentially built at the side of higher chemo-attractant concentration, which ultimately leads to polarization of the cell in the gradient [497]. The extension of lamellipodia and pseudopodia is often preceded by generation of filopodia. The formation of these structures is known to be linked, e.g. via lipid signaling and small G proteins, and fig. 24 depicts some fraction of the involved processes. Polarization can, however, also be acquired spontaneously, without a gradient, and depends on differential PI3K lipid signaling and the opposing PIP3 phosphatase PTEN, resulting in global cellular intra-membrane PIP3 and PIP2 gradients [497, 522]. A positive feedback loop of mutual activation of the small G protein Rac and the PI3K works as bistable system, establishing PIP3/PIP2 gradients much steeper than the extracellular gradient of the chemo-attractant molecule [508]. Rac and PIP3 induce actin polymerization via nucleation factors such as the Arp2/3 complex [526]. Another positive feedback within a complex of PAK1, binding and activating PIXα, which acts as GEF for Cdc42, which again activates PAK1, is now thought to be the main mediator of polarization ('the compass' [310]) within an extracellular gradient. Supported by mathematical modeling Wedlich-Soldner et.al. that Cdc42 activity can alone lead to - spontaneous or induced - cell polarization, by inducing actin polymerization, which again reinforces local Cdc42 activity by actomyosin-based delivery of more Cdc42 proteins to the site of its initial activity, thus also depleting Cdc42 from other sites[521, 523].

Focal Adhesion Turnover New integrin-ECM connections are build up at the leading edge of a lamellipodium, to form focal contacts. The integrin complexes link to the actin cytoskeleton via various structural proteins

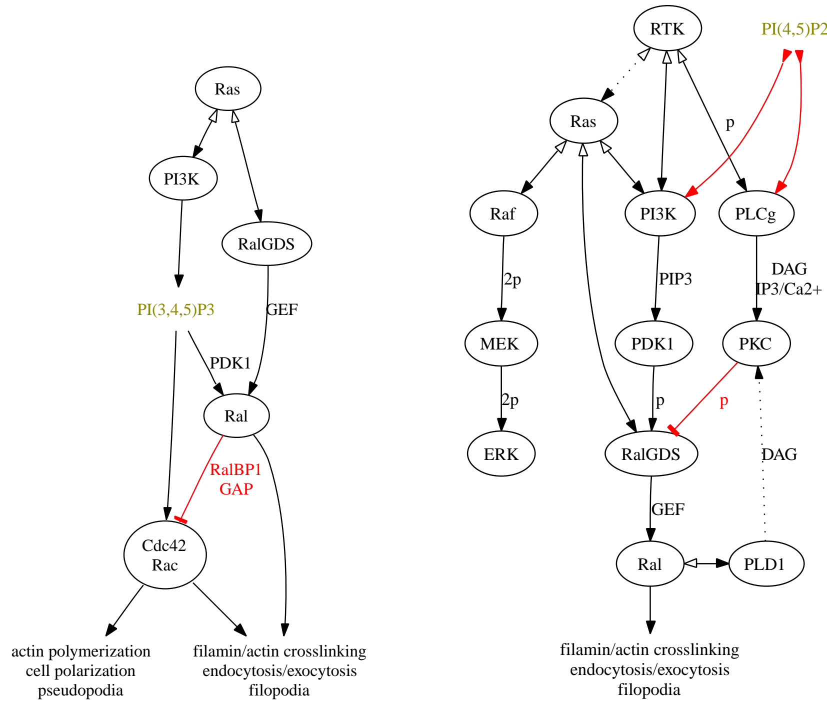


Fig. 24. Example for complex relations of small G proteins of the Ras and Rho subfamily with lipid signaling, and generation of filopodia and pseudopodia involved in directed cell migration or neurite outgrowth in PC12 cells [355, 149, 406, 475]. Left: Ral induces filopodia and suppresses Rac/Cdc42 induced formation of pseudopodia [355], which are sequentially build during directed cell migration. PI(3,4,5)P and Rac/Cdc42 determine cell polarity during migration. Right: Deactivation of RalGDS by PKC mediated phosphorylation can redirect Ras signaling towards the Raf and PI3K branches, as observed in NGF/TrkA signaling in PC12 cells [149, 406], while PI3K's product PI(3,4,5)P3 activates PDK1, which might be the second stimulus required for RalGDS activation by Ras. This relation was established in EGFR signaling in 293T and COS-7 fibroblasts [475]. PI3K and PLCγ consume the same substrate, while having opposing actions on RalGDS activity. Ral activates phospholipase D (PLD) to produce phosphatidic acid, that is usually converted to DAG, an activator of several PKC isoforms. This might constitute a potential negative feedback of the RalGDS branch, reinforcing the PI3K and/or Raf branches of Ras signaling.

(scaffolds), such as vinculin, talin, ezrin-radixin-moesin (ERM) proteins and α-actinin These complexes host e.g. the phosphatidyl inositol-5-kinase PIPKIγ, which enhances their membrane association via PIP2 production [43]. At the leading edge, the newly formed adhesion contacts link to newly polymerizing cortical actin skeleton. When the cell moves over, periph-

eral contacts can either mature into the larger focal adhesion complexes, or be disassembled. Such dynamic turnover involves the adapter and scaffold protein paxillin [488, 417], which provides a platform for the protein tyrosine kinases Src and FAK (the focal adhesion kinase) [325] mediating both formation and turnover at the front of the migrating cell. The role of ERK2 in this turnover is quite complex. Migratory signaling by EGF induces disassembly of focal adhesions and stress fibers, requiring MEK activity [543]. ERK2 is only transiently present at focal adhesion, compatible with a role in their disassembly [516], but are also involved in assembly of newly forming adhesions and their connection to the cytoskeleton. It has been proposed that ERK phosphorylation of cortactin switches on its ability to activate N-WASP for Arp2/3 activation, while Src phosphorylation inhibits this function of cortactin [299]. Hunger-Glaser et.al. observed, that in NIH 3T3 fibroblasts PDGF and FGF activate FAK via PI3K induced auto-phosphorylation at Y397 and at S910 phosphorylation by ERK2 with differential dose-dependence and kinetics [204]. The recent review by Mitra et.al. presented the following scenario: an initial auto-phosphorylation at Y397 creates a binding site for Src, which then further phosphorylates Y576 and Y577, thereby inducing maximal catalytic activity of FAK. Then a Cas/Crk complex binds and is phosphorylated by FAK to facilitate Rac activation and subsequent lamellipodia formation and migration. Src further mediates phosphorylation at Y925 in the paxillin binding FAT (focal adhesion targeting) domain of FAK, which could be involved in dissociation of FAK from paxillin and binding of Grb2 to the Y925, which could then activate Ras and the cascade, specifically ERK2. ERK2 phosphorylation of S910 of FAK further decreases FAK/paxillin interactions, while Src/FAK phosphorylation of paxillin at Y118 promotes ERK2 binding and further phosphorylation of paxillin, which facilitates FAK binding. This complicated meshwork of feedback mediated protein phosphorylation and complex formation and dissociation cycles let Mitra et.al. to conclude that *'Src- and ERK2-mediated phosphorylation of FAK promotes its release from focal contacts and ERK2-mediated phosphorylation of paxillin promotes the association of unphosphorylated FAK with paxillin at new or growing focal contact sites'* [325]. Detailed kinetic data for formation and disassembly dynamics of these protein complexes, including the p130Cas and the MLCK (myosin light chain kinase) proteins, have been measured in Webb et.al. [515] and could be helpful in designing of computational models of above and alternative models. In the same review, the PAK mediated activation of ERK has been considered as a 'secondary route' of PAK activity, while the 'primary route' is involved in cellular morphology regulation [325].

Uropod Retraction At the trailing edge (the uropod) PTEN consumes PIP3 to produce PIP2. Migrating cells establish a gradient of calcium, with a higher concentration at the rear end [170], and PIP2 at the rear could likely be involved in establishment of this gradient, as it is a substrate for PLC catalyzed production of IP3. Calcium has been observed to be involved in myosin II based uropod detachment and retraction [120, 113], e.g. via CaMK phosphorylation of the myosin light chain kinase (MLCK), see fig. 25. The review by Pettit and Fay (1996) provides several details on calcium and cytoskeletal interactions [369]. More recent observations include e.g.calcium modulation of focal adhesion kinase (FAK) mediated turnover of FA. Its local oscillations correlated with the FAK cycling between FA and cytosol [146, 145]. However, the main player at the rear is RhoA involved in formation and contraction of actin and myosin filaments, and subsequent disassembly of adhesion complexes. Cdc42 and Rac mediated F-Actin assembly at the edge and RhoA mediated microtubuli assembly at the rear of the cell mutually inhibit each other ([278, 548], reviewed in [310, 126]), thus constituting another - overlayed - positive feedback cycle of polarized activity.

Interestingly, The recent evidence that c-Raf might directly inhibit the RhoA effector Rok-α adds a further track to the cascade's connection with cytoskeletal remodeling, although it is not clear whether receptor activation - and maybe priming by ERK mediated feedback phosphorylations (see 7) - are required for this c-Raf activity [122, 19]. How does this all relate to above outlined scaffolding of the c-Raf/MEK/ERK cascade by PAK1 [139, 199, 457, 119, 121] and the experiments, that showed mitochondrial translocation of c-Raf, when phosphorylated at S338 by PAK1 [8]? A lot of further experimentation will be required to answer these questions. In the meantime, let's finish the outline of basic modules of cell migration by closing the cycle to apoptosis regulation. This might again intersect with migratory signaling at the level of c-Raf's inhibition of Rok-α. The conditional c-Raf knock-outs, where hypersensitive to Fas ligand induced apoptosis [207, 318]. Hyper-phosphorylation of Rok-α target ezrin was considered responsible for the bundling of cortical actin. This seemed to mediate clustering of Fas and inhibition of its internalization, and thus rendering the cells hyper-sensitive to Fas-induced apoptosis [19].

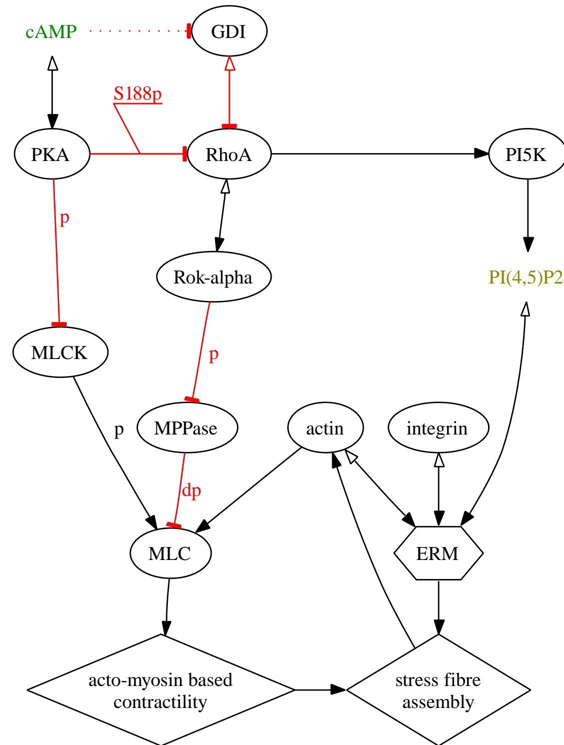


Fig. 25. Focal adhesions (FA) connect the extracellular matrix to actin stress fibers, e.g. via vinculin and the ezrin-radixin-moesin proteins (ERM). The formation and stability of stress fibers and FA depend on acto-myosin based contractility. The myosin light chain (MLC) promotes actin-activated myosin ATPase activity, resulting in contraction. MLC requires phosphorylation by MLC kinase (MLCK), which is opposed by the Myosin Phosphatase (MPPase). The small G protein RhoA activates the protein kinase Rok-α, which inhibits MPPase, as well as phosphatidyl inositol-5-kinases, that produce PI(4,5)P2, that is again required for focal adhesion assembly. cAMP and PKA cooperatively inhibit these processes at several points [420].

2.3.3 A Moving Gel

Useful and overlapping abstractions of the process of directed migration, identify several functional/dynamic modules of migration on a macroscopic (cellular) level, i.e. the oscillatory formation of pseudopodia, cell polarization and directional sensing and finally movement by leading edge extension and uropod retraction [497]. Again - similar to cell cycle - several feedback cycles, negative and positive and intermingled to produce the complex

macroscopic behavior of cellular migration. On a microscopic (macromolecular) level, these processes can be dissected into basic regulatory modules of protein interactions, that control the cycles of adhesion sites, and the dynamic organization of the cellular cytoskeleton with actin polymerization at the front and acto-mysoin based contraction at the rear [393, 522]. Adhesions sites couple the cell to the extracellular matrix, and ultimately, and integrated view will have to account for the basic physical forces that a cell exerts on the matrix and vice versa. An often cited review by Lauffenburger and Horwitz, was written in 1996 [268], at an only recent time, when however the basic molecular wiring of regulatory protein interactions were less well understood, outlines the latter questions, that are often neglected in more recent works. The *cellular tensegrity* concept was first proposed by Ingber [210]. It assumes that *'tensional forces are borne by cytoskeletal microfilaments and intermediate filaments, and these forces are balanced by interconnected structural elements that resist compression, most notably, internal microtubule struts and extracellular matrix (ECM) adhesions'* [209]. The mechanical properties of the cell itself and of its surroundings are surely crucial to multicellular live in general, and to mechanically highly complex organisms like vertebrates in special. Of course Ras/Raf/ERK signaling is mediator of mechanical signaling. Mechanical forces (e.g. shear-flow and blood pressure in endothelial vessels or interstitial fluid pressure during inflammation [532]) can be sensed e.g. via integrin-cytoskeletal associations, or stretch-activated calcium channels [334], and (of course) H-Ras is observed to be activated by mechano-sensing mechanisms [333, 267].

Fascinating movies of crawling cells are featured at the website of the Cell Migration Consortium at `http://www.cellmigration.org/science/sci_movies.html` and on laboratory websites linked from there. They impressively illustrate that the current knowledge on protein-protein interactions and regulation, the dynamic processes of adhesion assembly and disassembly and intracellular transport mechanisms miss important aspects to explain the cell-level 'behavior'. See e.g. the famous movie of a neutrophil chasing a bacterium between red blood cells, which was made in 1950s by David Rogers at Vanderbilt University (available e.g. at `http://www.biochemweb.org/fenteany/research/cell_migration/neutrophil.html`). The cell seems to act like an 'intelligent gel-sack', with fine sensors at each minute part of its surface, able to integrate the information into quick and smooth cell-wide responses. Some insights from the glycobiology of ECM polysaccharides offer several nice perspectives in this regard.

A biochemical Perspective : Gel-Sol Transitions Glucosaminoglycans are large polysaccharides of the extracellular matrix, usually negatively charged through sulfation, like chondroitin, heparan, keratan or dermatan sulfates, or merely by an acidic sugar residue, as the vertebrate specific ECM constituent hyaluronan (HA). Experiments and theory show that such molecules undergo a so-called gel-sol phase-transition upon slight changes of environmental conditions, such as a calcium increase [427]. At low calcium concentration they are highly hydrated, forming gel-like matter. When calcium concentration increases above some threshold, the two charges of the calcium connect each with a negative charge of adjacent matrix polymers, leading to abrupt expulsion of the hydration layer, thus an abrupt decrease in volume and a solid constitution. The process is fully reversible, in our example by decreasing calcium concentration. There are many means for inducing such gel-sol transition in various polymeric structures, and this topic will not be elaborated in detail at this place. Interestingly however, HA structure is found to be 'on the edge of instability under physiological conditions', e.g. at a temperature around $37°C$ [427]. A phase transition in an in vitro model of mesenchymal tissue and accompanying changes in viscoelastic properties has been studied, and possible roles in vivo been described by Newman et. al. recently [341]. Whatever physiological functions might be speculated, such gel-sol phase transitions are widely used in drug delivery and thought to mimic the function of secretory vesicles [246, 434], but also amoeboid cell migration [41, 534]. As outlined in a controversial and very informally written book by G. H. Pollack, such phase transitions have long been proposed to constitute a fundamental principle of cellular function, underlying all kind of (cell-morphological) processes - please see [371, 372] for short reviews on the hypotheses and underlying evidence. As shortly outlined above calcium concentration is graded in migrating cells [170]. Its role in myosin II based contractility and uropod retraction via protein interactions could likely be a specific regulation of a potentially very old cellular implementation of a rather physico-chemical phenomenon, where the bivalent calcium ion is involved in such a phase-transition through crosslinking negatively charged (phosphorylated) polymers.

From an evolutionary perspective, HA itself becomes interesting as a vertebrate innovation, that is central to vertebrate specific organismic structures and functions (e.g. the neural crest cell migration and chondrogenesis, endothelial growth and migration, or the dynamic skin) [444]. HA integrates biophysical aspects of morphology and development with the establishment of new functionality on cell level. Its synthases (Has1-3) are most probably

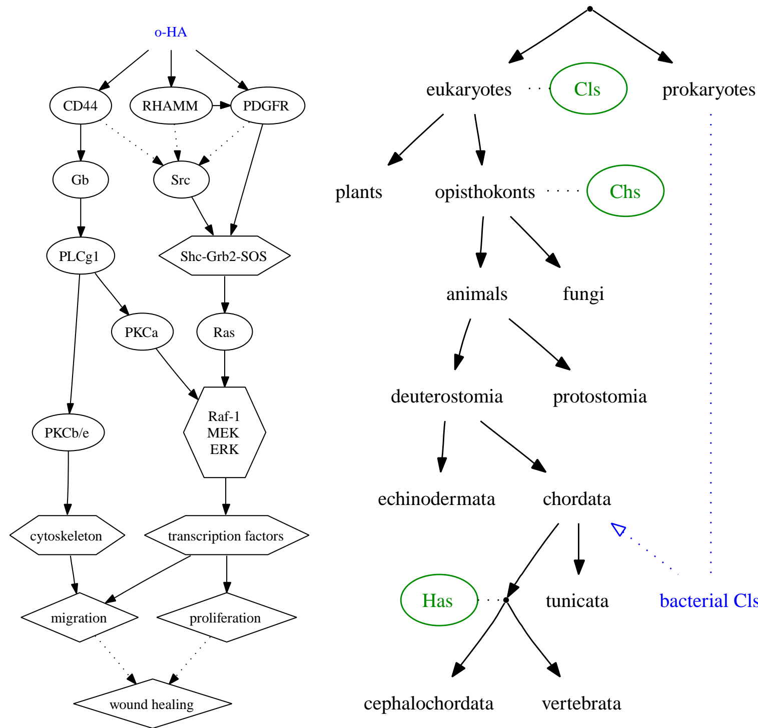


Fig. 26. The Vertebrate Hyaluronan System. Left: mitogenic and migratory Raf/MEK/ERK signaling can be initiated by Hyaluronan oligomers and their receptors, after figures in [439, 444]. Right: The evolution of the vertebrate hyaluronan synthase (Has). The HAS genes are probably derived by gene duplications from a single corresponding gene in the common ancestor of vertebrates and cephalochordates (A.P. Spicer, personal communication), that has itself been derived by mutation from the only remaining metazoan chitin synthase (Chs) [444]. The only close relative of the Has and Chs are the cellulose synthases, of which a bacterial can be found in the Ciona intestinalis genome.

derived from a single chitin synthase, that is e.g. present in the urochordate Ciona intestinalis (see phylogenetic tree in fig. 26). Has' product differs from chitin, in that a negatively charged glucuronic acid is incorporated instead of an N-Acetylglucosamin monomer, which leads to the highly hydrated mesh-work structure of HA polymers. The Has proteins themselves have several transmembrane domains and reside in the plasma membrane, directly involved in pushing the growing HA polymers out of the cell. They seem to act relatively autonomously, as e.g. demonstrated by HA production by a transgene in Drosophila. In contrast, other (evolutionary older) negatively charged glucosaminoglycans of the ECM (see above) require complex

endosomal production cycles and exocytosis pathways. In vertebrates HA polymers and oligosaccharides (degradation products) also act as essential developmental signaling factors. They have acquired their own receptors, which have been integrated into an old cellular regulation network, including the Raf/ERK1 cascade, to transduce migratory as well as mitogenic signals [439, 444] (see left graph in figure 26).

2.4 Signal Amplification : Catalytic Cycles

However complicated the spatio-temporal organization and the upstream and downstream dynamics — and their evolution — might be, the phosphorylation cascade itself can conceptually be described as a catalytic amplification step in signal relay. A minute signal can be enzymatically converted into a full activation of available ERK proteins. Several mathematical models have addressed such phenomena and investigated interesting new properties that emerge from the underlying biochemistry of the catalytic cascade, such as a switch-like or all-or-none response to a continuous signal concentrations. Such a behavior can be described within theory of chemical kinetics under the term *ultrasensitivity*. The next chapter will review this concept and both, experimental evidence and theoretical analysis of such properties of the MAP kinase phosphorylation cascade.

2.4.1 Ultrasensitivity

The first published mathematical model of the MAP kinase cascade, was presented in 1996 by Huang and Ferrell. Based on the assumption that the three-tiered kinase system has evolved to allow signal ramification or amplification, they reasoned that such a system could not only amplify a signal but also show **ultrasensitivity**, a term coined a decade earlier by Goldbeter and Koshland for the steady-state behavior of (a) positively cooperative allosteric enzymes, (b) enzymes near saturation ('zero-order ultrasensitivity') or (c) multiple step enzymatic cascades ('multi-step ultrasensitivity') [153].

For an explanation of ultrasensitivity, but also for basic understanding of modeling of reaction networks, we have to take a short detour into the history of biochemistry and enzyme kinetics. The phenomenon of **positive cooperativity** was first recognized by C. Bohr in 1904 [37] for hemoglobin's binding and dissociation of multiple O_2 molecules. In the case of cooperative enzymes, the binding of multiple substrates, enhances the catalytic step.

This leads to a deviation from the hyperbolic Michaelis-Menten plot of a reaction's velocity against the substrate concentration [316], which can be expressed quantitatively in terms of the **Hill coefficient**, an extension to the classic Michaelis-Menten equation introduced 1910 by A. Hill [188, 189]: the exponent h of substrate concentration in equation 1. Hill coefficients greater than 1 turn the hyperbolic curve of reaction velocity vs. substrate concentrations into a sigmoid (s-shaped) curve.

$$v = \frac{dP}{dt} = v_{max} * \frac{S^h}{K_{0.5}^h + S^h} \tag{1}$$

where v is the reaction velocity, dP/dt denotes the time change of the product P's concentration, S is the substrate concentration, v_{max} is the maximal velocity at enzyme saturation with substrate. The constant $K_{0.5}$ is the substrate concentration where $v = v_{max}/2$, and is thus similar to the $Michaelis - Menten\ constant$. Hill found that this coefficient can be estimated from titration experiments by the relation:

$$h = \frac{log(81)}{C90/C10} \tag{2}$$

where C90 and C10 denote the substrate concentrations required to obtain 90% or 10% of the maximal product concentration. Analyzing the effects of cyclic enzyme activation and deactivation by covalent modification, it was found that the Hill equation can also be abstracted to describe the general relation between a 'switch-like output' of a metabolic system to a continuous range of 'input strength', the stimulus for an activation:

$$\frac{P_{active}}{P_{total}} = \frac{S^h}{K_{0.5}^h + S^h} \tag{3}$$

where P_{active} and P_{total} are the steady-state concentration of activated and total product concentration, respectively, S is the concentration of a stimulus, and $K_{0.5}$ represents the concentration of S, where P_{active}'s concentration is half-maximal.

The Hill coefficient h controls the 'sigmoidity' of the response. Please consult the original works by e.g. Stadtman and Chock [447] or Goldbeter and Koshland [151, 256, 152, 153], for detailed derivations. Conceptually spoken, the system doesn't respond to low levels of a stimulus, but at some threshold quickly shows maximal response. In the conceptual framework of cellular signal transduction, a sigmoid response curve of a signal transducer's activity

to an incoming stimulus can be interpreted as a **all-or-none** or **switch-like** response to a stimulus. Such a sigmoid stimulus/response curve can already be seen in the first MAP kinase papers for MAP-2 phosphorylation upon stimulation with increasing insulin concentrations [383].

Xenopus laevis oocytes undergo maturation upon stimulation by the hormone progesterone mediated activation of a Cdc2/Cyclin B complex via a Mos/MEK1/ERK2 cascade. Huang and Ferrell tested ERK1 / ERK2 activation in dependence of the strength of an activating signal both in theory and in experiment, using Xenopus laevis oocyte extracts, and found that this pathway indeed can act as an ultrasensitive switch, i.e. that MAP kinase activation's relation to stimulus strength can be described by the Hill equation with a Hill coefficient of 4-5 in their model and in oocyte extracts, treated with varying concentrations of Mos, the upstream kinase in Xenopus (see fig. 27). This behavior depends on a two-collision mechanism of the dual phosphorylation of the enzyme and is lost when a processive (single-collision) mechanism is assumed in the model. A two-collision mechanism was supported by measurements of mono- and di-phosphorylated MAP kinase during the reaction in oocyte extracts [130]. An additional requirement is partial saturation of the reactions that activate or inactivate the M2K [202], i.e. there should be more M2K than M3K and/or M2K phosphatase (M2KP). Thus cascade dynamics can be influenced by expression levels.

2.4.2 Bistable (... or not bistable)

Later, Ferrell and Machleder tested the dynamics of ERK activation also in intact oocytes treated with different concentrations of progesterone and found Hill coefficients of at least 42, much higher than that measured in oocyte extracts and calculated by the previous model. Such a high coefficient depends on progesterone-induced and Mos/MEK1/ERK2 mediated protein translation of the upstream M2K kinase Mos. The system, apart from some intrinsic ultrasensitivity of the cascade, thus depends on a positive feedback cycle for true in vivo ultrasensitivity. Moreover this feedback will lead to a 'true switch', with stable off and *on* states [131], [546]. Such bistability is a phenomenon well known in the theory of complex dynamic systems as *hysteresis*. Depending on parameter values of a bistable system, a transition from one to the other state can also be irreversible, and such a property has been discussed in relation to the (in many cases) irreversible differentiation of cells during development. Bistable switches based on positive feedback can account for a kind of 'biochemical memory'. The systems stays *switched on*

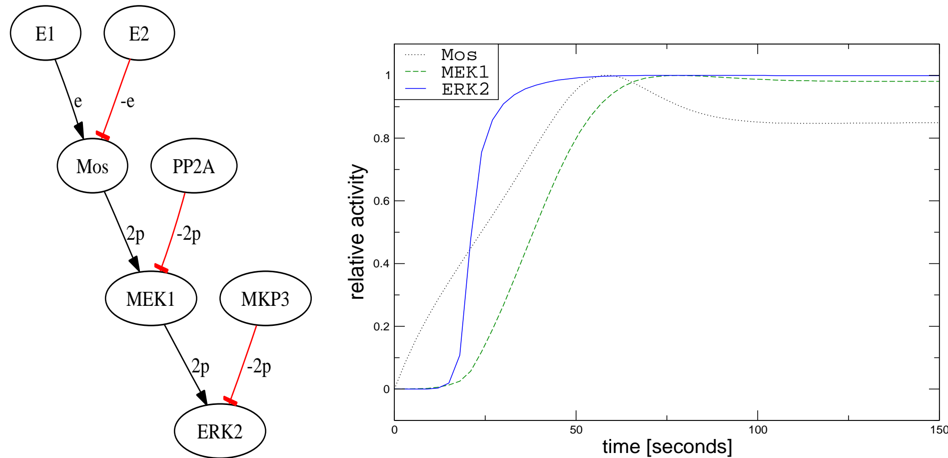


Fig. 27. Left: schematic representation of the mathematical model of ERK activation in Xenopus oocytes, Huang and Ferrell 1996 [202]. Right: simulation results for the model shown in the left graph. The relative activities of the three kinases (active kinase / total kinase concentration) shows the increasing sigmoidity of the concentration/time curves with every step of the cascade.

long after the initiating signal has vanished [30, 31]. Importantly, in the absence of the positive feedback via Mos - e.g. by blocking translation with cycloheximid or specifically inhibiting Mos translation with antisense RNA constructs, the pathway shows graded response to increasing signal strength [546]. Ferrell further speculates that nuclear translocation of ERK and its upstream activator MEK - or in general the translocation of two or more components of a signaling cascade to a smaller compartment, can also result in an increase of the Hill coefficient [132], an idea that is supported by some evidence for the MEK/ERK system (see chapter 2.2.2). In the nucleus however, ERK can activate transcription of different sets of genes. Among them is an ERK phosphatase (MKP), which thus acts as a negative feedback inhibitor. In a complex model of PDGF signaling in fibroblasts, Bhalla, Ram and Iyengar explored a positive feedback loop between the cascade, DAG, calcium and PKC signaling which also leads to bistable behavior. As outlined above, the cell 'remembers' a short pulse of PDGF, in that this feedback cycle stays active afterwards. However, sustained (nuclear) ERK activity induces MKP expression and stabilization (see 'feedforward sensors' in chapter 2.2.2), and the high phosphatase activity pushes the cascade out of the bistable regime, and also abolishes the switch-like behavior. The cascade shows a graded response to carying signal strength [31].

A recent work by Hornberg and coworkers analyzes, both in theory and experiment, the different consequences of kinase and phosphatase concentration (or inhibitors thereof), leading to the conclusion that kinases and phosphatases are equally important for signal amplitude, but that *phosphatases are more important than kinases for signal duration and integral signal intensity* [195]. The 'integral intensity' denotes the area under the concentration/time curve, and this integral would be proportional to the total number of 'affected' downstream molecules, i.e. substrates of the MAP kinase. In this regard, another aspect has been tested in theory: the potentially significant consequences of differential localization and diffusion of kinases and phosphatases, which consequently establish intracellular gradients of phosphorylated proteins [49, 240].

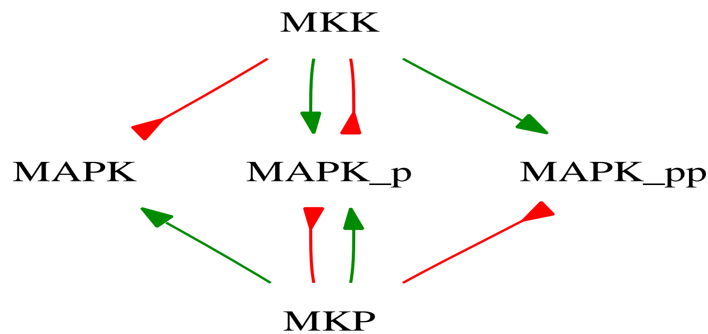


Fig. 28. A single level of the cascade: a dual specificity upstream kinase activates and a dual specificity phosphates de-activates the kinase. In theoretical models a single level of the cascade can show bistable behavior, due to 'apparent feedback' [295]. See text for details.

Shifting Balances Important for Koshland and Goldbeter's concept of multi-step ultrasensitivity in enzymatic cascades and networks is the presence of reversible modifications of the enzymes, which in the MAP kinase cascade is achieved by kinases and corresponding specific phosphatases. The individual enzyme cycles between active and inactive state and the ratio of (active) kinase to phosphatase for each level of the kinase cascade determines the actual output of the system. A very interesting theoretical observation has recently been published by Markevich et.al. 2004. The two-collision, dual phosphorylation and dephosphorylation cycle of a single level of the cascade can show bistability arising through what the authors called an

'apparent feedback', but in the absence of 'real' positive feedback (contradictory to Thomas's conjecture ([468]) [295]. The 'apparent feedback' of the dual specificity converter enzymes is caused by substrate saturation of the kinase (or the phosphatase, or both) that leads to competitive inhibition of the second step by the product of the first step (= substrate of the second step). The parameter space domain for bistability is restricted by product inhibition, which was modeled in detail for the phosphatase reactions. This is a purely theoretical model. However, it fits into above outlined picture, that the cascade is built for bistability on many levels, and single sigmoid signal/response effects add up, to observed amazingly high Hill coefficients. It would be a fascinating perspective, if the dual phosphorylation mechanism by itself, was evolution's initial innovation that lead to this ubiquitous use of the MAPK cascades in cellular signaling. The work by Markevich et.al. also analyzes differences arising between models of elementary steps of catalysis, vs. Michaelis-Menten like descriptions of enzyme kinetics, that don't explicitly account for enzyme-substrate or enzyme-product complexes, and are thus only valid for steady state calculations, but not for time-courses [295]. SBML versions of some of the models of this publication are available at `http://www.tbi.univie.ac.at/~raim/mapk.html`, and have been accepted and annotated at the SBML model repository `http://www.biomodels.net`.

2.4.3 'Interconvertible Enzyme Cascades'

Cellular coordination networks make intensive use of such reversible modification cycles, e.g. acetyl-transferase/deacetylase in the regulation of chromatin structure, methylation/demethylation of proteins or DNA, or the PI3K / PTEN system in lipid mediated signaling are all mediated by specific enzymes. We also met complex modification cycles of Ras, the nuclear shuffling of many proteins and protein complexes. Many of the properties studied for the MAP kinase cascade could be relevant and compared to the uncounted other systems of the ubiquitous mechanism of specific reversible protein modifications. In Cornish-Bowden's text-book on enzyme kinetics 'interconvertible enzyme cascades' (such as the one outlined in this work) only fill a small subsection. The following subsection, however, features 'the metabolic role of adenylate kinase' (AK) as another example for ultrasensitive regulation and it is proposed here as a putative sensor of receptor phosphorylation events and to point to the metabolic connection, which is rarely accounted for in theoretical models of cellular signaling networks.

AK catalyze the interconversion of the three adenine nucleotides:

$$2ADP \leftrightarrow ATP + AMP \quad (4)$$

with very high activity and in both directions. The ratio of these three metabolites are held constant (by other mechanisms) at roughly 100 ATP : 10 ADP : 1 AMP. The enzyme will thus convert small changes in the ATP/ADP ratio into a relatively large change in AMP, that could effect AMP sensitive proteins (see chapter 12.9.3 in [84]).
Neurites, the long processes of neuronal cells, are inhabited by an alternatively spliced isoform of AK1, AK1β [212]. In PC12 cells the expression of AK1β is enhanced by the neurogenic factor NeuroD [344]. AK1 is cytosolic, but AK1β is a fast diffusing membrane species [405]. In Val5 fibroblasts this isoform — featuring a consensus signal for N-terminal myristoylation — was found 'to play a relevant role' in p53 mediated establishment of a reversible cell cycle arrest [78] (lower right graph in fig. 29). Thus, AK1β could act as membrane metabolic sensors [221], a function that could be quite useful especially for neuronal cells and the high energy usage in actively signaling and/or outgrowing neurites. A sensor for AK's AMP pulse could be right at hand. The AMP-activated protein kinases (AMPK) are AMP sensitive — actually ultrasensitive [176] — enzymes with various mostly metabolic targets. AMPK forms can be targeted to the membrane by N-terminal myristoylation [323, 513, 214]. AMPK is also considered to act as a metabolic sensor, responding to ATP deficiencies. In the membrane, together with AK1β, it could constitute an efficient sensor for local adenosine phosphate turnover in neurites and drive e.g. fatty acid synthesis [440] for new membranes. Of course, AMPK has been found to modulate Ras/ERK signaling. Dependent on the varying energy status of cells, AMPK was observed to inhibit Ras activation or stimulate Ras-independent ERK activation [241]. If this connection is too far out in the land of speculation, maybe a hands-on warning will catch your attention. The commonly used MEK specific inhibitors U0126 and PD98059 strongly activate AMPK by increasing AMP/ATP and ADP/ATP ratios - potential side-effects can not be excluded [110].

MAP Kinase Cascade and Nucleotide Signaling A possible usage of AK1β and AMPK for sensing massive phosphorylation events at the membrane could be a direct connection of such signaling events to metabolic regulation. The available models involving phosphorylation mediated signaling consider ATP as a constant species in quick and stable equilibrium (which it

is in part due to AK's activity). ATP consumption is not a question in these models, which usually implement only an abstracted fraction of the known phosphorylation events, anyways. The upper graph in fig. 29 shows a sketch of adenosine-phosphate converting and sensing enzymes around the adenosine phosphorylation cycle. An interpretation of signaling cascades from the point of metabolism can lead our concepts to evolutionary old principles of cell function. Generally spoken, MAPK cascades with their amplifying and 'switch-like' dynamics are an ideal module for eliciting quick large scale responses to small signals and have probably always been used for various stress signaling systems. At the same time different sets of the cascade are commonly employed in chemotactic cell-cell signaling, such as mating factor pathways in yeast. The border to metabolic stress is blurred and metabolic state can probably be considered one of the oldest 'signals' that had to be sensed (the ur-signal?).

The distinction between signaling, gene-regulatory and metabolic networks is a conceptual one. One, that evolution maybe doesn't know about. The former networks however, employ exactly such mechanisms, that had to be excluded for theoretical analysis of metabolic networks. The complex multicellular signaling networks can viewed as gradual extensions of a basic cell metabolic regulation to achieve the ultimate 'function' of a single cell, to survive and/or replicate. A putative AK/AMPK sensing mechanism was pointed out only to emphasize this connection, that will surely have to be considered for evolutionary scenarios for the origin of the MAP kinase cascades, and to close the circles to above discussion of the PC12 cell model for spatio-temporal control of ERK signaling in neuronal differentiation.
The upper graph in fig. 29 sketches the adenosine phosphate cycle and some of the responsible enzymes. cAMP was introduced as an important second messenger, tightly networked with phosphorylation cascade, above. We can close another circle at this point: cAMP is degraded by so called phosphodiesterases (PDE). And the PDE4 proteins have recently been shown to constitute another highly localized branch point for cross-regulation in the cAMP/ERK networks. PKA, the cAMP activated kinase, inhibits PDE4, constituting another positive (double negative) feedback cycle. In contrast, active ERK can activate some spliceforms of PDE4 ([198, 197, 196], see lower left graph in figure 29). Vertebrate cells can switch between expressing PDE4 forms, where ERK can deactivate cAMP signaling, and such that can't (at least via this way). The vertebrate specific process of osteoclast formation, has e.g. been shown to involve PDE4 isoforms and an interaction with ERK [462].

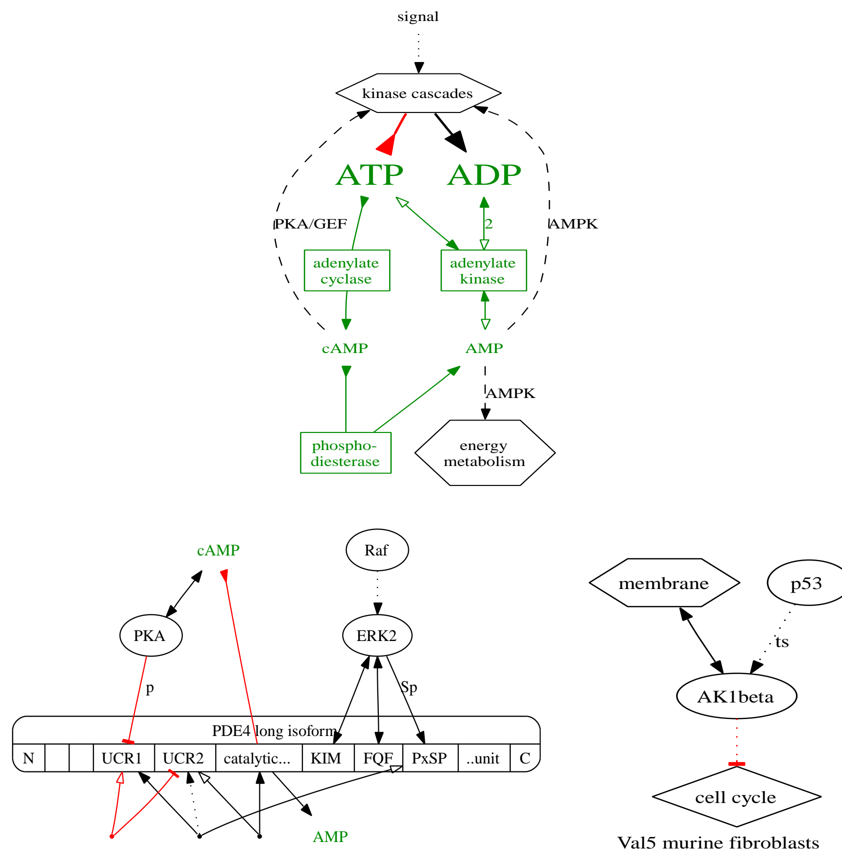


Fig. 29. An old connection of kinase cascade and adenosine phosphate metabolism? Note e.g. that adenylate cyclases and phosphorylation cascades could locally compete for ATP. See text for details.

3 bioLog and SBML Models (Methods)

3.1 bioLog diagrams

The MAP kinase pathways are only one of many modules of signal interpretation, but probably the best understood. A Pubmed search for the MeSH terms of MAPK revealed almost 19.000 publications, by the end of 2003, only 17 years after identification of the first MAPK (see figure 2). The cell biological and medical research communities have coped with such an exponential increase in experimental observations - available mostly in natural language description - by adopting graph-like notations, that comprehend models of physical interactions, abstract activation/inhibition relations, spatio-temporal developments, and integration into higher-level processes (of biological function).

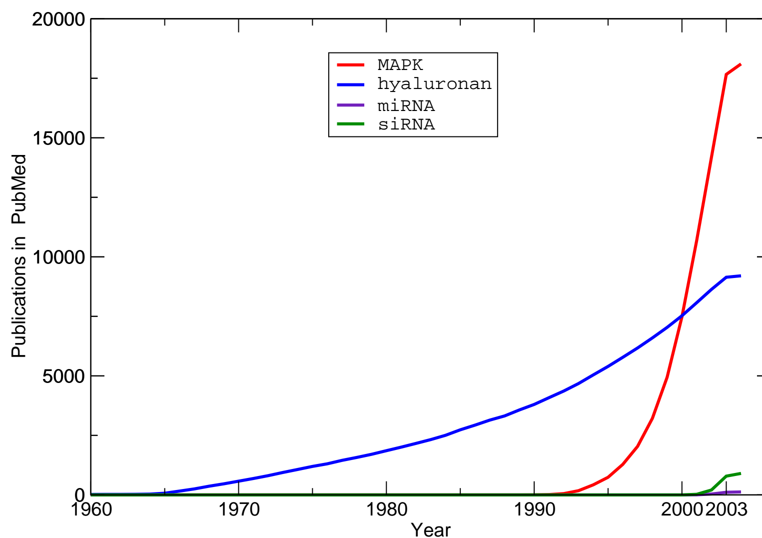


Fig. 30. Literature appearance of the MeSH terms for the 'Mitogen-activated protein kinase', compared to 'Hyaluronan' and 'siRNA' and 'miRNA', until February 2004

The tradition, necessity and ability to depict molecular processes in abstract

diagrammatic form is old. Biochemical text books couldn't live without graphical notations, which lead from well-defined notation of chemical reactions and structures of small molecules directly to higher/level notation of modulating, activating and inhibiting interactions. The reader has to infer the meanings of nodes and edges not only from the text, but also from previous knowledge. A second kind of graphical convention involves representation of structure, i.e. primary, secondary and tertiary structures of RNA, DNA and polypeptides, which are well defined, if referring to a specific (consensus) sequence, but entering the realm of interpretation with symbolic depiction of tertiary structures and huge macromolecular complexes.

The requirement for standard notations of protein and higher level interactions to cope with vast and growing literature knowledge, has only recently been discussed and interestingly compared with e.g. the general origins of written language or the development of musical notation by B. Franza [135]. The so far existing approaches are often derived from an initial attempt by K. W. Kohn to capture mammalian cell cycle in a graphical standard notation [251, 252, 55, 7, 351].

Strengths and Drawbacks of a General Graph Notation: The graph notation of the diagrams adopted in this work, does not attempt to strictly define such a language, but represents the results of a rather experimental approach, trying to capture specific topics from referring literature in a private interaction 'database'. Definitions are loose, and that is exactly the expressional strength of such diagrammatic models. They can comprehend multi-hierarchical knowledge (e.g. 'from a sequence to pathologies'), and can often include vast areas of incomplete knowledge (e.g. 'mutation at serine xx is correlated with 'decreased migrational efficiency'). The approach herein employs however the common, yet dangerous approach of mixing content and layout, as frequently interactions have been abstracted or neglected for layout reasons, only. Importantly nodes that represent proteins, often represent not a single static molecule, but rather symbolize cycles of catalytic modification and complex formation. The basic edge division into 'activation' and 'inhibition' (and thus labeling of edges with $-$ and $+$ signs) implies causal interactions, but should often rather be regarded as temporal correlations, especially when the exact mechanistic details of the interaction are not known or explained in detail. The most significant convention - in fact the only one, that was followed (almost) restrictively within this document - is that activation and inactivation paths are consistent within one graph description. While this approach makes them highly context-dependent -

they can e.g. not just be merged -, it allows to deduce the global sign(s) of paths by merely counting the negative interactions. In a path with an odd number of negative interactions, the source of the path 'exerts a negative influence' on the sink of the path. This feature is incredibly useful for the identification of (the sign of) possible feedback cycles.

Figure 1 of the introduction is the first example of this approach, and shall thus be discussed shortly: a diverse variety of signals activate one or more MKKK and thereby start a specific phosphorylation cascade, culminating in accumulation of one or more active MAPK. At each step, each single activated kinase can activate many of its substrate proteins, the products being again activated enzymes, each able to catalyze many reactions. Dual phosphorylation of a MAP kinase is thus conceptually interpreted as 'activation' by an 'upstream' MAPK kinase (MKK). The cascade can be described as a catalytic amplification of an initiating small change in environment, a 'signal', whatever its nature. Figure 1 shows an activation scheme of the pathway, a diagram abstracting causal interactions - initiated by a signal, similar to above mentioned diagrammatic representations in cell-biological literature, but generated automatically from a textual graph description by the graph layout tool graphviz [143]. The edge labels 'Sp', 'Tp' and/or 'Yp' in this case denote the phosphorylations at serine (S), threonine (T) and tyrosine (Y) residues, respectively. Such diagrams will be extensively used throughout this document. Figures 31 and 32 give rough definitions of nodes and edges used.

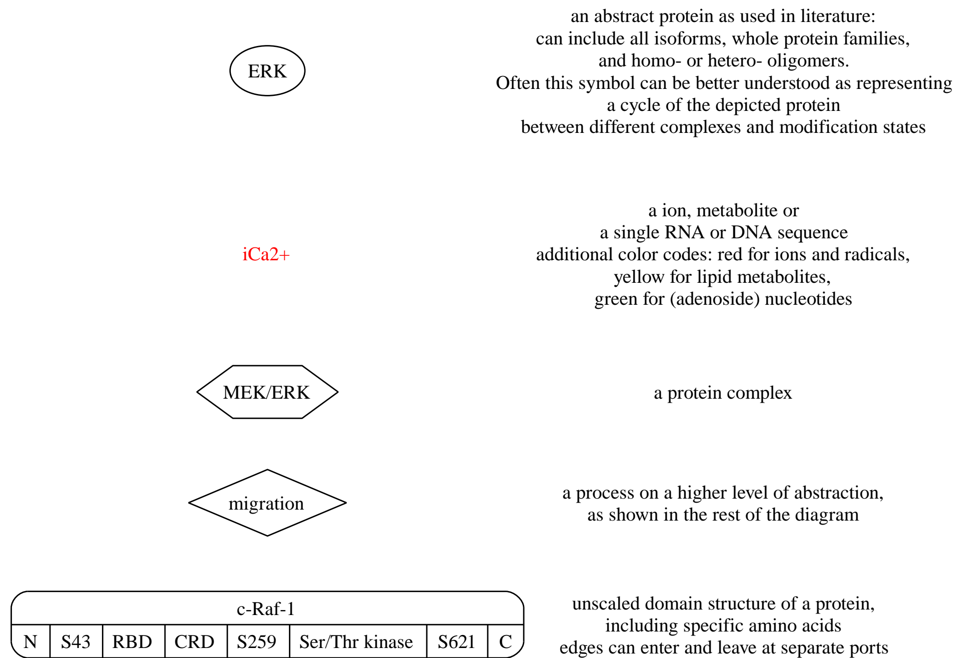


Fig. 31. Index for node types used in interaction graphs within this document.

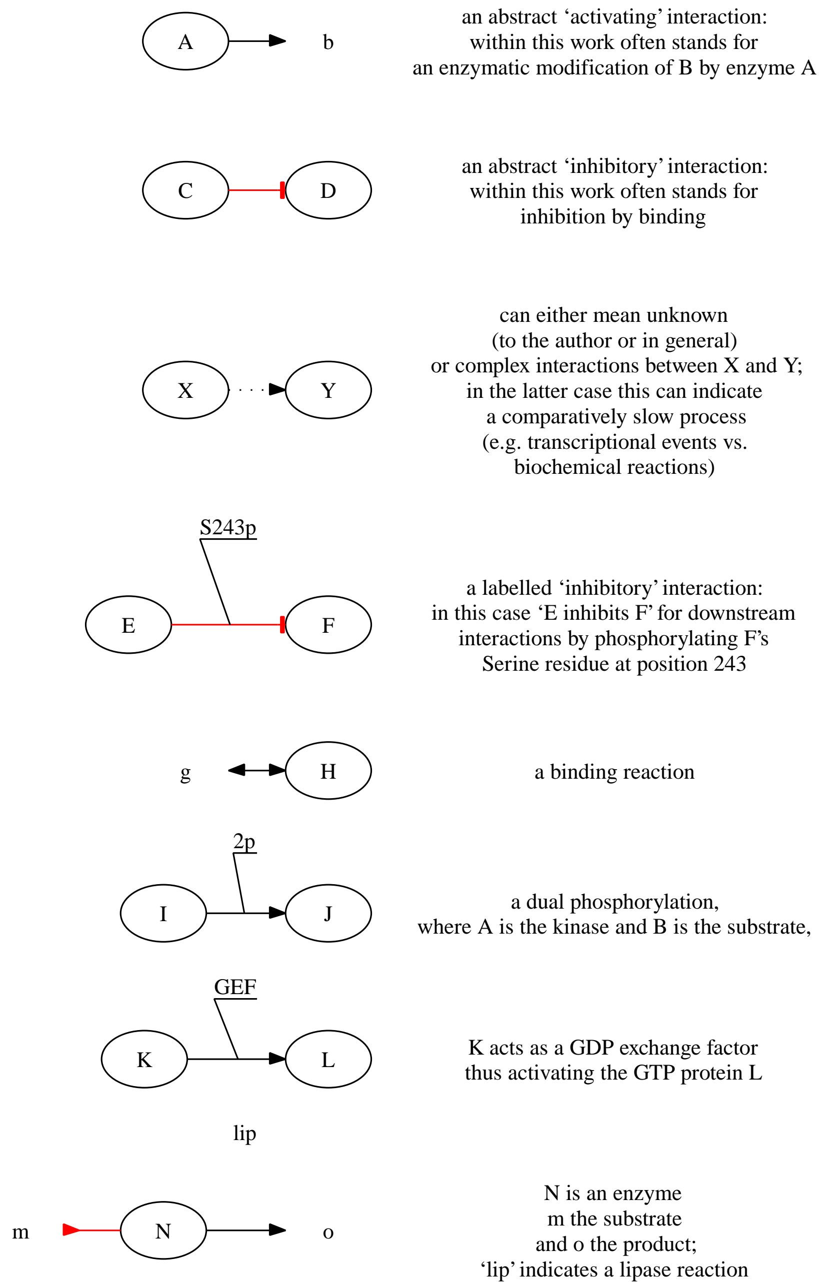


Fig. 32. Index for edge types used in interaction graphs within this document.

3.2 SBML Models of the MAPK pathway

Some of the published mathematical models of the MAP kinase pathway (section 2.2.3, chapter 2.4) have been converted into SBML, the Systems Biology Markup Language [203], and integrated numerically with the SBML ODE Solver, which is described in chapter 4. Here, the SBML models are shortly introduced, and problems of the conversion to SBML are described.

WARNING: The SBML models presented here are 'wrong' in the following biological sense: The models have a volume of size 1, which avoids conversion of rate constants applicable to SBML's kinetic law units [substance/time], default [micromol/sec].

3.2.1 Huang and Ferrel 1996: Ultrasensitivity

Ultrasensitive by Nature [202]

SBML The model was constructed by hand, from instructions in the original paper.

Description First published ODE model of the MAP kinase pathway! It was used to analyze the intrinsic ultrasensitivity of the cascade, that can (in part) account for a switch-like or all-or-none response to a progesterone signal of the Mos/MEK1/ERK2 pathway in Xenopus oocytes. Importantly the ultrasensitive behavior depends on a two-step dual phosphorylation mechanism. Ferrell later showed that actual ultrasensitive behavior additionally depends on a positive feedback via expression (translation) of the upstream kinase Mos, and is possibly further supported by co-translocation of MEK and ERK to the nucleus (where concentration increases due to smaller volume). Figure 27 in section 2.4 depicts simulation results and a sketch of the structure of this model.

3.2.2 Kholodenko 2000: Oscillations

Oscillations by Negative Feedback [238]

SBML The model was obtained from the official SBML model repository.

Description Potential for oscillatory behavior of the MAP kinase pathway through a negative feedback form MAP kinase to MKK kinase, which however is unlikely/unknown to cause oscillations in in vitro or in vivo MAP kinase activation. The MAP kinase pathways are however, integrated - as a driving input - into many oscillatory systems, such as the cell cycle [546], the somitogenesis clock [392], or Dictyostelium cAMP signaling [288]. Figure 37 in section 4.3.3 'Visualizing Structure and Dynamics' shows the structure and simulation results of this reaction network.

3.2.3 Markevich et.al. 2004: Bistability

Bistability by Dual Phosphorylation [295]

SBML Two alternative models of Figure 1 of the publication were handwritten.

Description A model of the dual protein phosphorylation/dephosphorylation cycle of the MAP kinase. The model shows bistability in the absence of 'real' positive feedback, contradictory to Thomas' conjecture [468]. This depends on an 'apparent feedback' of the dual specificity converter enzymes: substrate saturation of the kinase (or the phosphatase, or both) leads to competitive inhibition of the second step by the product of the first step (= substrate of the second step). The parameter space domain for bistability is restricted by product inhibition, which was modeled in detail for the phosphatase reactions. The work also analyzes differences arising between models of elementary steps of catalysis, vs. Michaelis-Menten like descriptions of enzyme kinetics, that don't explicitly account for enzyme-substrate or enzyme-product complexes.

3.2.4 Schoeberl et.al. 2002: Internalization Dynamics

EGF Receptor Dynamics [419]

Description Model of the effects of EGF receptor activation/internalization dynamics on SHC/GRB2/SOS adaptor complexes and Ras mediated activation of the Raf/MEK/ERK pathway. Quote: "It shows that EGF-induced responses are remarkably stable over a 100-fold range of ligand concentration

and that the critical parameter in determining signal efficacy is the initial velocity of receptor activation." The model's dynamics were well supported and documented by an experimental system in HeLa cell culture. Please see figure 38 in section 4.5 'Integrated Result Visualization' for simulation results for this model.

Comment The initial velocity of EGF receptor activation in fibroblasts was shown to depend on a positive (double negative) feedback cycle in lateral signal propagation, see Reynolds et.al. 2003 below. Differential dynamics of receptor internalization also account for the differential response of PC12 cells to EGF (proliferation induced by a transient ERK signal via Ras/c-Raf-1) and NGF (differentiation into a neuronal phenotype through sustained ERK activation via Rap1/B-Raf). The elucidation of differential activation profiles and fine tuning of ERK activity by Ras/c-Raf-1 and Rap1/B-Raf cooperation, and an important cross-link to the ancient cAMP signaling system, are only recent fascinating insights in the complex immediate upstream events of MAPK function.

SBML The model was obtained from SigPath and intensively modified by hand. It is not complete! The original Mathematica model compared complex formation on activated receptors (with the same reaction parameters) for both membrane-bound and internalized receptors. In this version of the model, only receptor activation at the membrane is included and internalization is thus treated as a sink for receptors. Reaction names, e.g. v18_65, indicate that this reaction was modeled two times in the original model. Reaction names generally correspond to the figure in the original paper. Thanks to Martin Ginkel for clarification! A full model will be available as soon as possible.

3.2.5 Reynolds et.al. 2003: RTK Lateral Signal Propagation

Bistability of RTK Lateral Signal Propagation [391]

Description Experimental study in fibroblasts, showing that EGF receptor lateral signal propagation depends on a positive (double negative) feedback cycle, potentially via ROS (reactive oxygen species) mediated inactivation of PTP - protein tyrosine phosphatases that inactivate EGF receptors. See Paek et.al. 2004 for a pathway database of ROS signaling, ROSPath [361].

SBML The paper included a small reaction network model of the feedback cycle, that was used for interpretation of the results. The SBML was written by hand.

3.2.6 Bhalla et.al. 2002: Phosphatase controls Bistability

Negative Feedback controls Bistability [31]

Description A big model around the MAP kinase pathway, activated by PDGF receptor activation (see figure 33), showing bistable behavior and hysteresis via positive feedback cycles between MAP kinase and PLA2/PKC activation. The bistable behavior could constitute an autonomous cellular memory mechanism, where a transient signal leads to sustained activation of the pathway. This behavior has also been analyzed - with a different kind of positive feedback - in Ferrell's work for the MAP kinase in Xenopus oocyte. In this paper, an additional negative feedback via expression of the MAP kinase phosphatase MKP1 was shown to 'turn off' bistability.

SBML The model was constructed computationally by a quick and dirty Perl script (`www.tbi.univie.ac.at/~raim/kkit2pl/`, using Perl bindings for the SBML library, and a text file description of the KinetiKit/Genesis model to Figure 1b of the article, available from the Upinder Bhalla's supplementary material website. **Known Errors**: The original model assumed a cellular volume of 10^{-12} liters, and a nuclear volume of $0.2*10^{-12}$ liters for the transcriptional regulation. Here the model has a default compartment of 1, and no nuclear compartment is used! To use compartments, you have to multiply all(!) reaction parameters, with the volume of the reaction's compartment. Detailed active PKC species: The Genesis/Kinetikit export file was additionally edited by hand, because the 'pool' construct in the original Kinetikit/Genesis model, comprehending several forms of PKC into an 'active' pool, cannot be expressed in SBML, and needed detailed reactions for each of the different PKC forms.

bhalla_02.xml : Active PKC as separate species
The left graph in Figure 34, shows the relative activities of the Raf/MEK/ERK cascade in this model. I am not sure what is wrong with the model, but it doesn't need any PDGF to activate the positive feedback cycle. PDGF has been set to zero to indicate this fact.

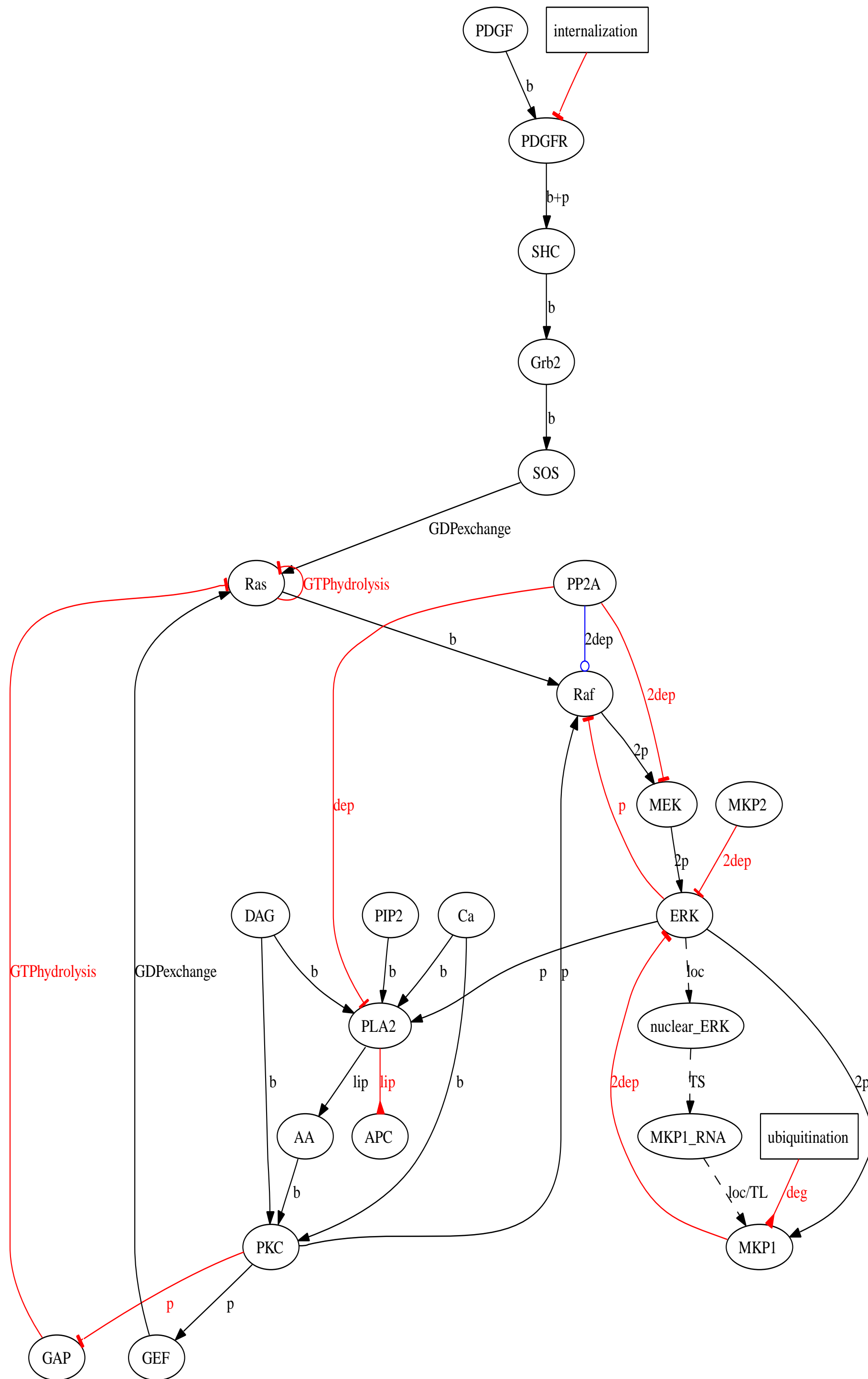


Fig. 33. Sketch of the model by Bhalla, Ram and Iyengar 2002 [31]

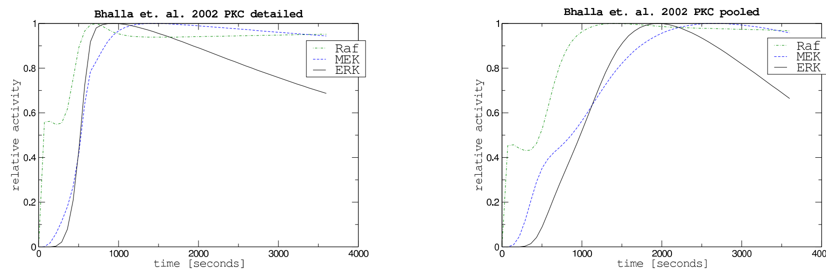


Fig. 34. Simulation results for two different SBML versions of the model by Bhalla, Ram and Iyengar [31]. Relative activities are the ratios of active species to total concentrations, normalized to the highest value during the simulation run. Please see text for details.

bhalla_02pool.xml: Active PKC Pool as an SBML assignment rule The right graph in figure 34 displays results obtained by a version that uses SBML assignment rules to comprehend all active PKC species into 'PKC_a_pool', and uses this abstract species for PKC mediated phosphorylation of GEF, c-Raf-1 and GAP. This construct is not correct in the context of the model, as active PKC species are consumed by formation of enzyme-substrate complexes, which should not be available for dissociation of the active species. If the phosphorylation reactions would be modeled with Michaelis-Menten instead of elementary step mass action kinetics for substrate binding and product dissociation, the model would be correct for analysis at steady state, but couldn't account for possible competition between PKC complex dissociation and downstream enzyme-substrate complex formation. However, this model needs a 300 seconds PDGF pulse to activate MAPK, and the active MAPK concentration time series, shown in the right figure, looks a lot more like the one in Figure 1b of the original article. The slope of MAPK activation seems somewhat less steep, though!

3.2.7 Sasagawa et.al. 2005: PC12, Differential ERK Activities

Description The model describes differential ERK activation by the NGF receptor TrkA (sustained) and the EGF receptor (transient) in PC12 cells. Sasagawa et.al. implement a negative feedback to the SOS protein and degradation of EGF receptors, responsible for switching off ERK activity in EGFR signaling, and a cascade activation via Rap1 instead of Ras and internalization of active NGF:TrkA complexes, responsible for sustained ERK

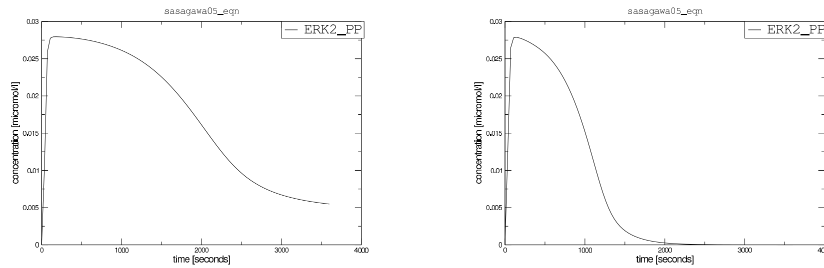


Fig. 35. Mathematical modeling of PC12 signaling. Left: sustained ERK activity upon NGF activation of TrkA signaling. Right: transient ERK activation upon EGF receptor signaling.

activity in TrkA signaling. Several other branches, such as lipid and calcium signaling via PLCγ and PI3K or cAMP signaling, have however been implicated in differential signaling but are not accounted for in this model. Please see chapters 2.1.1 and 2.2 for a detailed description of this phenomenon and its consequences for cell cycle.

SBML Sasagawa et.al. used the Genesis/KinetiKit tool for construction of their model. The same Perl script as above (Bhalla et.al. 2002) was used for conversion into SBML. Again, some post-processing by hand was necessary. Curiously the input file (output of Genesis) indicated a volume size of $1.6667^{-18} l$, which is however far too small for a eukaryotic cells. Integration of the SBML version with the SBML ODE Solver requires to use this volume to obtain the results given in the paper. This might indicate a serious problem of the used parameters in this model.

Comment Several models were handwritten, and initially had severe syntactic errors. Thanks to Nicolas Le Novère for reporting. Hand-writing SBML is NOT recommended!

4 SBML and CVODE based ODE Solver (Results II)

4.1 Summary

Abstract The *SBML ODE Solver* is a programming library, accessible also as a simple command-line tool, for (1) constructing a system of ordinary differential equations (*ODE*) from chemical reaction networks and (2) numerically integrating the time course of concentrations of chemical species and (3) basic visualization of model structure and integration results. It is based on *SBML*, the recently developed standard for description of biological reaction networks, the *SBML* library *libSBML* for parsing *SBML* and constructing the *ODE* system, and on *CVODE* for numerical integration of the derived system of *ODE*s. Optional data visualization modules allow printing of integration results directly to Grace and drawing graphs of the reaction network, and a Jacobian interaction graph of the *ODE* system via graphviz' graph drawing library.
The *SBML ODE Solver* is written in ANSI C - and therefor platform independent, and provides bindings for SWIG and Perl5.

Availability `http://www.tbi.univie.ac.at/~raim/odeSolver`

4.2 Introduction

Background Mathematical modeling of chemical reactions, and especially biochemical reaction networks involves a variety of techniques and theories and has long been applied for various purposes in research and technology. Diverse but potentially complementary approaches have been taken to analyze networks of chemical reactions, roughly dividable in 'dynamical' and 'structural' analysis.

Dynamical analysis tries to understand the time-dependent development of reaction rates and molecular concentrations, including intuitively hardly recognizable properties that 'emerge' due to complex feedback cycles within reaction networks. Given a complete reaction network, including a rate law description for each reaction, one can either derive a system of ordinary differential equations (ODE) for the time-change of the participating chemical species, or a so-called chemical master-equation for discrete stochastic modeling. Both formulations assume a well-stirred homogeneous solution of all reactants. If interested in diffusion regulated processes the researcher can set up a series of partial differential equations (PDE), additionally describing space-dependence of the concentration of chemical species. Several other approaches adapted from various mathematical and computational techniques have been explored, including multiple agent systems, petri nets [387, 192], which naturally resemble a bipartite reaction graph and it's stoichiometry, or the π-calculus for analysis of concurrent parallel processes [389], and grammar models, semantical and logic descriptions.

Some of the latter methods overlap conceptually with the second class, the 'structural' network analysis. Those methods include graph theory based approaches to describe global network structure, that are essentially 'graph walking' and 'graph partitioning' problems. More specialized techniques - derived from theoretical chemistry, such as mass conservation analysis, metabolic control or regulation analysis, allow to identify e.g. sensitivities of the reaction network to a subset of parameters or minimal steady state modules such as so-called 'elementary flux modes' or the related 'extreme pathways'.

Another interesting class of computational models of reaction network would be constituted by the already wide range of metabolic pathway databases, such as KEGG or MetaCyc. At least for the former, an $SBML$ export already exists. A very recent development provides for curated databases of signal transduction and regulatory pathways, as derived from experimental knowledge in literature. 'Domain experts' extract the most established knowledge on signaling networks and comprehend them into activa-

tion and inhibition diagrams. Adequately, such approaches are taken by or in collaboration with the big journals, such as Science's Signal Transduction Knowledge Environment STKE, `http://www.stke.org`) or 'the signaling gateway' of the 'Alliance for Cellular Signaling' (AfCS) and Nature (`http://www.signaling-gateway.org/`). Both of the latter are currently implementing *SBML* export of their models.
And finally, the 'biomodels initiative' will provide curated quantitative models of biological reaction networks of any kind (metabolic, signaling, and gene regulatory) at `http://www.biomodels.net`.

SBML - the Systems Biology Markup Language Accordingly, many tools for all kinds of computing platforms have been created, each relying on their own data format for describing reactions networks and their parameters. The need for exchange and merging of models motivated collaborative efforts to develop a standard format for describing the common chemical reaction networks underlying the various derived mathematical descriptions. Of two competing XML based formats, *SBML* (Systems Biology Markup Language) [415, 133, 203] and CellML (Cell Markup Language) [280] the former now seems to be widely accepted in the modeling community and is supported by a growing number of long-existing as well as newly emerging tools.

Motivation The available tools (see e.g. website [415]) cover a variety of methods to edit and analyze a reaction network and its dynamics and/or structure. However, they are designed either as - often platform specific - standalone tools whose functionality is only accessible via more or less complex user interfaces (Jarnac/SCAMP, Copasi, Genesis/KKit, ...) or depend on commercial tools for mathematical analysis (the 'SBML Toolbox' for Matlab, 'MathSBML' for Mathematica).
The *SBML ODE Solver* in its first released versions (1.0 and 1.5) is a minimal *ODE* construction and integration tool with some additional (optional) features for graph drawing and result visualization, entirely written in C and based mainly on *libSBML*, the C/C++ library for parsing and editing *SBML* [38], and *CVODE*, a stiff and non-stiff *ODE* solver in C [408], the same tool that is also used in SCAMP, a classic tool for model simulation and metabolic control analysis [413]. The *SBML ODE Solver* is targeted at bioinformaticists, biomathematicians and 'command-line friendly' biochemists and biologists.

Possible Applications Through it's easy-to-use and stripped down functionality, the *SBML* ODE Solver offers itself for a variety of purposes, both as a stand-alone tool for quickly exploring system structure and dynamics and as a simple and reliable programming library, surrounded by other additional and higher-level analysis or visualization tools. The program might be most interesting for a use in batch integration of models, e.g. via a calling script or program that interprets results and changes *SBML* structure or parameters accordingly. Such a use is indicated by the green path in figure 36. Examples for a possible usage of the program via short Perl scripts, depending on the Perl5 binding for *libSBML*, are included in the distribution.

1) High-throughput simulation: While many users will only study a few models, with a few simulation runs, other applications will require high-throughput numerical analysis of automatically constructed models. The study of **evolution of network structure and dynamics**, will e.g. require quick identification and classification of specific dynamics such as oscillations or multiple steady states (multi-stationarity) of large series of models, derived from each other by mutations. Another obvious use would be the test parameter sets, derived from optimization techniques, for the desired dynamics, as e.g. measured in experiments. **Parameter optimization/identification** and the **inverse problem of chemical kinetics** would be the buzzwords for this area of research. Besides heuristic 'black-box' methods, such as neural networks or genetic algorithms - several analytic methods exist, which employ an *ODE* system's 'Jacobian matrix' and derivatives thereof. The *SBML ODE Solver*s formula manipulation routines include formula evaluation and symbolic differentiation, which will be highly useful for such approaches.
2) A general ODE solver: At this point it is worth pointing out that the *SBML ODE Solver*'s use is not restricted to chemical or biological problems. Through *libSBML*'s formula parsing and data structure, the *SBML ODE Solver* opens *CVODE* for a use with general *ODE* systems. *SBML* can encode any system of *ODE*s. In fact the program itself produces such a model to represent *ODE* systems (see chapter 4.3.1). Thus the program qualifies as a general *ODE* solver, opening CVODE's capabilities to library use, without the need to hard-code *ODE* models.
3) A low-level tool for education: Last but not least, it should be emphasized that the *SBML ODE Solver*'s development has always considered its potential as a convenient tool for **educational purposes**. The programs' command-line usage, including the optional data visualization

modules, comprises a very low-level interface to *SBML* models and their structure. Without 'blinding' of back-end functionality, as within complex GUI tools, it allows an introduction the principles of chemical reaction networks and the standard *SBML*, as well as to *ODE* construction from such reaction networks. For bioinformatics programing courses, the source-code exemplifies the use of *libSBML* for handling reaction networks, and the use as a library extends *libSBML* natively for manipulation and theoretical analysis of *SBML* models. Plans for further development of the tool will especially consider easy and quick, and informative visualization of model structure and dynamics.

4.3 Usage and Basic Architecture

The *SBML ODE Solver* is a very simple, command-line driven ANSI C program and programming library, stripped down to the basic functionalities of

(1) **construction of an *ODE* system**
from an *SBML* encoded reaction network
(2) **numerical integration of an *ODE* system**
encoded in a defined subset of (semantically incorrect) *SBML*
and
(3) **printing and basic visualization**
of model structure and integration results

It is distributed as source code under the LGPL (GNU Lesser/Library General Public License) and can be compiled via the usual 'GNU-style' configure/make procedure requiring the automake tool to be installed. See the file INSTALL in the distribution for detailed instructions.

Table 1 lists all available procedures and the command-line options to call them. Figure 36 shows the program's work-flow of of data parsing, data conversion, *ODE* construction, *ODE* integration, and output of the program. The steps (1-3) are labeled as above. Plain-text nodes represent accessible data, while elliptic nodes represent program functionality. The green path indicates a possible use by an external script or program. Each step is described in detail in the following chapters 4.3.1-4.3.3. For more details, please consult the extensive documentation of the source code.

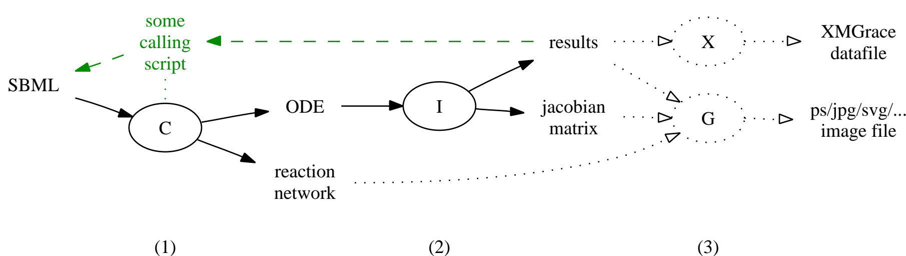


Fig. 36. Basic data-flow architecture of the *SBML ODE Solver*, see chapters 4.3.1-4.3.3 for details

4.3.1 Constructing ODEs from Reaction Networks

See node **C** in figure 36: *SBML* parsing, *ODE* construction, data conversions; depending on *libSBML*.

The simple *SBML ODE Solver* makes heavy use of the ANSI C/C++ *SBML* library libSBML [38] for parsing *SBML* encoded reaction networks and constructing *ODE*s and other formulas and finally for evaluating their current values, e.g. during an integration run. The *libSBML*'s Abstract Syntax Tree (AST) convention for representation of mathematical formulas was especially useful for the latter purpose.

The steps implemented by the functions subsumed in node **C** of figure 36 can be outlined as follows (*italic* symbols *ODE.xml* and *SBML.xml* resemble nodes *ODE* and *SBML* in the figure):

- **C.1 Load, validate and parse SBML file**
  The input file is an *SBML* encoded model *SBML.xml* of chemical reactions and all other possible *SBML* definitions. LibSBML provided functions are used to parse the model, and access its data in the following steps. The data can optionally be validated towards *SBML*'s schema definitions before anything else is done.

- **C.2 Copy predefined ODEs**
  A new model *ODE.xml* with compartment size 1 is created; predefined *ODE*s, i.e. *SBML* 'rate rules' are copied from *SBML.xml*, and their variables added to the new model. Note that parameter values or the compartment sizes can be described by a 'rate rule' in *SBML.xml*. Thus, in *ODE.xml*.

- **C.3 Construct ODEs from reactions**
  For all yet undefined species, that have their ‘boundaryCondition’ and ‘constant’ fields set to ‘false’, an *ODE* is constructed from all reactions that consume or produce the species, i.e. where it appears in the list of reactants or products of the reaction definition. The *ODE* is constructed directly as a *libSBML* AST, combining *SBML*’s ‘kinetic law’, ‘stoichiometry’ or ‘stoichiometry math’ definitions and the species’ compartment. Local parameters, definable for ‘kinetic laws’ are replaced in the formulas, i.e. their name is replaced by their value.

  As an example consider the two reactions in a homogeneous and continuously stirred compartment of size V:

  $$\text{A} + \text{B} \rightarrow \text{C; kinetic law} = K1;$$
  $$2\ \text{C} \rightarrow \text{D; kinetic law} = K2;$$

  The resulting *ODE* for the concentration of C, denoted [C], would add up from the two reactions’ kinetic laws each multiplied with the species’ stoichiometry and set positive for producing or negative for consuming reactions:

  $$d[C]/dt = (\ +\ 1\ *\ K1\ \text{-}\ 2\ *\ K2\ )\ /\ V$$

  Please consult basic text books like [501] for the details on constructing *ODE*s from reactions. One notable difference between the usual process and *SBML* specific *ODE* construction lies in *SBML*’s ‘kinetic law’ formula that differs from the usual rate law in that its units are amount/time instead of concentration/time. This facilitates *ODE* construction from multi-compartmental models and, according to the *SBML* Level 2 Version 1 specifications [133], only requires the division of the resulting *ODE* by the compartment volume to obtain the usual concentration/time description. The new *ODE*’s AST is added as a ‘rate rule’, i.e. an *ODE* describing the concentration of a species, and the corresponding species to *ODE.xml*. The species’ compartment is the default compartment and its initial values are set to initial concentration.

  The new model *ODE.xml* now constitutes a usual ‘initial condition problem’, it consists of *ODE*s and the initial values of their variables.

- **C.4 Copy incompatible *SBML* structures**
  SBML’s ‘algebraic rules’, that are needed in systems of differential

algebraic equations (*DAE*) cannot be interpreted in terms of *ODE*s, and neither can discrete 'events'. Such structures are just copied to the new model, for print-out and analysis with other tools.

- **C.5 Replace constants, assignments and defined functions**
  User defined functions, assigned variables and constant parameters, species and compartment of *SBML.xml* are replaced in all *ODE*s ('rate rules') and the copied incompatible structures (from step C.4) of the new model *ODE*.

At this point the contents of the input *SBML* model as well as the derived *ODE* system can be printed out to inspect reactions, initial conditions and equations. The new model can be printed as *SBML*, and this way the program can essentially be used as a conversion tool, condensing an *SBML* encoded reaction network to an *ODE* system, encoded in a defined small subset of SBML.

4.3.2 Integrating ODEs Numerically

See node **I** in figure 36: Jacobian matrix construction, *ODE* integration; depending on *CVODE* and *libSBML*.

LibSBML's abstract syntax tree (AST) represents formulas in their correct precedence encoded in tree structure. A simple recursive function, that is also included as an example program in the *libSBML* distribution, is used to evaluate AST formulas in the functions described below.

The simplified *SBML* model *ODE.xml* is used to fill an internal data structure used by the integrator function. The Jacobian matrix of the *ODE* system is generated in symbolic form, again as an AST. Note that, at the moment, in the exact procedure of the program, this functions takes the old model *SBML.xml* and calls the above described function to obtain *ODE*.

An integrator function then initializes and calls *CVODE*, an ANSI C tool for solving non-stiff and stiff *ODE* systems [408], and provides *CVODE* with a function that evaluates the AST representation of the *ODE*s and (optionally) the Jacobian matrix of the *ODE* system with current values (current species concentrations), whenever this is requested by *CVODE*'s integration method. The integration methods employed by *CVODE* are variable-coefficient forms of the Adams and *BDF* (Backward Differentiation Formula) methods, and simple functional (or fixed point) iteration or

a variant of Newton iteration for non-stiff and stiff problems respectively. Please consult *CVODE*'s user guide [408] for more detailed information about method and implementation. The *SBMLODESolver* uses the BDF method and Newton iteration with the *CVODE* dense linear solver which can solve both stiff and non-stiff systems. The integrator function has been derived from *CVODE*'s example program 'cvdx.c'. It requires the current values of the Jacobian matrix. These can either be calculated from their AST representations or by *CVODE*'s internal approximation of the Jacobian. The latter occurs if (a) the *ODE*s include expressions, whose differentiation is currently not implemented, (b) the solver produces errors with the generated Jacobian but not with the internal approximations (the reasons of which have yet to be determined in detail) or if (c) the user chooses so explicitly via command-line options or *CVODE* settings. *CVODE* uses absolute and relative error tolerances for each calculated time step. The absolute and relative error tolerances are set to 10^{-18} and 10^{-14}, respectively, and can be set via a command-line option. The accuracy required by published tests (see 4.6) could be achieved easily by setting the absolute error in the range of 10^{-21} to 10^{-18}. For some problems the user will also have to adjust the maximum number of steps that *CVODE* tries to reach the next requested time step within the error tolerances. Table 1 lists all available command-line options. If *CVODE* integration fails an error message is printed. The given error flags are explained in table 4. In any the final output of the *CVODE* module is a set of statistics. e.g. how many internal steps, how many calls to *ODE* or Jacobian evaluation were needed. Please consult table 3 for interpretation of this output.

Discrete Events SBML allows to specify discrete events, in which a variable's value triggers the resetting of other variables. Such discrete events can lead to discontinuities and are not defined in the realm of *ODE* systems. The *SBML ODE* Solver currently (versions 1.0 and 1.5) implements a provisional event evaluation which can be activated via a command-line option or settings (see 1). At each printed time step, the event triggers are evaluated. Upon triggering of an event, the integrator stops and is restarted with new values. This event detection is not exact. The accuracy of event detection depends completely on the chosen print-step interval!

4.3.3 Visualizing Structure and Dynamics

The odeSolver prints all data to stdout, and messages to stderr, as a default. The data should then be processed by other tools. However, it also offers some additional functionalities for quick and easy exploration of structure and dynamics of a reaction network model. Via command-line options the program can be used to print model contents instead of integrating. Two optional modules that depend on additional libraries are used to support visual exploration of the model. In the interactive mode the user has some additional possibilities for processing of data.

Interactive Mode Via an interactive mode the user has access to most functions that are available via command-line options. The user can inspect a loaded $SBML$ model, construct and view the ODEs, integrate them, store and view integration results. Additionally the interactive mode allows to set alternative initial conditions and print phase diagrams for two species to XMGrace. The interactive mode is especially helpful when exploring a new SBML file with a structure unknown to the user and in the lack of other tools. It might especially find appreciation for educational purposes as outlined above.

Result Visualization using XMGrace See node **X** in figure 36: result visualization with XMGrace; depending on the grace library np_grace.

Instead of printing integration results to a file the user can choose to directly visualize concentration/time graphs in XMGrace [547]. See Table 1 for other output data. The interactive mode additionally allows to select 2 species to draw 2-dimensional phase diagrams to XMGrace (see lower images in figure 37).

Graph Drawing using graphviz See node **G** in figure 36: reaction network and Jacobian matrix graph drawing with graphviz; depending on the graphviz library.

The reaction network can be drawn as a bipartite graph of molecules and reactions, based on graphviz' algorithms for graph layout (graph drawing, graph embedding) [143]. Edges from chemical species to and from reactions are labeled with the corresponding stoichiometry. The generated graphic files can easily be used for exploration of the structure of the reaction net-

work.
A species interaction graph based on the non-zero entries of the Jacobian matrix can be constructed via graphviz. Edge colors and labels are set by the value of the corresponding entry in the Jacobian matrix at some chosen time point of integration. Negative influence of a species on the ODE of another species is represented by a red arrow, positive influence by a black arrow. The exact values are the labels of this graph. This graph is well suited for visually exploring the dynamic regulation of the network, e.g. to get a first impression on possible and relevant positive or negative feedback loops within a reaction network.The upper left image in figure 37 shows such a graph for the MAPK pathway's phosphorylation cascade with a theoretical negative feedback. The upper right image is the reaction network of the same model. This model by Kholodenko et.al.[238] has been obtained from the official $SBML$ model repository at `http://www.sbml.org/models`.

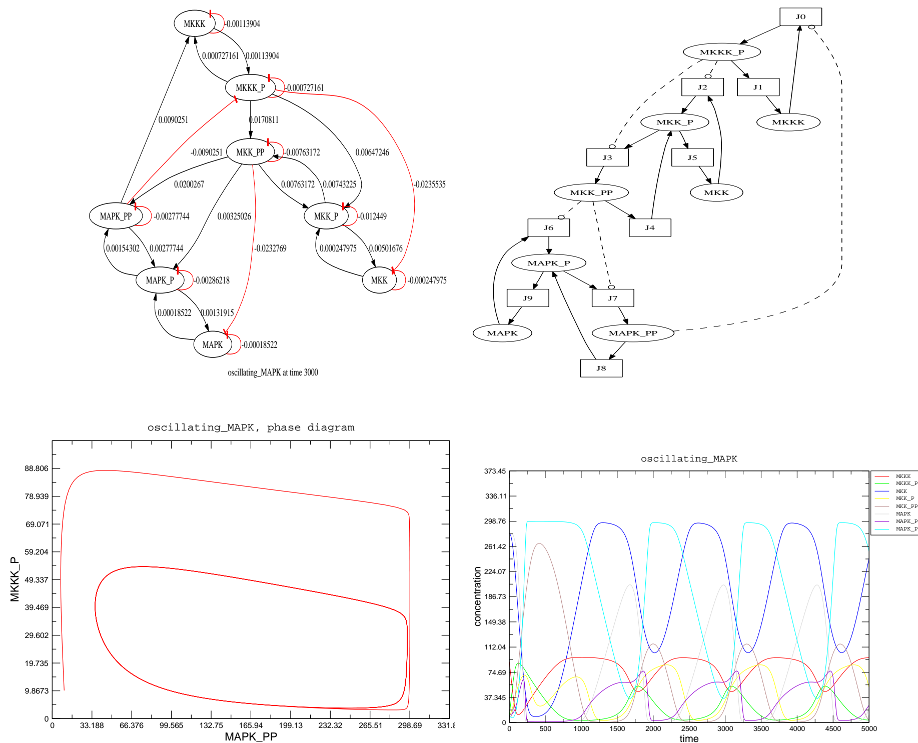


Fig. 37. Example results for a model by Kholodenko et.al. 2000 [238], (taken from $http://www.sbml.org/models$)

Compilation without optional modules Both functionalities, grace- and graphviz- dependent, are optional. The configure script recognizes if the necessary libraries are available and, if not, the program can be compiled without these functions. Compilation without the Grace library will cause the program to just ignore the command-line option and print out results as text, while compilation without graphviz will lead to printout of graphs as text files without calculated coordinates in the graphviz' 'dot' format.

4.4 API Functionality

The *SBML ODE Solver* is completely written in ANSI C and its functionality is available as a library. Moreover it currently provides bindings for *SWIG* and *Perl*5.

4.4.1 High-level Interface Functions

While any of the public functions can be used, version 1.5 provides three easy to use main interface functions:

SBMLResults
Model_odeSolver(*SBMLDocument_t* ∗ *d*, *CvodeSettings settings*);

This function takes any *SBML* Document, plus the settings for CVODE integration as an input. It returns a special data structure *SBMLResults*, that contains time-courses for species, and for variable compartments and parameters.

SBMLResults ∗
Model_odeSolverBatch(*SBMLDocument_t* ∗ *d*, *CvodeSettingssettings*, *VarySettings vary*);

As above, but additionally takes the structure VarySettings, which holds instructions for the variation of a parameter between a start and an end value. The *SBML ODE Solver* will search for this parameter in the model, set it accordingly, and simulate for each value of the parameter. It will return an array of *SBMLResults*, containing time-courses for each parameter value.

SBMLResults ∗ ∗
*Model_odeSolverBatch*2(*SBMLDocument_t* ∗*d*, *CvodeSettings settings*, *VarySettings vary*1, *VarySettings vary*2);

As above, but the function takes a second structure VarySettings, and will

simulate for each pair of parameter values, and return a 2-dimensional array of *SBMLResults*.

Please, see the files in the examples directory of the source distribution for the usage of above interface functions.

4.4.2 External Function Evaluation

The *SBML ODE Solver* provides a simple means for including external data into an integration run. If an *SBML* input model contains a function, without an associated function definition, the formula evaluation routine will look for an available function returning a double value. A programmer can provide this function, which should take the name (AST_NAME) of the used function in the formula, and it's (potential) arguments - which can for example be the current simulation time.
We use this functionality to include an external time-course, as it could e.g. result from experimental measurement. The external function takes the current simulation time as an argument, and interpolates the current value from an external time series.
Please see the file 'processAST.c' for the needs for such an external function.

4.5 Integrated Result Visualization

The Perl wrapper script 'bioLog_resultVisualizer' (*rV*) exemplifies a very simple use of the program for both direct and higher-level visualization of simulation results. The script uses *SBML ODE Solver*'s and Perl's graphviz modules to generate SVG based graph drawings and embeds them in a set of cross-linked html files. The SVG files (chemical species, reactions) are animated by the results of a simulation run and link to sites with detailed model and result information for each species and reaction.

BioLog Result Interpretation Additionally the script searches for two other, already existing files, which can be created to embed the *SBML* model and display the results of an animation within some higher-level, hand-written, representation of the reaction network model.
A hand written *indexfile*, that is parsed by the *rV* script, lists all proteins or otherwise defined higher-level (modular) entities in the model system, and each protein/module is accompanied by a list of chemical species in the *SBML* model that represents different states of the protein (e.g. chemically

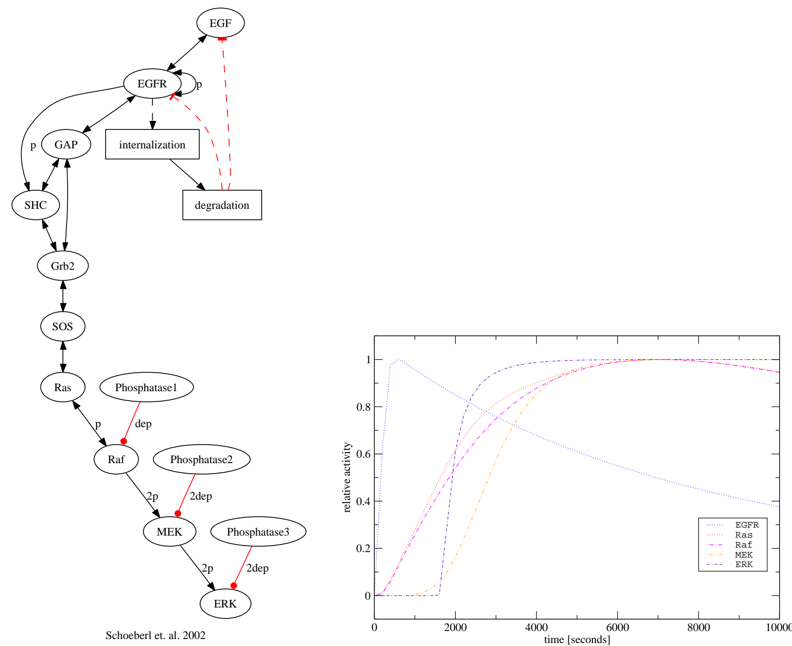


Fig. 38. Example bioLog schema of Schoeberl et. al. 2002 [419]

modified or bound within a complex). For each entity there must be at least one chemical species, that is marked as 'active'.
The second file is an SVG based diagrammatic representation of interactions between the above defined modular entities, similar - in the example models - to the well-known diagrams in cell-biological and medical literature describing cellular regulation processes and experiments. This *bioLog* activation/inhibition diagram is encoded again as a graph file and graphviz was used to create the SVG encoded images of the graph drawings. The modular entity list (proteins or defined processes in the example models) and its activity tags are used to to calculate active/non-active ratios from simulation results, and visualize their time series within the graph SVG file by using transparency values of SVG objects.
The example shown in figure 38 is a such a *bioLog* activation/inhibition schema of a published model of receptor mediated activation of the so-called 'Mitogen Activated Protein Kinase' [419], a eukaryotic module of cellular signal transduction pathways used as a 'switch' or 'amplifier' of externals and internal signals in diverse contexts of cell regulation, like growth, cell-cycle, differentiation, migration, adhesion and apoptosis. The simulation

of this model is based on an *SBML* model initially obtained from SigPath and adapted by hand. The *indexfile* and the *bioLog* diagram are hand-written. An animated and hyper-linked set of result files for this model can be browsed at `http://www.tbi.univie.ac.at/~raim/schoeberl_02/index.html`.

The *rV* wrapper script is written in PERL5 and dependent on Perl modules SVG::Parser, GraphViz, GD::Graph, and the newly developed LibSBML bindings for the *SBML* library *libSBML*.

4.6 Accuracy and Testing

The accuracy of the simulation can be set via the two error tolerances. See Figure 39 for integration of the pendulum equations with very high error tolerances. CVODE uses the absolute and the relative error tolerances for each time-step integrated. Errors can thus accumulate.
The *SBML ODE Solver* has been extensively tested with the first published version of the 'SBML Semantic Test Suite' provided by the *SBML* team (see website [415]). All of the *SBML* test models that do not include
(a) algebraic rules, which can only be solved with methods of *DAE*s (Differential Algebraic Equations),
(b) events, that would require approximations of the exact event time and
(c) delays, that would require methodology of solving '*ODE*s with delays'
could be successfully integrated, except for one including a cube root expression for which at the last tested time point the *SBML ODE Solver*'s result deviates from the target results produced with MathSBML, a *SBML* package for Mathematica [430]. The test suite includes models at the extremes of low numerical values, and they were solved without problem. However, models handling very low amounts, in the range of circa 0 to 1000 molecules (per cell), which will require stochastic approaches.
Detailed results of this test run, and instruction how to reproduce the tests are distributed with the source code.

4.7 Shortcomings of ODE models

The ODE models require several significant simplifications, that (again) exclude some of the most central aspects of cellular signaling. First, the ODE approach assumes a 'continuously stirred reactor', a homogeneous solution in which all reactions occur. For an account of diffusion-regulated pro-

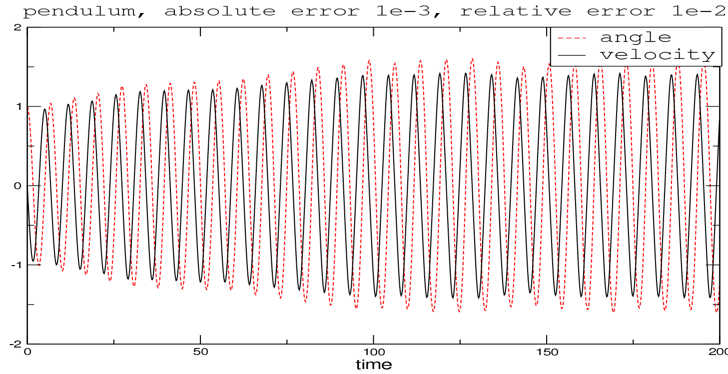


Fig. 39. Accumulation of Numerical Errors: Simulation of equations for a friction-less pendulum (d(angle)/dt = velocity; d(velocity)/dt = - sin(angle)) with a very high error tolerance.

cesses within fine-structured and highly compartmentalized cells, one would have to employ partial differential equations (PDEs), which brings along a huge increase in computational demands, as well as a decrease in analytical approaches. The $VirtualCell$ project would cover such techniques [438]. Second, several cellular reactions are known to involve minute amounts of reactants and often stochastic effects are speculated to be involved in central regulation mechanisms [237]. The ODE approach is a deterministic one, not accounting for potential contribution of stochastic effects. The StochSim tool, would be one of the SBML compliant solvers for stochastic problems [270]. And third, from the opposite perspective, quantitative models require detailed quantitative descriptions of individual reactions, including reaction parameters (rate constants). The required parameters are in most cases not available. If literature offers measured parameter values for e.g. single enzyme reactions, they often pose two more problems. First, they are mostly based on in vitro measurements, which are unlikely to resemble the cellular situation. Second, these parameters often come in the form of steady-state relations for enzyme reactions, such as the Michaelis-Menten constant.Michaelis-Menten-like descriptions subsume elementary steps of enzyme reactions and don't account explicitly for enzyme-substrate complexes. However, if an enzyme is involved in more than one reaction, enzyme-substrate complexes must be considered. Equilibrium constants of enzyme

kinetics must be converted back into elementary step constants, and this again can only be estimated, as e.g. described by Bhalla and Iyengar in [30].

4.8 Outlook SBML ODE Solver

At the moment the tool can process all *SBML* Level 1 + 2 definitions and numerically integrate the dynamics of models that are interpretable within the realm of ordinary differential equation systems and thus numerically solvable with *CVODE*. Some possible further developments, through additional internal functionality or integration with other tools, are outlined in the following.
The maintenance of the tool will include a more detailed use of *CVODE* and its various methods for integration, result printing and processing, and extensive testing of use and communication with other programs.

Events, DAEs, Steady State Analysis Discrete 'events' and 'delays' cannot be interpreted by *CVODE*. The detection of discrete events - or at least a useful approximation thereof within the chosen error tolerances for integration - is, however planned as an internal extension of the presented tool for the near future. *SBML* models containing definitions for 'algebraic rules' call for a separate module solving systems of differential algebraic equations (*DAE*). The official *CVODE* is now part of the *SUNDIALS* package, which also features *IDA*, for solving such *DAE* systems, as well as *KINSOL*, which can be used to identify fix points (steady states) of a system of *ODE*s. Their implementation is similar to *CVODE*, and an implementation of these tools within *SBML ODE Solver* should be straightforward.

PDEs Level 3 of *SBML* will also come up with some definitions, including spatial models, that won't be interpretable by the tool at this stage. Separate modules for constructing and solving *PDE* systems, describing e.g. morphogenic activity during development or the interpretation of chemical gradients by chemotacting cells ranks high on our interest- and todo-lists. However, there is no *PDE* Solver available, that could be used similar to the tools of the *SUNDIALS* package, and thus *PDE*s are probably out of the scope of the current approach of the *SBML ODE Solver*.

Structural Analysis Tools for mass conservation analysis [388, 412] would allow to reduce the amount of equations. That however could happen independently of the *SBML ODE Solver*, which then would just get passed this reduced model. The information necessary for above described biologic result visualization, the *indexfile* could be automatically generated, given only the active state(s) of an entity, while all other (inactive) states of the entity should be deducible by mass conservation. Moreover, even the causal interactions of the *bioLog* interaction diagram could be automatized, if additionally 'input' and 'output' species can be defined, when interpreting the network as a module - of signal transduction in our examples. Such a higher-level embedding of a reaction network could employ graph search and partitioning algorithms to identify relevant higher-level causal relations - e.g. dominant cascades or feedback cycles - in the reaction network but also in the Jacobian matrix of the derived *ODE* system.
Identification of relevant parameters and elementary flux modes by methods of metabolic regulation analysis, would help to extract interesting subsystems form large models. This can again be useful for theoretical parameter optimization approaches (see below) but also offer interesting possibilities for the (graphviz dependent) model visualization module and the result visualizer wrapper (chapter 4.5).

Dynamic Analysis Having the *ODE* system and its Jacobian matrix in symbolic and interpretable form, motivates for approaches to identify and analyze positive and negative feedback cycles of a system. Such tools would provide a platform for automatic classification of the dynamic structure of large series of models. Moreover they could help reducing the system's dynamics to higher-level discrete or logic models of system behavior. Such biochemical feedback cycles constitute basic biological regulation modules [469, 470] realizing both stable oscillatory behavior, e.g. in cell cycle or cell migration, or stable stationary states, leading to differentiation, cell adhesion in (epithelial) tissues or e.g. directed migration. Cell-biological and medical experimentation operates much closer to this higher-level descriptions of function than to basic reaction networks as encoded e.g. by *SBML*. Again a 'bioLogic' annotation as described above could be incredibly useful to map dynamic time-course data onto a temporal-logic description of the interactions of biological entities.

The Inverse Problem of Chemical Kinetics The 'inverse problem of chemical kinetics', i.e. parameter optimization towards desired system dynamics,

as e.g. measured in experiment or conjectured in theory, would constitute an obvious application for a refinement of the internal batch integration and parameter variation functionalities. The interface to external function evaluation will be useful to integrate the *SBML ODE Solver* with sophisticated parameter optimization algorithms that are currently developed with collaborating groups.

(Collaborative) Experiment Design Cell biological and medical knowledge of the gene regulatory and signaling reaction networks is mostly closer to above mentioned higher-level logical (*bioLog*) models and this knowledge is often represented in activation/inhibition schemes. Such diagrams in literature are poorly defined in their node and edge meaning, but interpretable representations (for an in the context educated reader) of a specific process and the current understanding thereof. The lack of definitions is actually their power in representing the diverse mechanistics of cell-biological phenomena. Thomas and Kaufman have proposed methods to derive such logic models from underlying reaction networks and their feedback regulation [470]. A top-down approach of such methods might help to extract possible network structures and relevant parameters from an experimentally known or theoretically conjectured higher-order logic model, as represented by such 'causal graphs' in cell biological and medical literature. A 'Computer-aided experiment design' software could allow researchers to draw similar models as used in literature, but add temporal information. A detailed reaction network model could be constructed from such information, while 'inverse methods' (see above) could identify parameter sets, that produce the temporal dynamics, which could again be tested in experiment. Moreover, such models could be discussed, developed and integrated with experimental data in web-based community frameworks, to support large-scale collaborative projects of organized experimentation.

Last words The *SBML ODE Solver* was programmed and will be maintained and extended for our own purposes in one or more of the many named directions. We hope, however, to raise some interest for the application and find users, who will be welcome to participate in further development. The program is written very close to *libSBML* and some of its functions might be of interest to other *libSBML* users.

5 Discussion

5.1 The Cascade

The MAP kinase cascade has been studied in several mathematical modeling approaches (see chapter 2.4). A range of models studied the consequences of the cascade's basic architecture: the three-tiered layer of dual phosphorylations can result in ultra-sensitive ('all-or-none' or sigmoid) response to an activating signal [202, 131], represented in enzyme kinetics by the Hill coefficient (see equation 3). If such a system is supported by positive feedback it can remain in an active state even after the signal has vanished ('switch', bistability, hysteresis). Such systems have often been described as a cellular 'memory modules' [30, 31, 546]. Interestingly, one single layer of a dual phosphorylation/dephosphorylation cycle alone can in theory cause ultrasensitivity and bistability, by an *apparent feedback* through enzyme saturation [295]. It is thus tempting to speculate for an evolutionary scenario, where a single enzyme, that is activated by such a dual phosphorylation/dephosphorylation mechanisms was found useful to introduce a threshold response to some sort of signal. By adding several layers of dual phosphorylations the threshold can be sharpened; and more so by the potential additional contributions to the sigmoidity of the signal-response curve by co-translocation of upstream components to a compartment with smaller volume, as speculated for MEK and ERK translocation to the nucleus [131]. In the case of Xenopus oocyte maturation, nuclear ERK activates transcription of the upstream activator (M3K) Mos, which contributes even more to ultrasensitivity and bistability of ERK activation [546]. Importantly, ultrasensitive and bistable signal response depend on the level of counteracting phosphatases [195]. Rising phosphatase concentration can abolish the threshold response and convert the switch into a graded responder, proportional to signal strength. In NIH-3T3 fibroblasts, bistability of ERK activation by PDGF can be established by a putative positive feedback cycle via lipid/cPLA2 and Ca^{2+} mediated PKC signaling. Sustained ERK activation then leads to transcription of the ERK phosphatase MKP, closing a negative feedback cycle: the cascade can render itself into a graded responder [30, 31]. Negative feedback is widely used in cascade regulation. The upstream M3K of the Raf family — shown for mammalian c-Raf and B-Raf proteins — are regulated by direct negative feedback through inhibitory ERK mediated phosphorylations, and so are e.g. the upstream components SOS in EGF mediated signaling [67], or the FRS2α adaptor for SOS in FGF and NGF signaling [269], see fig. 8. A

range of negative feedback modulators affect the cascade on different levels in developmental FGF signaling, from receptor inhibition to direct dephosphorylation of ERK [485] (left graph in fig. 8). Direct enzymatic feedback occurs on another timescale as transcription dependent feedback, and yields fundamentally different consequences for pathway dynamics. Transcription dependent feedback can again occur on all levels of the cascade. Do these mechanisms just co-operate in shutting down the cascade, or do they differentially modulate cascade activity in different developmental or physiological contexts? What are the consequences for upstream branching of parallel pathways?

Depending on the mechanism of negative feedback, upstream *nodes* can become available for redirecting a signal towards parallel pathways, as e.g. studied in theory for adaptor targeted feedback in RTK and cascade signaling [13], while enzyme targeted feedback had in this model no consequences for upstream branche. The former situation was observed in experiment for RalGDS/Ral vs. PI3K and Raf branches of the Ras *node*, resulting in sequential formation of filo- and pseudopodia [149, 406], as depicted in the left graph of fig. 13, and in fig. 24).

Such questions will certainly benefit from further modeling approaches, that could e.g. compare the different temporal profiles of ERK activity and potential branching points of the pathway at the different levels. But what are the above mentioned contexts? One fundamental character of biological research and knowledge are their multi-scale nature. While our understanding of biological phenomena has huge gaps on each *stratum* (level of organization or temporal and spatial scale), it is usually embedded in a bigger 'story' (or narration). While we might not understand the detailed mechanistics of e.g. Raf kinase activation, we know that only a couple of amino acids in B-Raf are mutated in 60% of human tumours [314]. Biological research, but also evolution itself usually happen on such *ligne de fuite* (*Fluchtlinien, lines of flight* cf. [99]), from individual atoms in a molecule to complex organismic or even ecological phenomena (e.g. [22]). In the case of the Raf/MEK/ERK cascade we can zoom out a step to get a broader view of the cascade's spatio-temporal coordination and its integration with other pathways that branch off downstream or feed into its upstream regulators, forming various interwoven negative and positive feedback cycles which ultimately define system level properties. The Raf proteins provide a never-ending story of cross-connections and alternative functions. So, let's take a look at ...

5.2 The Ras/Raf Interface

5.2.1 Coordination: Morphology, Metabolism and Cell Cycle

The cascade can be initiated by extracellular signals at RTK complexes (or by GPCR cross-activating RTK pathways [530]), but the cell's state, encoded specifically in expression levels of transducers and modulators and generally in Ca^{2+}, lipid and cAMP levels, coordinates whether and — importantly — where the cascade is allowed to strongly amplify a signal, in a potentially ultrasensitive manner, starting off a cell-wide serine and threonine phosphorylation wave, that prepares the cell for the global morphological and metabolic requirements of cytokinesis or cell migration. While ERK1 and ERK2 have a wide range of cytoplasmic and nuclear targets [276, 275], MEK1 and MEK2 seem to be mainly involved in ERK activation. Regulation of cascade and thus ERK activity is coordinated at the interface of Raf with small G proteins at cellular membranes [528].

Just as for several genes, vertebrates have three copies of a single invertebrate gene for Raf. B-Raf is the most similar to the single invertebrate Raf gene in its primary sequence. B-Raf is the main mutational target in human cancer and is now considered the main activator of the cascade, while for A-Raf and c-Raf cascade independent functions have been observed [314, 360, 122, 19]. There are however several indications that B-Raf cooperates with both A-Raf and c-Raf to obtain specific variations of the time-course of cascade activation, that has so far been dissected into different versions of an initial peak and a sustained activity. The latter was in one case further distinguished in intermediate and late ERK activity, and is suspected to depend on B-Raf, while the initial peak also involves c-Raf [51] or possibly both c-Raf and A-Raf [313]. B-Raf and c-Raf have been found in a 400-kDa protein complex [326], and a direct cross-regulation of c-Raf by B-Raf has recently been observed [507]. The exact mechanistics of this cooperation are however not understood in molecular detail.

In PC12 cells, the upstream events involve either receptor internalization and endosomal receptor signaling and/or cAMP production to modulate the small G proteins Ras and Rap1 for differential c-Raf and B-Raf activation phases [560, 559, 232, 540, 451, 50, 33, 350] (see fig. 11 to 16). GTP bound Ras is the initial stimulus that can activate all Raf proteins for cascade phosphorylation. Mouse and rat B-Raf, as well as Drosophila's single D-Raf can furthermore be activated by Rap1 [560, 322], which also binds to, but does not activate c-Raf — and thereby could inhibit it by sequestration

[451]. A large group of GEFs/GRFs and GAPs regulate these two small G proteins, see e.g. fig. 4, 6, right graph in fig. 11, left graph in fig. 12, and the left and middle graphs in fig. 15.

At the interface between Raf and Ras or Rap1, a large and diverse range of data is available that links the cascade with a variety of other important and well-studied pathways. The available data of coordination of Raf and parallel pathway activity open fascinating perspectives for integrative models of cellular signaling networks, but also highlight some of the most serious problems to the noble goals of a 'system level' understanding by means of computational modeling. Figure 40 comprehends some general interactions, where small G proteins take center stage in integrating diverse signals into Raf and cascade activation. The figure could serve as a flexible framework guiding the quest for such general principles of cellular coordination. It is likely, that such cell-wide integration networks cannot be appropriately described in quantitative detail by the yet available methods. The amazing complexity of c-Raf regulation exemplifies that similar details might arise for other players. Considering the evolutionary time-scale and some physicochemical aspects of signaling complexes, the Raf/MEK/ERK cascade might nevertheless give some important hints towards an at least conceptual outline of some general principles of cellular coordination networks (see e.g. [227]). First, a useful abstraction of cell function in general will be necessary, and might be approached within the triangle of cell morphology, metabolism and cell cycle coordination.

Morphology: Adhesion vs. Migration Membrane lipids are tightly connected to the cytoskeleton via the actin cortex [339, 428]. At the same time, they direct RTK and Ras/Raf clustering. In all these processes small G proteins, bound to membranes by lipid-anchors or membrane binding domains, play a central role. Generally, small G proteins of the Ras superfamily (see fig. 5) seem to be involved in coupling membrane systems to cytoskeletal rearrangements, as seen e.g. in the interconnected phenomena of migration, endo- and exocytosis and vesicular trafficking [461]. GEFs and GAPs regulate these G protein switches, and a widely used theme are positive feedback switches between downstream effectors (often kinases or phosphatases) and events (cytoskeletal polymerization events) and upstream second messenger modulators of the GEFs and GAPs. Raf and the MEK/ERK cascade have various connection to cytoskeletal regulation within the dualism of cell adhesion (section 2.3.1) and cell migration (section 2.3.2):

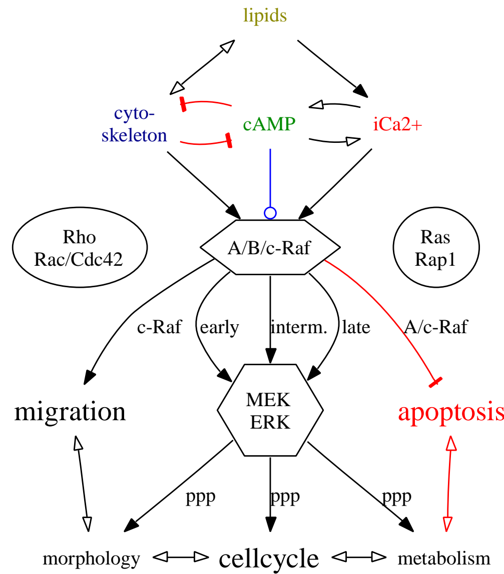


Fig. 40. Coordination of the Raf/MEK/ERK phosphorylation cascade

First, loss of adhesion renders cells insensitive to Raf/MEK/ERK activation by RTK signals, leading e.g. to growth-arrest in fibroblasts or even anoikis (loss-of-adhesion induced apoptosis) in epithelial and endothelial cells. This decoupling of the cascade from activating signals seems to depend, at least in the initial phase of cell suspension, on loss of c-Raf phosphorylation at serine 338 by PAK1 or PAK3, effectors of the PIP3 activated Rac/Cdc42 G proteins (see below). The deactivation of PAK is mediated by the cAMP-dependent kinase PKA [199] (see fig. 23), and cAMP is a long-known messenger of cytoskeletal disintegration, especially of the actin cortex [420] (see fig. 25). Other effectors of cAMP are the Epac proteins (exchange protein directly activated by cAMP) that activate Rap1, which besides its putative direct interaction with Raf, is also famous for its role in endo- and exocytosis and vesicular trafficking processes [40, 452]. Again, this points to the tight integration of the cascade with cytoskeletal processes and this role of Rap1 might well be connected indirectly to the sustained ERK signaling via B-Raf in PC12 cells from internalized endosomes [560, 559, 232, 538]. Finally, the differential ERK modulation of different PDE4 isoforms (cAMP phospho-diesterase 4) could close another highly localized and cell-type specific feedback cycle [198, 197, 196] (see fig. 29).

Second, the cascade is also involved in the dynamic turnover of integrin adhesion sites ('focal adhesions' and 'focal adhesion complexes', FA) during migration. On a cellular and developmental scale, RTK can mediate transition from an adhering to a mesenchymal cell-type (epithelial-mesenchymal transition, EMT) and directed migration. The cascade's parallel lipid pathways induce formation of filopodia and pseudopodia via the above mentioned Ras/RalGDS branch, and the processes of spreading, and establishment of cell polarization via the Ras/PI3K branch [149, 406], see fig. 24. Rap1, on the other hand, is involved in stimulation of integrin adhesion formation during initial cell spreading on the ECM substrate that usually precedes migration [380, 124], and Rac/Cdc42 activation at the front of the cell during cell polarization [40, 11, 452]. At the front of the cell PI3K produces PIP3 from PIP2, while at the trailing edge PTEN degrades PIP3 to PIP2, generating a gradient that is much steeper than the inducing external gradient [525, 497]. PIP3 locally activates Rac/Cdc42 G proteins and their effectors of the PAK kinase family. Rac enforces local PIP3 production — a bistable positive feedback module [525], while Cdc42 regulates location and stabilization of the leading edge [445]. Cdc42 activity itself works as an independent bistable module, by actin-mediated transport of more Cdc42 proteins to Cdc42 induced actin polymerization sites [522, 521, 523]. Both, Rac and Cdc42 induce actin polymerizations via WASP and WAVE/SCAR activation of the Arp2/3 complex [526, 497], leading to formation of the pseudopod and the leading edge of a migrating cell. The closely related G protein Rho acts at the trailing edge to induce acto-myosin based contraction, e.g. by activating Rok kinases (see e.g. fig. 25). The Rac and Cdc42 modules at the front and the Rho module at the tail inhibit each other [278, 548, 310, 126] — again a positive (double-negative) feedback module supporting bistability of global cellular morphology. The cascade feeds into these multiple feedback modules at various points (the first identified substrate of ERK was the microtubuli-associated protein 2 (MAP2)). An Src/FAK pair is centrally involved in adhesion turnover via modulation of integrin/paxillin associated modules [488, 417]. ERK2 seems to be involved in a feedback cycle, phosphorylating both paxillin and FAK sequentially during dynamic turnover of anterior adhesion sites in migration [325]. One recently identified alternative function of c-Raf is at the trailing edge (the uropod), where it acts a scaffold that forms an inhibitory complex with the Rho/Rok-α modules [122]. All these modules act via interlinked positive feedback cycles that underlie various sequential combinations and cyclic repetitions of EMT/MET, spreading, filopodia and pseudopodia formation, stable polarization, FA turnover and uropod retraction.

Metabolism Signaling pathways include membrane lipids and their soluble derivatives and cyclic guanosine and adenosine nucleotides (there are also a range of cGMP sensing proteins). Such second messengers, have always been treated in opposition to protein mediated signal transduction, as they can be thought to diffuse rapidly across the cell and are produced and consumed in large amounts by enzymes. They can also be considered as substrates and products of the basic metabolic networks. Adenosine and guanosine nucleotides are part of the nucleotide metabolism — representing both pairs of the double helix — and as signal mediators they probably represent an old and very fundamental connection between general metabolism and cell cycle coordination. While receptor signaling and phosphorylation cascades certainly convert large amounts of ATP to ADP and GTP to GDP, other processes such as cytoskeleton polymerizations or DNA and RNA synthesis certainly require orders of magnitude more of these nucleotides. Membrane lipids and their enzymes are involved in complex modification cycles during cellular signal transduction, but their initial synthesis requires the two old cellular reductants NADH and NADPH. However, cellular signal transduction can be considered as gradual extensions of a very old basic coordination of metabolism and morphology with the cell cycle, and this connection could still be reflected in some general principles of such highly complex but intensely studied mammalian signaling networks.

In this work, only one hypothetical connection to metabolic regulation was followed. Inspired by theoretical insights in enzyme kinetics, Cornish-Bowden mentioned in his classic text book [84], that the adenylate kinase (AK) can convert a minor decrease of the ATP/ADP ratio into a relatively large change in AMP concentration and could therefor generate a signal for AMP sensitive proteins. A potential implementation of such a module could be identified herein by literature search. Neuronal cells form large networks of dendrites and axons, and neuronal signaling also involves Raf/MEK/ERK phosphorylation cascades [30, 467]. Local neuronal activity can be expected to require local increase of metabolism (mitochondrial activity). An isoform of mammalian adenylate kinase (AK1β) is known as a 'fast diffusing' membrane species in neuronal cells [212, 344, 405]. The AMP-sensitive kinase (AMPK) would constitute a perfect sensor for phosphorylation cascade induced AK activity. It can also be found at the plasma membrane [323, 513, 214], and is known to be highly expressed in the (developing) nervous system [89, 500, 307]. Indeed AMPK is known to respond ultrasensitively to AK activity [176]. AMPK has also been observed to influence Ras activity depending on the energy status [241]. It is to note however, that

AMPK is strongly activated by the MEK inhibitors U0126 and PD98059, which increase the cellular AMP:ATP ratio, but not by PD184532, pointing to a potentially confounding side-effect of these commonly employed inhibitors [110].

Cell Cycle: Proliferation vs. Differentiation vs. Apoptosis Certainly, the metabolic state can be suspected to be interlinked with signaling pathways at many levels. Literature will provide tons of other metabolic cross-connections of the cascade. Most importantly ERK activity can not only influence morphological dynamics, but also DNA synthesis and cytokinesis, which can be considered the most fundamental of all (cell-) biological processes. The metabolic state will have to exert control over either ERK activity or readout and cell-cycle influencing consequences of ERK activity. Again literature provides a wealth of data about involved proteins, comprehended into several more or less well-defined and often highly conserved modules of the cell cycle. J.J. Tyson recently gave an excellent review on mathematical models that integrate various experimental insights on cell cycle coordination [490]. Minimal modules of cell cycle regulation can be described again in terms of complex systems analysis. An autonomous Cdc2 oscillator is coupled to a bistable trigger, that mediates the transition from interphase to mitosis and prevents slipping back [429, 373]. In Xenopus oocytes the system is coupled to above mentioned Mos/ERK positive feedback cycle to even render this bistable transition irreversible, i.e. converting a transient stimulus into a self-sustaining pattern of kinase activation [546].

Spatio-temporal variations of ERK activity are measured by nuclear feed forward sensors [336, 335], which in our PC12 cell model system can mediate either cell cycle arrest and a neuronal differentiation program or cell cycle progress (proliferation). Cell cycle progression has been observed to require cyclic ERK and PI3K activities, but each branch during different phases [394]. At the same time, the cascade and the parallel PI3K/PDK1/Akt branch protect against apoptotic signals. Another recently discovered kinase independent function of c-Raf is at mitochondria, where it can again act as a scaffold inhibiting another serine/threonine kinase, namely MST2 [360]. To close the circle to above outlined adhesion signaling, PAK mediated phosphorylation at S338 is involved in translocation of c-Raf from the membrane to this mitochondrial location and activity, at least in vascular apoptotic protection [8], see fig. 9.

5.2.2 I: c-Raf's and other Cycles

The c-Raf protein is one of the best studied eukaryotic signal transducers. It is likely that the hub-like cross-connections from c-Raf to cytoskeleton and membranes, to Ca^{2+} and cAMP pathways — mediated via small G protein and upstream kinases — will only be prototype for several such complex regulation stories among cellular signal transducers. At the level of the Raf kinase and scaffold, important questions for now would be, how these alternative functions of c-Raf are related to each other and to its function in the cascade. Does c-Raf (have to) activate the cascade before translocating to its other sites of action? Does it go back again, i.e. is their a cycle of c-Raf activities? Can we expect a direct connection between these pathways, e. g. c-Raf needs to be tagged by a combination of specific phosphorylations, or is the only effect a mere titrational one, i.e. sequestering c-Raf at one location will keep it unavailable for the other functions? How many such cycles does an individual protein undergo before (proteasomal [423, 290]) degradation?

Figure 41 depicts a sketch of a fragmentary model of the c-Raf cycle, extended from and tying in with a series of previous sketch models in c-Raf literature, e.g. in [254, 257, 184, 19] and thus comprehending almost two decades of collaborative research in Raf biology. Inputs from inhibitory cAMP/PKA, PIP3/PDK1/Akt pathways, from activating RTK/Src and integrin/PAK branches, as well as feedback desensitization by ERK and resensitization by PP2A are represented. The recent observation, that an inactive fraction of c-Raf is already present at the membrane [184], is also included. Alternative functions of c-Raf at mitochondria, with a possible role of PAK mediated S338 phosphorylation mediating translocation, and at the trailing edge of migrating cells are indicated. The model neglects c-Raf's potential direct interactions with its sibling proteins A-Raf and B-Raf [519, 326], which might however be crucial for spatio-temporal control of ERK activation.

The figure converts the static interactions depicted in fig.7 into a cyclic model. As the known interactions involved in c-Raf activation reveal a quite complex picture, sketches will likely not suffice anymore to capture all possible states and dynamics of c-Raf cycles. Quantitative models of Raf cycles will be required to handle above outlined and other questions appropriately. Such dynamic models will not attempt to represent the exact mechanistic process, but rather our hypotheses of c-Raf's specific wiring into global cellular networks. The figure has been created with the graphical SBML editor *CellDesigner* [203, 141], that offers representability beyond SBML, as it al-

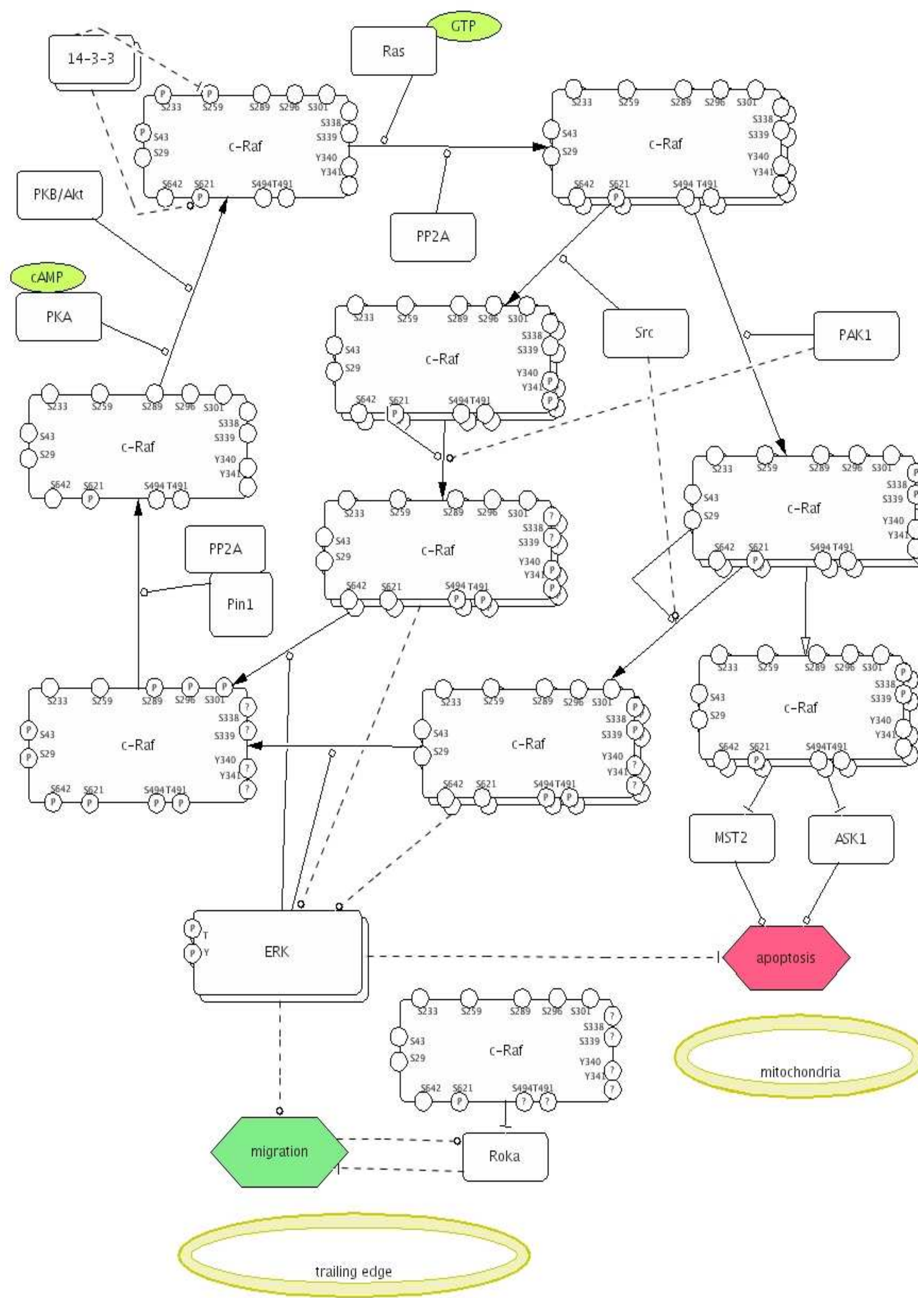


Fig. 41. Fragmentary c-Raf cycle. See text for details. Compare with fig.7.

lows to depict protein structure and specific biochemical modifications in *process diagrams* [247]. In the problematic dualism of content and layout, the model is still rather close to the latter and can at this point not be used as a detailed reaction network in SBML. A series of more detailed models could capture different aspects and cycle possibilities, and should then be completed by reaction parameters calculated from experimental data such as the spatio-temporal dissections of c-Raf states in Hekman et.al. [184], to allow an analysis of possible dynamics in parallel with further experimentations. Embedding such models into community-based webtools would allow a collaboration between different groups and render such models into frameworks for organized collaborative experiment design and hyper-linked data integration via RDF+XML technology [512].

Within the herein adopted framework of coordination between morphology, metabolism and cell cycle, several signaling modules that feed into this cycle and/or are modulated by diverse states of c-Raf, could be identified by an organized literature review. A set of modules are proposed here, where mathematical modeling might yield significant insights about Raf's and the cascades' integration into cellular coordination networks:

Proposed Models I: Morphology, Metabolism and Cell Cycle

(1) The epithelial-mesenchymal transition (EMT) of an adhering to a migrating cell-type as well as its reverse process (MET) are crucial not only for pathogenesis, but also for development [466] and its evolutionary transitions [368]. The basic modules that coordinate these processes are quite well conserved [497]. An integrated model would couple above outlined multiple feedback cycles, which commonly involve receptors (RTK, integrins, GPCR), lipids (PTEN/PIP2, PI3K/PIP3), a set of small G proteins (Ras, Ral, Rap1, Rac, Cdc42, Rho), and effector kinases (Raf, PAK, Rok, Src, FAK, ERK). While these modules are often observed to work independently, their sequential activity underlies the complex processes of EMT and migration. Such a coupling of bistable feedback modules can lead to complex behaviors, that requires mathematical modeling for a detailed analysis [490, 129]. Accounting for major cytoskeletal remodeling processes, will allow to interpret such models in terms of basic biophysical requirements such as integrin/ECM adhesion forces (cf. the concept of *tensegrity* [208, 209]).

(2) Outgrowth of neurites from a differentiating neuronal cell can be considered a migrational (chemotactic) process that decouples the leading edge modules from tail retraction and adhesion turnover [319]. Sustained activation of ERK in PC12 cells can lead to cell cycle arrest and neuronal differ-

entiation. This crucially depends on cytoplasmatic signaling via NGF:TrkA receptor complexes at internalized endosomes, which are transported retrograde along such outgrowing neurites. As observed in high-resolution confocal microscopy, e.g. by Gerhard Schütz and colleagues, such vesicles travel continuously with *random interruptions by periods of diffusive motions with concomitant pathway changes* [425]. Such a process could likely be captured by reaction kinetics in restricted dimensions (Noriko Hiroi, personal communication), such as applied to movement of DNA binding proteins on their substrate [27, 502, 222, 431, 171].

(3) Sustained ERK signaling from NGF induced endosomes [297, 232], but also e.g. in B cell receptor signaling [51], occurs via differential modulation of Ras/Rap1 and c-Raf/B-Raf activation of the cascade. The model of receptor internalization and endosomal signaling by Birgit Schöberl et.al. [419], as well as the recent PC12 model by Sasagawa et.al. [411] have been encoded in SBML for this work (subsections 3.2.4 and 3.2.7) and would be an elegant starting point for a refinement according to the known interactions in the PC12 system and accounting for vesicular trafficking. A combination with cell cycle models — which are available in SBML at databases — could further try to account for the feedforward sensors of ERK/Rsk activity [336, 335], expression of cell cycle regulators, and interpret the differential timing of cyclic PI3K and ERK activities during cell cycle progression [394].

(4) Finally, the AK1β has been observed to be involved in cell cycle arrest [344] and PC12 neurogenesis [344], and thus might be one other target of the nuclear feedforward sensors [336, 335]. An AK1β /AMPK module [175], hypothetically co-expressed in neuronal membranes, could sense neurogenic activity (via sustained phosphorylations) and drive local metabolic activity — e.g. fatty acid synthesis for new membranes [440] — to enable neurite outgrowth, but also directly attenuate Ras/Raf activation upon ATP depletion [242].

An integrated version of the models 1-4 could close the circle at this point, by coupling neurite outgrowth, vesicle transport and sustained B-Raf/ERK activity to this hypothetical metabolic module. However, the exact details of B-Raf and c-Raf relations are not understood very well. Has e.g. the bi- or trimodal regulation of ERK activity always been possible via modulation of the single Raf protein, or was that a vertebrate innovation, depending on the three Raf paralogues? In which developmental and physiological contexts is differential ERK activity required? A look at the possible evolutionary origins of this cooperation might help to clarify the picture:

5.2.3 II: Kinase/Scaffold Evolution

Scaffolding of the cascade has long been recognized as a central mechanism to impose specificity and allow plasticity of this ubiquitous signaling module [19], and is thought to be essential for evolutionary, but also artificial rewiring of the cascade [362, 379]. Raf proteins are thought to di- or oligomerize during activation [283, 128, 326, 184], a property that is likely shared with all members of the serine/threonine protein kinase (STPK) family [562]. Duplication of a gene whose protein acts as a di- or oligomer yields hetero-oligomers and the mutation of one of the duplicates, introducing e.g. activating or inactivating amino acids or a potential for regulation by post-translational modifications, can modify activation dynamics, redirect sub-cellular localization and connect a given module to other pathways [362]. Mutation of regulatory sequences can yield cell-type specific differences of signal response. Cooperation of the duplicated Raf proteins, potentially by direct interaction [519, 326, 184, 483], can modulate the time-course of cascade activation in a cell-type specific manner [297, 254, 314, 528]. Certainly several of the known cross-regulations of STP kinases arose from such duplication and diversification events. Raf itself seems to act both as a scaffold and a kinase [19], a combination that is also found e.g. in the cascade's direct regulators Ste20/PAK and KSR, both of which contain STPK domains and act as scaffolds at the same time. At least the latter shares similarity to the Raf kinase and can be considered a paralogue of Raf, while its kinase activity — it can phosphorylate c-Raf at T269 [545] — seems not to be required for its function in the cascade [317, 544, 404]. Is this a general theme? Kinase/scaffold pairs might result from an initial gene duplication resulting in hetero-dimers which can both bind the substrate and modulate each other. One kinase could then e.g. loose its enzymatic activity and act as a scaffold, bridging the remaining kinase with its substrates.

However, a rough phylogenetic analysis of >500 human protein kinases (>100 in the yeast *kinome* [65, 291, 64]) features the c-Raf inhibited kinases on separate of the 7 identified subgroups. MST2 clusters with PAK, MEK and some M3K proteins and Rok-α with PDK1, Rsk, PKA, Akt/PKB and PKC families [291]. This raises the question, whether the various direct interactions of c-Raf with other SPTK and tyrosine kinases (YK) has always existed — descending from an ancient duplication and diversification processes, or whether c-Raf has newly acquired these functions. STPK share a flexible structure for diversification of regulation mechanisms and contain similar dimerization motifs [206, 562]. Is their a general tendency of STPK

to interact with each other? Specific SPTK interactions could either be re-acquired along diverse evolutionary paths or result from a duplication and diversification process.

Proposed Models II: Phylogenetic Pathway Reconstruction

(5) The phylogenetic analysis of the human *kinome* included STPK and tyrosine kinases [291], and while the 5-7 groups likely reflect evolutionary relations, the details are certainly not reflected. To clarify potential scenarios implicated by above hypothesis, a more detailed phylogenetic analysis of protein and nucleotide sequences should concentrate on specific pathways and known interactions. With above mentioned (Raf, STE20/PAK, KSR, Rok, TAK, MST, ERK, Rsk, etc.) or alternative interacting STPK systems such as the recently identified kinase networks around AMPK (involving the STPK members of the CaMKK, CaMK [536, 205], Rsk (p70 ribosomal S6 kinase) [243], LKB1 [228] groups), several nice pathways with some known evolutionary differences lie at hand. Such analysis should especially account for binding and target sites in each of the proteins, especially the regulatory and dimerization motifs, but also the set of interacting small G proteins and known cascade scaffolds. Ultimately such an analysis could try to outline or predict potential evolutionary transitions of known and yet unknown pathway dynamics.

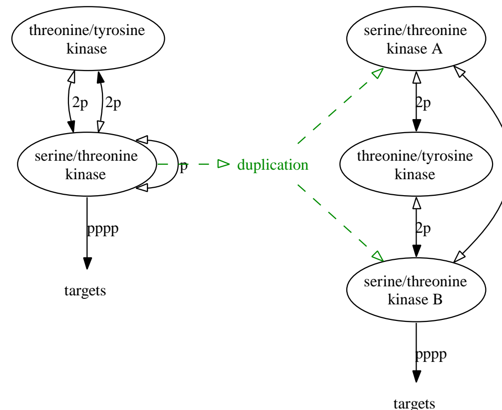


Fig. 42. **A Putative Origin of the MAP Kinase Cascade?** See text.

However, such a hypothesis could even go several steps further back in evolutionary time. So far, analyses of cascade evolution only looked at (co-) duplication and rewiring of an existing cascade [54, 362, 379]. Can the evo-

lutionary invention of the three-tiered kinase cascade itself also be suspected in a duplication of initially single STPK? This putative single kinase, acting as a di- or oligomer, could have been involved in mutual regulation with a T/Y kinase, or a RTK and have had a broad range of cellular target. Upon duplication of this STPK, one became the upstream and the other specialized as the downstream kinase. In yeast osmolarity response, the Psb2 protein acts as both a scaffold for the M3K Ste11 and the MAPK Hog1, but at the same time as the intermediate M2K [362], and thus could be derived from an enzyme that initially regulated only one other (dimeric) kinase. The Ste20/PAK activation of Raf can also be observed in yeast and thus can be considered as a very old connection [92]. On above mentioned phylogenetic tree, PAK groups with the MEK1/2 [291]. This *kinome* tree is based on too many sequences — including all tyrosine kinases — to be reliable in such detail, but inspires speculation of a scenario involving co-duplication of the M3K/MAPK with a common ancestor of PAK and MEK. Both properties of the cascade, signal amplification and dual-phosphorylation dependent ultrasensitivity could be increased by repeated additions of dual phosphorylation layers which would allow a quicker or stronger response and a sharpened threshold, i.e. a smaller range of initiating signal concentrations, respectively. Together with upstream and downstream STPK the cascade actually consists of 4 to 5 layers and a growing number of possible regulation levels. A sharp threshold of signal response is of immediate consequence for the developmental usage of signal factors in morphogenesis [182], and the FGF proteins are widely used morphogens in metazoan development. This would be a fascinatingly simple scenario of the cascades' origin and further evolution, but will be hard to test. A putative conservation of the original functional role might provide some further hints.

The cascade's original function is often seen in separate but interconnected pathways of osmolarity and cell cycle regulation [259, 258, 54]. A possible origin of this dual roles can be suspected, as dynamic regulation of osmotic pressure is certainly crucial for cell volume coordination during cell growth and cytokinesis. This connection has recently been elegantly described in an ODE model of yeast osmotic shock response and cell volume coordination with glucose metabolism [249]. A putative 'cascade-less' ancestral MAP kinase can be expected to have been (or still be, wherever still existing) involved in coordination of osmolarity regulation and/or life cycle related processes (mating, proliferation, migration), that involve cell growth or generally cell morphological transitions. Candidates could be suspected within the bacterial family of eukaryote-like STPK. Interestingly, such bac-

terial STPK are usually expressed at the membrane, as they contain a single membrane spanning domain coupled to diverse extracellular domains. As the single transmembrane domain is unlikely to transmit conformational information from the extracellular domain, the latter are probably involved in co-localization and thus support of oligomerization of the intracellular kinase domains [562]. As an example, the PnkB protein of *Mycobacterium tuberculosis* is linked to four extracellular PASTA (penicillin-binding protein and S/T kinase associated) domains, and tandem PASTA domains have been proposed to target *Streptococcus pneumoniae* Pbp2x protein to sites of cell growth [154, 562]. Continuing on this quite uncertain road of speculation, further insights into PknB biology would propose even more links to todays eukaryotic MAP kinase cascades. PknA and PknB are expressed predominantly during exponential growth phases, while over-expression (of kinase active proteins only) slows proliferation, diminishes viability and alters cell morphology, namely by causing long, broad and sometimes branched cells that suggest defects in septation, cell wall synthesis and/or cell division [230]. It is clear, that these phenotypes do not reflect homologous functions of MAP kinase cascades in yeast or even metazoan protein-protein interactions in cell wall and cell cycle regulation. The eukaryote-like STPK in bacteria might have even been acquired through a horizontal gene transfer, early after the separation of eukaryotic cells [273, 173, 562]. The consequences of STPK (over)expression in bacterial cells might however point to a general biochemical of biophysical function of protein phosphorylation. In bacteria a single kinase could achieve what requires an amplification via a phosphorylation cascade in bigger eukaryotic cells. Let's reflect shortly on both, complex formation and protein phosphorylation cascades from such a biophysical perspective:

5.2.4 III: Complex Formation, Phosphorylation & Calcium

Signaling pathways, at least eukaryotic, are often mediated by the local nucleation of (clusters of) super-molecular multi-protein complexes, or 'signaling particles' [326, 286, 183, 184], which assemble on various scaffold proteins. Initial (Y) phosphorylations mediate formation, while later on additional (S/T) phosphorylations further lead to inactivation of the complex. In cascade activation at RTK, two or three macromolecular complexes can be distinguished. First, RTK themselves can oligomerize, and sequester a range of adaptor molecules, (tyrosine) kinases, phosphatases and lipid modifying enzymes. Second, numerous binding partners, scaffolds, other kinases

and phosphatases are involved in Ras mediated assembly of Raf containing complexes at membrane sub-compartments [183, 184]. One experiment could distinguish a B-Raf complexes from a third large (60-75 S) complex containing MEK1 and ERK1b. This latter complex was not soluble in non-ionic but required high-salt preparations, while the activated B-Raf containing complex was detergent-resistant [286], hinting towards differences in their electrochemical nature. Cytosolic (inactive) c-Raf proteins on the other hand have been found in 300-500 kDa complexes, containing 14-3-3, heat shock and other proteins [134, 514, 169].

Such complexes will lead to a combinatorial explosion of species numbers in the common approaches of analyzing reaction network dynamics by systems of coupled differential equations. Nucleation or clustering processes at cellular membranes call for integration of biochemical and biophysical aspects of polymerization reactions, membrane biology and protein properties.

Phosphorylation at Membranes Cellular membranes are lipid bilayers with negative charges at each side. Negatively charged sugar polymers are a major constituent of the extracellular matrix. These glucosaminoglycans are either sulfated during their complex endosomal synthesis or directly incorporating deprotonated glucuronic acid monomers in the case of the cephalochordate– and vertebrate–specific hyaluronan, which is probably directly synthesized at the plasma membrane [444]. In both cases, negative charges are immobilized on polymers, in contrast to their soluble bivalent and monovalent counter-ions. Eukaryotic plasma membranes nevertheless have an electrochemical potential which is negative on the inside, unless reversed by depolarization. The nuclear membrane is likewise negative towards the cytoplasma [304]. The (eukaryotic) plasma membrane is further supported by a very densely packed actin cortex. Actin itself has an isoelectric point around pH 5 (as can be easily calculated with the tools available e.g. at SwissProt database, `http://www.expasy.ch/sprot/`), and thus is negatively charged at physiological pH [464]. Thus, the negative charge of lipid bilayers is supported by immobilized negative charges (polyelectrolytes) on both sides. Within this matrix of fixed negative charges, a diffuse layer of counter-ions (e.g. Ca^{2+}, Mg^{2+}, K^{+}, Na^{+}) will concentrate to form a so-called *Gouy-Chapman cloud*. The electrostatic profile of this *series of plate capacitors* has been reviewed in more detail by M. Olivotto et.al. 1996, who employed the Gouy-Chapman theory on surface potentials [95] to predict field strengths in the order of 100 kV/cm, *with predictably crucial effects on the physico-chemical reactions at the boundaries of the membrane* [358].

While the consequences of such biophysical phenomena are hardly understood in terms of protein-based coordination networks, they can well be imagined to be of immediate relevance for our case of receptor tyrosine kinase mediated activation of the Raf/MEK/ERK cascade. Activation of RTK signaling leads to further immobilization of additional negative charges (the γ-phosphate of ATP) to the newly forming receptor complexes. Initial (Y) phosphorylations could support complex formation through mere electrostatic repulsion and inflow of solvent, i.e. a kind of local swelling of the actin cortex region around RTK complexes. Newly transferred charges can be quickly covered by adaptor proteins, that get again phosphorylated and continue to sequester additional proteins to the complex. Inhibition of these complexes by hyper-phosphorylations via ERK, such as observed in feedback inhibition of c-Raf (predominantly at S) [112] or the adaptor protein FRS2α (at T) [269] could subsequently lead to dissociation from the membrane, again by electrochemical or osmotic forces. It can be further imagined that PLC (PIP2 degradation) mediated changes of intracellular pH and the local ionic milieu via proton and ion pumps [329, 215, 6, 187] and of detachment of the membrane from the actin cortex [382] would be involved in such a general phenomenon. Charge driven or supported processes could finally be involved in internalization of phosphorylated receptor complexes. If such a scenario is considered worth further research, one other interesting and only recently discovered signaling mechanisms at RTK [391, 229] should be accounted for. RTK signaling has been observed to induce formation of reactive oxygen species (ROS) via activation of NADPH oxidase enzymes see fig. 17). The ROS inhibit protein tyrosine phosphatases that de-phosphorylate RTK. This system has been proposed to form a bistable feedback mechanisms, responsible for the phenomenon of cross-activation of non-ligand bound receptors, the so-called *lateral signal propagation* [391]. Such a REDOX based signaling pathway, drawing an again highly speculative cross-connection, could furthermore be involved in receptor internalization processes. Oxidation of phospholipids leads to a cone like structure, as opposed to the rod like structure of their reduced form. When applied externally, they immediately localize to the curved membrane structures of caveolae and clathrin coated pits and are internalized with the respective pathways (Reinhard Grurl et.al. presented at the GEN-AU meeting in Litschau, 2005). It is thought, but not yet shown, that endogenous oxidization of phospholipids might be causally involved in formation of membrane invagination and internalization processes.

Protein Charge and Localization Some additional evidence that the *electrical dimension of cells* [95] might play a fundamental role in differential and highly localized complex formation, shall be mentioned. This evidence might also hint towards a potentially quite simple theoretical handling of such a phenomenon on proteomic levels. A couple of recent works employed simple isoelectric point (pI) calculations to proteomic databases and found (although yet little) evidence that the trimodal (eukaryotes, see figure 19) or bimodal (prokaryotes) distribution of protein pI values generally corresponds with subcellular localization [426, 32, 524]. Protein charge at specific pH values can be easily calculated. Well known protein pI calculations, based purely on amino acid composition, generally yield good results, as the contributing amino acids are polar and usually lie on the outside of a protein – except for cysteine residues which might form disulfide bonds. A comparison with calculations for available molecular structures is required and has recently been made possible by the *H++* webserver [155]. Such calculations could be refined with respect to subcellular electrochemical milieus to determine whether the involved charges and — importantly — charge changes upon phosphorylation and other post-translational modification and signal induced pH changes could at least theoretically lie in ranges that would allow mere force effects on cellular morphology. Isolation and further electrochemical characterization of above mentioned 'supermolecular signaling particles' would help to test such hypotheses experimentally. This could lead to a very general and simple model of receptor tyrosine kinase mediated signal transduction.
As pointed out by M. Olivotto et.al. for plasma membranes [358] and extended to nuclear membranes by Matzke and Matzke [305], electric fields at membranes might be an important player not only in neuronal and muscular excitation processes but for cell function in general. Trans-epithelial potentials could repeat such putative principles on organismic scale in developmental [348, 395] or physiological contexts [347], where *galvanotaxis* might guide neural crest cells [162] or keratinocytes during wound-healing [390], and embryonic Ca^{2+} waves can be observed in increasing resolution [147, 518]. While above hypotheses remain pure speculation, the main motivation here is to point out that biophysical aspects of signaling are in the opinion of the author accounted for much too little by both cell-biological and theoretical approaches to cellular signaling, while on the other hand they would provide a simple framework for a more profound understanding thereof. Localized immobilization of negative charges by e.g. protein phosphorylation could lead to localized swelling of cell structures and thus directly induce morphological changes. While such a process would

be sequence-independent, specific protein-protein interactions could refine these general processes into species– and sequence–specific subcellular variations. The involvement of the (ultrasensitive and bistable) MAP kinase phosphorylation cascades in cell-morphological changes during cell growth, cytokinesis and migration could well be grounded in such a direct local regulation of osmotic pressure.

However, the monovalent and bivalent counter-ions of above outlined negative matrices might further be functionally differentiated. The bivalent ones, of which Ca^{2+} is the best known in signaling context, are well understood as cross-linkers of negatively charged polymers. A subtle change of mono- or bivalent counter-ion concentration — but also of other conditions such as pH or temperature — can cause a spontaneous displacement or inflow of solvent molecules (e.g. water), which are called gel-sol or sol-gel phase transition, respectively. Such processes are known to be important e.g. for vesicular secretion and are technically applied for drug delivery via artificial vesicles [246, 434]. They have also been proposed to underlie e.g. amoeboid cell migration [41, 534] and recently have even been suspected to constitute a fundamental driving force of cellular life in general by G.H. Pollack [371]. Again, the problem will be to explain how such general phenomena are integrated with specific protein-based signaling networks. So finally, let's take a look at ...

Calcium Signaling RTK, GPCR signaling, and more specifically e.g. also Ras, can induce PLC proteins (PLCγ, PLCβ and PLCϵ, respectively), whose product IP3 can induce a stable increase of intracellular calcium (iCa^{2+}), but also autonomous Ca^{2+} oscillations, via the 'Calcium Induced Calcium Release' mechanism (CICR), with potentially quite complex patterns, arising through diverse feedback mechanisms up to IP3 degrading enzymes [260, 424, 561]. Besides the classic iCa^{2+} sensing proteome, that includes again (evolutionary very old) members of the STPK family, the Ca^{2+}/calmodulin dependent kinases (CaMKI - IV), the phosphatase calcineurin (also known as PP2B), and the scaffold calmodulin, now many other sensors are known, and were recently reviewed by Berridge et.al. [29]. Like MAP kinase protein phosphorylation cascades, iCa^{2+} release acts both locally and globally and has diverse connections to all three corners of above triangle of morphology, metabolism and cell cycle. By its nature as a bivalent cation, iCa^{2+} release can be considered a mechanism of cellular signal transduction that is implicitly different from, but putatively complementary to phosphorylation cascades.

Calcium is stored in the endoplasmatic reticulum (ER) and actively balanced by pumps and channels between these ER and other vesicular stores, the cytosol, mitochondria and extracellular media. Highly localized transients ('puffs' and 'sparks') and oscillations that can spread as global waves of calcium release [29] are known to occur on single cell as well as on organismic levels during development [517, 518]. Calcium signals are sensed via many mechanisms, and several examples are known in which frequency and amplitude of iCa^{2+} oscillations encode the relevant information (reviewed in [150] from a theoretical perspective).

... in Morphology: Depleted calcium stores are refilled by a direct coupling of the ER to yet undefined 'Store Operated Channels' (SOC) at the plasma membrane, and this requires disassembly of cortical actin [365, 284, 402]. Actin disassembly usually involves cAMP signals [420]. A communication of the calcium with the cAMP system was one of the earliest studied cross-talk systems [381] and is now known to be mediated e.g. via phospho-diesterase 1 proteins (PDE1A - C) and direct activation of a range of adenylate cyclases [29]. Migrating cells establish a gradient of iCa^{2+}, with a higher concentration at the rear end [170]. While the exact role is not understood, iCa^{2+} has been observed to be involved in myosin II based uropod detachment and retraction [120, 113], e.g. via CaMK phosphorylation of the myosin light chain kinase (MLCK), see fig. 25. The review by Pettit and Fay (1996) provides several details on calcium and cytoskeletal interactions [369]. More recent observations include e.g. iCa^{2+} modulation of focal adhesion kinase (FAK) mediated turnover of FA. Its oscillations correlated with the FAK cycling between FA and cytosol [146, 145]. However, within the set of interactions reviewed in this work, one other possible relation shall be noted: as the initial cell polarization is guided by PIP3 at the front and PIP2 at the rear, the establishment of a iCa^{2+} gradient can well be speculated to involve PLC catalyzed production of IP3.

... in Metabolism: iCa^{2+} distributions are directly influenced by the metabolic state, with one route via the ADP ribosylcyclase being modulated by both major glycolysis products, ATP and NADH/NADPH [29]. Via CaMK, calcium feeds into the recently outlined CaMKK/CaMK/AMPK cascade [205, 536] to drive metabolic activity but also activate p70 S6k (of the Rsk family) to modulate translation [243] or e.g. induce p53 mediated cell cycle arrest [473]. The balance of calcium concentrations between mitochondria, endoplasmatic reticulum and cytosol is also known to be directly involved in the cellular apoptosis program and likely represents a very fundamental connection of mitochondrial metabolism and apoptosis [28].

... and in Cell Cycle: The most direct influence of iCa^{2+} on nuclear transcription events are via calcineurin mediated dephosphorylation and activation of the NFAT (nuclear factor of T cells) transcription factor [29]. However, here only the Raf/MEK/ERK cascade shall represent its influence on transcriptional events and thus also on cell cycle coordination. A well-known iCa^{2+} responsive or sensitive family are the diverse PKC isoforms. Importantly PKC activates Ras/Raf via a mechanism distinct from RTK mediated Ras activation [292]. A bistable feedback cycle that in theory could account for *cellular memory* includes PKC, PLA2 and CaMK/calcineurin in neurons [30] and fibroblasts [31], see fig. 33. A range of so-called CalDAG-GEFs activate either Ras or Rap1, and are themselves activated by binding to iCa^{2+} and DAG (and thus both branches of PLC activity), e.g. in some version of PC12 neuronal differentiation [551], also see fig. 12 and 13. Interestingly however, Ras has been observed to be modulated via an iCa^{2+} sensitive GAP (CAPRI) and a GEF (RasGRPI), inducing an inhibition of early Ras activity at the membrane, while activating Golgi located Ras, respectively [33], see fig. 6. It has recently been proposed that Ras and Ras/Raf activation cycles are coordinated with iCa^{2+} oscillations, and that the latter's frequency might even be optimized for Ras/Raf and cascade activation [261]. On the other end of the cascade, ERK has recently been observed to activate iCa^{2+} release in human platelets [403]. While a cellular function of these observations of ERK cascade and calcium cross-connections remains elusive, a clarification of potential dynamics certainly calls for computational models.

Phosphorylation and Calcium waves It is clear however, that one can again get lost in observed cross-connections, which depend on protein-protein interactions and are thus subject to species– and cell-type–specific variations. Evolutionary and physico-chemical perspectives might again provide an alternative *ligne de fuite*, along which above interconnections could find a more general definition. Both, iCa^{2+} increases and ERK mediated phosphorylations are regulated locally, but can spread throughout the cell. As mentioned above, bivalent cations can cross-link negative charges on large polymers and thereby mediate gel-sol phase transitions. Do e.g. iCa^{2+} waves that propagate through the cell interact with local ERK (or general MAP kinase) targets, especially cytoskeletal ones? Cellular iCa^{2+} waves could check for the local structural integrity during phosphorylation induced cell-morphological changes. Local osmotic swelling by charge immobilization could be counteracted by pulses of iCa^{2+} induced contraction. Such a hy-

pothesis is relatively far away from todays understanding of cellular signal transduction and experimental accessibility, but certainly would provide a fascinating perspective for integrated models of cell function.

Proposed Models III: Calcium & the Cascade

(6) Model (1) could be extended by the putative relation of the PIP3/PIP2 gradient, with PTEN providing the PIP2 substrate for a PLC at the rear, where its product IP3 could activate release of iCa^{2+} from local stores, which could further have an impact on contraction via a CaMK/MLCK branch [369, 120, 113] (compare with fig. 25), and in focal adhesion turnover via the observed correlation with FAK cycles [146, 145]. Does iCa^{2+} also have a direct contribution to acto-myosin based contraction by supporting gel-sol phase-transitions?

(7) RTK, FRS2α and c-Raf phosphorylation time-courses of model (2) could be fed into refined protein charge and pI calculations that can account for e.g. phosphorylated residues. The H++ webserver would allow some structural considerations of kinases at membranes, where their activation might be influenced by electrical dimesion of membranes, which can be handled by the Gouy-Chapman theory [358]. c-Raf is nearly neutral and thus less soluble [32] when 'de-sensitized' by ERK feedback phosphorylations (see section 2.2.1). Can local charge changes have electrostatic or osmotic effects? Is protein charge a general localization signal? Accounting for ROS signaling [391] in NGF:TrkA lateral signal propagation [229] could allow to test whether putative lipid oxidations could result in significant changes of membrane curvature. Are such effects involved in internalization processes?

(8) A reaction network model of the localized interactions of iCa^{2+} with Ras modulators, as observed by Bivona et.al. [33] and related works [88, 503, 504], could be employed to test the conclusion of Kupzig et.al., that iCa^{2+} *oscillations are optimized for* Ca^{2+}*-mediated activation of Ras and signaling through* the Raf/MEK/ERK cascade [261]. Including potential effects of additional calcium sensitive GEFs could extend model (3) to study effects of iCa^{2+} on sustained signaling via c-Raf/B-Raf cooperation. Finally, such a model could test putative dynamics of ERK feedback on iCa^{2+} release [403] to explore potential dynamics. The link from CaMK to AMPK could test an impact of iCa^{2+} on the putative metabolic sensor in outgrowing neurites proposed in model (4).

(9) Finally, the most speculative hypothesis is the proposed general interaction of bivalent cations and negative polymers: do cell-wide waves of

protein phosphorylations interfere with iCa^{2+} pulses, by e.g. local swelling and contraction phenomena? This could only be tested experimentally, by employing confocal microscopy to visualize e.g. local ERK activity by (a) FRET based peptide sensors [159], (b) the general phosphorylation status by phospho-serine or phospho-threonine fluorescence markers [450] or (c) ATP analogs carrying a fluorophor instead of the γ-phosphate and construction of ERK enzyme that utilize this analog [190]. While for visualization of iCa^{2+} a variety of fluorescence markers are available, the eventually very small changes of local morphology will be hard to detect. Maybe flexible nano-patterned surfaces, as used to study integrin clustering in cell adhesion [10], would allow a fine-grained detection of local force generation.

It has to be stated again, that this last model emigrates far into the land of speculation, but would certainly provide a fascinating perspective for an integrative understanding of the cell. A general model of cell function could be based on a seizable set of variously old general principles, that must account for basic metabolic (biochemical) and morphological (biophysical) requirements, on which evolution can act via sequence– and thus species– and cell-type– specific implementations, that e.g. modulate their subcellular organization. A specific framework will then be required to understand evolutionary implementations of these putative general principles. Usually, conceptual frameworks arise from pathological or developmental contexts. An exceptionally nice context, that would allow to interpret all processes and hypotheses above, could be identified in vertebrate — and with interesting variations also cephalochordate — somitogenesis:

5.3 IV: Cascade & Calcium in Context : Somitogenesis

Fascinatingly, calcium waves also occur on multicellular scale, e.g. during and beyond gastrulation, and both between coupled cells and within the extracellular matrix. While these waves can be visualized with increasing resolution [148, 147, 518], their potential source as well as their functions remain mysterious, but are often suspected in a structurally integrative role in morphogenesis [517, 459, 505]. They are likely generated by potential differences across epithelia: so-called trans-epithelial potentials [395]. One obvious link to a morphologically integrative role can be suspected to involve calcium-dependent cell-cell adhesion via cadherins [250], which provide on their own much evidence for a role in morphogenesis [164]. Recently however, one direct role of calcium in developmental signaling has been clarified.

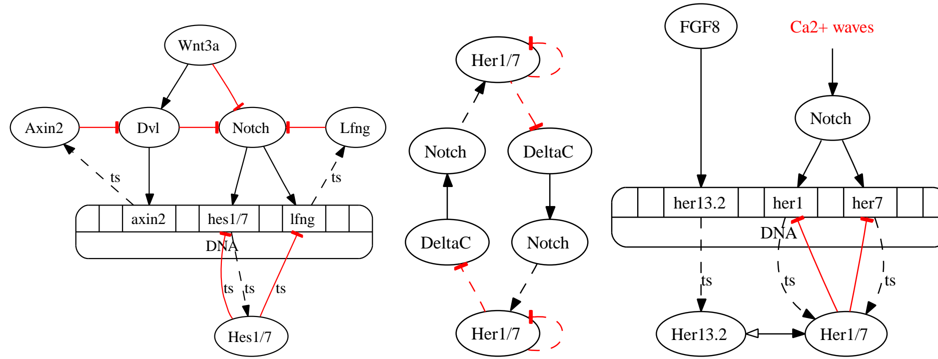


Fig. 43. Different implementations of the vertebrate somitogenesis clock after Rida et.al. 2003 [392]. Left: entrainment of the clock within the tailbud by a Wnt3a signal, as known from mouse (mainly) and chicken. Middle: coupling of oscillations between two neighboring cells within the presomitic mesoderm via Notch/DeltaC signaling, as known from zebrafish (mainly) and Xenopus. Right: In Zebrafish FGF8 feeds positively into the clock by inducing expression of Her13.2 a binding partner for Hes1/7 [234]. Global embryonic calcium waves might feed into the cell-cell coupled Notch1 oscillations, as known for the initial L-R patterning, that is known to be tightly integrated with somitogenesis.

The Clock & Calcium Waves During vertebrate left-right (L-R) determination, the Notch receptor for cell-cell signaling — the neighbor cell activates Notch via a membrane-bound Delta protein — has recently been observed to act as a sensor for the extracellular calcium gradient between the future left and the right sides of the embryo [386]. Vertebrate L-R differentiation is intricately linked to the subsequent process of somitogenesis via sequential utilization of the retinoic acid (RA) pathway, which interacts with the Notch pathway in both systems. Shortly on this linkage: cephalochordates develop assymetric somites and so do vertebrates, if RA signaling is impaired [44, 499, 233]. However, oscillatory activation patterns of the Notch pathway are part of the 'somitogenesis clock' (see fig. 43), a molecular oscillator whose temporal pattern is converted into the spatial pattern of somites by outgrowth of the tail. While a coupled system of an oscillator and a determination front (see below for the latter) has been predicted some 30 years ago [83], in the recent years central parts of the molecular machinery behind the clock and the front have been identified. Cell-autonomous oscillations of the Hes (hairy and enhancer of split) transcription factors are started in the outgrowing tailbud [90], probably by prior oscillations of the Wnt3a/axin2 signaling system which also initiates the front [17]. In cells that leave the tailbud, Hes oscillations keep being coupled between neigh-

boring cells by oscillations of the Notch pathway [392]. Calcium waves have been speculated to play an integrative role in a recent review on the subject. Interestingly, such waves measured from before somitogenesis until the somite 16 stage, which is also a known mechanistic transition in somitogenesis. Is the calcium wave accompanied by a kind of multicellular contraction via enforcement of cadherin based cell-cell adhesion, such as proposed for the preceding process of convergent-extension movement during gastrulation [459, 505]? It would be a fascinating perspective, if above proposed role of intracellular calcium in sensing of structural integrity would be repeated on the embryonic scale. A possible direct connection of the calcium waves to the somitogenesis clock via Notch has however not been proposed and least of all tested experimentally. How would the coupled Notch/Hes oscillators of the somitogenesis clock respond to the extracellular calcium waves? Besides the Wnt pathway, which is thought to entrain the cell-autonomous Hes clock in the tailbud, and Notch, which couples the clock between adjacent cells, this third rhythm — extracellular calcium waves reinforcing both cell-cell adhesion and Notch signaling — could further coordinate somitogenesis within the process of global embryonic growth.

The Front & the Cascade Notch signaling is generally known for formation of epithelial boundaries between adjacent groups of Notch and Notch ligand expressing cells. Somite boundaries can form between presomitic mesoderm (PSM) cells whose Notch oscillations have been frozen in different phases [414]. The so-called determination front is like the clock initiated at the tailbud. It is now understood to be established by a gradient of FGF8 mRNA, synthesized at the tailbud and gradually degraded in cells that have left the tailbud [115]. By mere outgrowth of the tailbud, the FGF8 gradient moves posteriorly, with anterior cells being exposed to less and less FGF8. An individial cell that falls below a certain FGF8 concentration will freeze its oscillations in their current state. Some cells will keep expressing Notch, while their posterior and anterior neighbors will express Notch ligand. Between them, a cell-cell boundary can form. Thus, the temporal pattern of Notch/Hes oscillations is converted into the spatial pattern of the somites. See fig. 44 and the animated output of theoretical models on Hans Meinhardt's website at `http://www.eb.tuebingen.mpg.de/dept4/meinhardt/somites.html` to get a feeling for this fascinating mechanism. While the FGF8 gradient is accompanied by a gradient of Akt activation, ERK activity is not graded, but just 'on' before and 'off' after the determination front [115]. FGF8 conducts its family's business as a 'competence factor' keeping

cells in a mesenchymal state during morphogenetic processes [167]. Is the ultrasensitive and bistable behavior of the cascade or the differential modulation via differential Raf expression involved in the threshold response within a gradient? Young PSM cells could e.g express only c-Raf and/or A-Raf to respond transiently to FGF8, while later B-Raf expression could be involved in a differentiating sustained ERK signal, shortly before MKP3 feedback and RA supported expression would turn off the response.

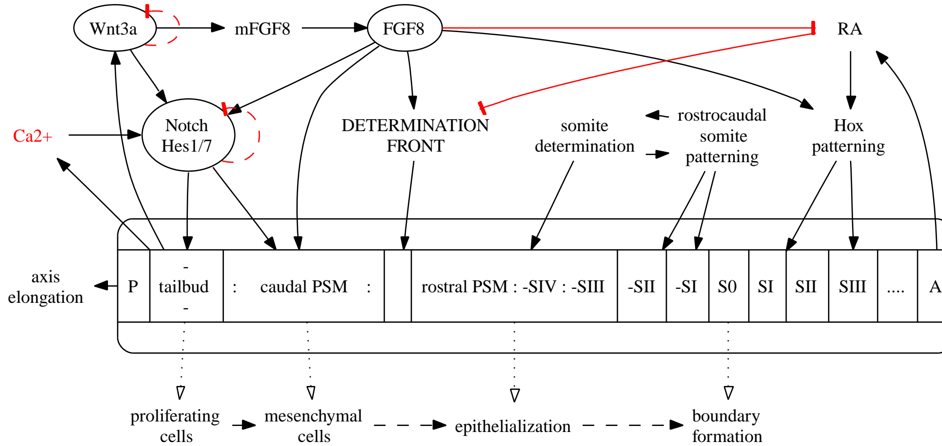


Fig. 44. The vertebrate somitogenesis combines several expressional oscillators, that are coupled between neighboring cells (somitogenesis clock, see fig.43), with a threshold response in a spatial FGF8 gradient (the wavefront or determination front) to control the cell cycle oscillator (mediating arrest and differentiation) and cell morphology (mediating a complete epithelia-mesenchymal-epithelial transition cycle). P: posterior pole; A: anterior pole; PSM: pre-somitic mesoderm; SI – SIII: somites; S0: currently forming somite; -SI – -SIII: determined cells with frozen somitogenesis clock undergo further rostro-caudal patterning before somite boundaries develop. See text for other details.

Proposed Models IV : Vertebrate Somitogenesis

(10) A basic model of Raf/MEK/ERK signaling as used in models 3 and 8, should here be adapted to FGF8:FGFR signaling, which e.g. shares the FRS2α adaptor protein with NGF:TrkA signaling. Besides FRS2α [269] and c-Raf targeted feedback, transcription based negative feedback via Sprouty, Sef and MKP3 are known to be involved in developmental patterning of the body axis [485, 486] (see fig. 8). This specific implementation of the cascade module would allow a detailed analysis of various feedback and cross-talks of cascade activation in a developmental context. Within the FGF8 gradient, ERK seems to be activated in non-graded manner, while

Akt activation is graded. MKP3 deactivates ERK after passing of the cell through the determination front. Its expression is regulated by ERK [486], but also by the opposing RA gradient [330], which thus can further guide the exact positioning of the front. In parallel, a reaction network model of the somitogenesis clock, consisting of coupled Wnt3a/axin2, Hes, and Notch oscillators, and eventually also cell cycle progression within the tailbud [376] could be employed to test potential coupling scenarios.

(11) Well tested versions of model 10 could finally be incorporated into a multi-cellular model with spatial representation. In the Cellular Potts Model (CPM) cells are represented as connected membrane pixels on a 2 or 3 dimensional grid [315]. While it is thought to represent a minimal biophysical model of cell morphology, multi-scale combinations of a CPM with an intracellular gene regulatory network has recently been employed for studies of evolutionary dynamics [14] and developmental morphogenesis [217, 315]. Here, the CPM could serve to represent the spatial constraints and relations of cells during axial growth. Cells proliferate in the tailbud and are continuously pushed out and undergo an EMT to finally form somites via a MET [114]. Each single cell could 'run' the signaling network model via the SBML ODE Solver, with external input to the model calculated from cell-cell contact within the CPM. This combination could represent cell-cell adhesion via cadherins, the synchronization of the clock via Notch signaling, cadherin-based adhesions, but also establishment of FGF8 gradient by FGF8 mRNA degradation [115]. Lax et.al. observed that other growth factors that activate ERK can desensitize the cell for FGF activation by deactivating FRS2 by extensive hyper-phosphorylation [269] (see right graph in fig. 8). Other growth factors from adjacent structures (e.g. EGF) could thus restrict FGF8 induced ERK activity. What then is the role of the graded Akt activation [114], which is usually interpreted as a survival signal? Diminishing FGF8, in cooperation with the increasing RA concentration towards the anterior pole will lead to deactivation of the somitogenesis clock in different phases of Notch expression, finally inducing somite boundary formation. FGF8 and RA cooperation in axial Hox patterning [108] could be included as a gene regulatory network that further coordinates A-P patterning of the axis. In the most interesting test however, tailbud cells but also other observed sources could emit pulses of extracellular calcium waves. Both, their transmission and possible resonance between different sources, and their impact on cadherin-based cell-cell adhesion and Notch signaling could test the proposed role of these waves in tuning of the somitogenesis clock and integration with global embryonic morphogenesis.

6 Conclusion & Outlook

Within the ca. 20.000 publications that mention either of the MeSH terms for the MAP kinase, some detailed interactions were selected from three cornerstones of cell function: metabolism, morphology and cell cycle coordination. The vast amount of literature will certainly provide tons of other mechanistic links from the MAPK cascade to diverse cellular, developmental and physiological systems. As cancer and cell cycle researchers MV. Blagosklonny and AB. Pardee recently pointed out in a short comment:
'Enormous collections of data allow hypotheses to be generated and tested using pre-existing data. Many experimental discoveries arise from chance observations. Similarly, data-searching can reveal unexpected connections'. The according field of activity was denoted *conceptual biology*, while the data uncovered should be collected in a *conceptual review* [35].

This work tried to approach such a conceptual review and at the same time connects to another field that has recently gained much attention. *Systems biology*[6] has become *the* key phrase for the hope in conceptual as well as computational frameworks for handling the vast amounts of *...-omic* data and literature-*'encoded'* knowledge, as well as for recent grant proposals. The question arises whether this term makes sense as biology is *per se* a science of complex dynamic systems. *Systemtheorie* was first introduced as a scientific concept during the first half of the 20th century by the theoretical biologist Ludwig von Bertalanffy. Thus, systems biology is just a new name for long existing approaches of theoretical biology, maybe seen in the recent (twi-)light of -omic databases. Technically, this field can also be viewed as a combination of two other 'biologies', namely mathematical and (data-) integrative biology, employed to bridge knowledge*s* from genetics and cell biology, biophysics and biochemistry, developmental biology and physiology, ecology ... it gets clear that when talking about systems biology, people could merely talk about 'biology'. In singular; but based on yet to define fundamental theories.

[6]) a PubMed search on Sep. 13, 2005 retrieved 942 articles mentioning the term, 242 of which are reviews, please choose an adequate review for yourself. Personally, I appreciate Wang et.al. for pointing out one promising future application [511].

The construction of quantitative mathematical models of reaction networks (RN) is often seen as one core method of systems biology. It is derived from enzyme kinetics and usually starts with a drawing of the basic chemical RN [501]. In cell-biological interactions however, the underlying RN can be rather complicated, and thus the construction of such must be preceded by higher-level (protein-protein) interaction diagrams. Here, a hands-on approach towards reusable and formalized interaction diagrams was adopted to handle and collect information along a rather random walk on the graph of published causal and correlative interactions around the Raf/MEK/ERK cascade, as mainly studied in the mouse model. The *bioLog* graphs have a very weak and flexible definition. Employing the GraphViz language *dot* for general graph descriptions allowed to stay flexible in the description of literature derived experimental observations from multiple levels.

This way, 11 models — or hypotheses — could be derived, each of which features some connections that have not been proposed or tested before. While each of the models might be worth testing on its own, they are embedded into bigger 'stories' and connect to each other. Those cross-connections are however the most speculative hypotheses and often served rather as a guideline through literature jungle, but also try to approach a general *'big picture'* that might *'emerge from the sea of biological data'* [366]. Such a general theory of cell signaling will have to account not only for known protein-based coordination networks, but also for basic biochemical (metabolic, energetic) and biophysical (osmotic, morphological) requirements, as well as for cellular diversification during evolution and multi-cellular development. While it is unclear whether this work came any closer to such a theory (see below), or whether the 'big pictures' proposed here will hold further experimental and theoretical tests, some guidelines for the conceptual, integrative, systems, or theoretical cell-biologist can be proposed, that might help to muddle through literature jungle:

Cell Function A conceptual framework for cellular function is required. Specific cellular processes such as proliferation, differentiation or apoptosis are not very useful, as most pathways are involved everywhere, but often depending on cell-type or prior signaling history. Here a more general framework, that views cellular signaling networks as gradual evolutionary extensions of basic metabolic and morphological coordination with the cell cycle, was found better suited. The MAP kinase pathways, and in particular the Raf/MEK/ERK cascade, connect to all three corners of this triangle. When inducing cell cycle progress or migration, they obviously need to drive both, metabolic activity and cell morphological changes. While upstream coordination of Raf kinase/scaffold activity cycles has been studied in great molecular detail by various groups, the downstream targets of ERK phosphorylation are abundant. (Phospho-)proteomic studies will produce important lists of targets (e.g. [275]). At many points, the circle closes from ERK targets to Raf regulation, and such feedback cycles, passing through all kinds of morphological and metabolic checkpoints, will ultimately define an individual cell's response. Calcium signaling is often induced in parallel to the cascade with similar global consequences, and is the most interesting example for further theoretical studies. It modulates the spatio-temporal activity of the Ras/Raf interface [33], and its oscillations might even be 'optimized' for this activation [261]. Of course, ERK can feed back to calcium activation [30, 403]. In general, coupling of different feedback modules can produce very complex dynamics [129, 490], which can however be abstracted to two fundamental processes: multi-stationarity [470], and oscillations [150].

How do those two relate? Oscillatory behavior might be the default state. Cells 'want to' proliferate. Moreover, the circadian, the lunar and the solar cycles are older than life on earth and will have had a great impact already on RNA-based and proto-cellular life. Many examples are known, where transcription regulators induce their own inhibitor, and transcription introduces the delay, that is usually required for negative feedback to cause oscillations [193, 263, 90, 327, 85]; a principle elaborated into the quite complex oscillators of the cell cycle, the circadian or the vertebrate somitogenesis clock [150, 392]. Then, morphological and energetic requirements, relayed by signaling in multicellular organisms or ecosystems, can stabilize the system, i.e. freeze oscillations, and differentiate cell function.

Biochemistry and Biophysics Each protein seems to interact with almost each other protein, directly or via often short indirect paths, to regulate almost every cellular process. In graph-theoretical terms: protein-protein networks have a very high connectivity. Moreover, in complex organisms such as mammals these interactions are highly cell-type specific, and even more variation exists among the different species. Relieve comes from ions and *second messengers*. They are not object to mutation, diffuse rapidly through dense cellular networks, and might help to define evolutionary conserved general principles of cellular signaling networks.
Glycolysis and the citric acid cycle — by the way a compartmentalized and oscillatory process in eukaryotes [465] — yield ATP as the main energy currency and NAD(P)H as the REDOX equivalent for fatty acid (membrane) synthesis or more ATP generation via the respiratory chain. Interestingly, adenosine and guanosine — the two purines of the genetic code — are employed by two distinct but often directly interacting classes of protein-based signal transducers. GTPases of the Ras superfamily seem to be generally involved in membrane related processes, such as receptor signaling, vesicular trafficking and nuclear-cytoplasmatic transport. ATP — the main energy currency of the cell — is employed by lipid and protein kinase/phosphatase modules of diverse specificity and location. Kinases and membrane-linked GTPases are often coupled directly within positive feedback switches. Can such general observations be traced back to old relations of cell signaling and metabolism?

Cell growth and migration require osmotic volume regulation and local cytoskeletal rearrangements, coordinated globally. Here, some specific lipid and protein phosphorylations have been analyzed with respect to the 'electrical dimension' of the cell [95, 304, 358, 306], which is defined by immobilized negative charges at membranes and their diffusible counter-ions (the *Gouy-Chapman cloud*). ERK targets are often phosphorylated on 10 or more residues [269, 112, 21]. Many ERK targets can be classified among structural proteins [275]. Does further charge immobilization by phosphorylations have a biophysical impact? Does it modulate the 'electrical dimension'? What then, is the role of Ca^{2+} signaling? Can it interfere with negatively charged polymers, to induce local swelling or even rapid phase-transitions, that would then underly large-scale cell-morphological changes [371]?

Multi-scale Context I : Evolution Many questions, few answers. Biophysical and biochemical requirements can however be speculated to consist of a seizable set of rather old general principles, on which evolution acts by recombination and single nucleotide mutations of DNA polymers[7] to achieve an astonishing diversification by protein-protein interactions. Two different types of evolutionary transitions at the root of vertebrates were followed here: the innovation of a new enzymatic mechanism, and the duplication and functional diversification of a protein that acts as a di- or oligomer.

Hyaluronan (HA) is a negatively charged extracellular glucosaminoglycan (GAG), and the evolutionary successor of the neutral chitin. Only small changes were required to derive a hyaluronan-synthase (Has) from the single invertebrate chitin-synthase. While negatively charged GAGs existed long before, the unique HA synthesis — involving one membrane-standing synthase, as opposed to complex endosomal synthesis pathways of other GAGs, seems to be involved in virtually every vertebrate-specific developmental, structural and physiological process [177, 444, 478]. On a cellular level, the HA based peri- and extracellular matrix can probably be modulated much more quickly then the former matrix, with immediate consequences for epithelial-mesenchymal transitions (EMT and MET) and directed migration of single cells [479], potentially crucial for neural crest cell function, the most significant vertebrate-specific cell-type [432].

Unlike the three Has proteins, the three vertebrate Raf proteins constitute one of the best (yet still incompletely) understood examples of functional diversification after gene duplication. Raf sub-typing probably allowed a rewiring of the coordination network around Raf activation and a fine-tuning of spatio-temporal ERK responses to extracellular signals. Alternative kinase-independent functions of c-Raf, where it acts as a scaffold inhibiting other serine/threonine protein kinases (STPK), might point to a general principle of evolution of heteromeric kinase/scaffold pairs or groups.

[7]) one main cause of which is cytosine methylation, that can at least in plants be specifically targeted by RNA, and thus influenced by the phenotype; see e.g. [16, 495, 303] and [281] for some fascinating recent insights on RNA mediated DNA modifications.

Multi-scale Context II : Development Both, Raf/ERK signaling and HA production are involved in EMT and MET [479, 565] and directed migration of single cells. Were the three Raf proteins, and the possibilities for fine-tuning ERK responses involved in allowing the early vertebrate cell to make efficient use of the new type of highly hydrated peri- and extracellular matrix based on HA? However, besides accounting for basic physico-chemical constraints, as well as for evolutionary time-scales, a developmental or physiological (pathological) perspective can be very helpful for an interpretation within a specific functional context on the next organizational level. Here, the vertebrate process of somitogenesis, accompanying and succeeding the fundamental process of gastrulation, was found appropriate to provide such a specific context. This system consists of several coupled oscillators and multi-stable states and connects to very old considerations about biochemical mechanism of pattern formation. It allows to interpret and refine such theoretical developmental models in the light of recent observations of the molecular implementation and of theoretical models of intracellular signaling and gene regulatory networks.

A morphogen gradient can confer positional information on a cells [487, 83, 311]. FGF8 and RA are two opposing gradients, which define the freezing of the oscillatory somitogenesis clock, and thus the conversion of a temporal into a spatial pattern [392]. The ultrasensitive, bistable and amplifying properties of the Raf/MEK/ERK cascade could support the discrete 'determination front' within a concentration gradient of the FGF8 morphogen, while a parallel PIP3 pathway translates graded information into Akt activity [115]. The proposed role of calcium as a signaling factor that integrates local with global morphological processes might be repeated here at a multi-cellular scale. Embryonic extracellular calcium waves [148, 147] can repeatedly enforce local cell-cell adhesion via calcium dependent cadherins [517, 459, 505]. In this work, a direct connection to signaling was carried forward from an observed role in vertebrate left-right patterning to the intimately related process of somitogenesis. The Notch pathway has been observed to respond to the extracellular calcium gradient between future left and right sides [386]. Subsequently, calcium waves might further modulate the somitogenesis clock, that is coupled between neighboring cells via the very same Notch pathway.

A General Theory of Cell Signaling ? Systems Biology aims at a system level understanding of cells and organisms. So far, theoretical models capture only fractions of cellular signaling and gene regulatory or multi-cellular developmental and physiological networks. Some elegant recent approaches integrate basic metabolic or morphological phenomena with signaling pathways, to approach an integrated view [94, 285, 249, 343]. Such models will however always be limited to specific species or cell types as they are based on specific protein or nucleotide interactions and simplifications that capture only the studied properties. A true system level understanding will require a more general theory of cellular signaling. It is yet unclear, whether such a theory is feasible in the near future or even in general, but here some potential cornerstones were proposed, based on a detailed analysis of available knowledge around one of the best studied eukaryotic signal transducers.

The MAP kinase pathway is a widely used module in eukaryotic signal transduction. Its conserved role in osmoregulatory as well as cell cycle coordination networks might point to an old role in growth and morphology modulation, which could represent a fundamental physicochemical principle of eukaryotic cellular life. The perspective of the 'electrical dimension' of the cell, with negative charges immobilized at membrane polymers and counteracted by mono- and bivalent cations (the *Gouy-Chapman cloud*) [95], would allow a general interpretation of protein phosphorylations, especially those who are strongly amplified by inherently ultrasensitive and bistable cascades. Such an interpretation could likely be extended to all kinds of post-translational protein modifications. Sandwiched between polysaccharide– or polypeptide–based negatively charged polyelectrolytes, the plasma membrane maintains a resting potential that is negative on the inside [358], while the nuclear membrane is positive on the inside, sequestering DNA polymers via their negative backbone [304]. The bimodal proteomic distribution of isoelectric points in prokaryotes and the trimodal in eukaryotes [426, 524] strengthen the argument for a fundamental role of electrochemical modulation in cellular life. Fascinatingly, the developing embryo and the adult animal also maintain potential differences across epithelia, generating pulses of ionic currents, namely calcium waves, that can be observed in oscillatory, but irregular patterns during central phases of development [348, 147, 518, 346]. The electrical

dimension would thus be repeated on the multi-cellular scale.

Intracellular calcium signaling, often oscillatory both on local and global cellular scales, is commonly induced by pathways that in parallel can start off phosphorylation cascades. Charged polymers, in functional cooperation with diffusible counter-ions, are widely used in technologies that exploit one unique phenomenon: the gel-sol phase transition. Similar to the cascade, such phase-transitions can be interpreted as an ultrasensitive response to some change in environmental conditions. They are however able to generate force and are accompanied by large-scale morphological changes, which have already been proposed to underly all kind of cellular processes, from vesicular secretion to cell migration and cytokinesis [371]. On organismic scales very specialized structures would employ multi-cellular coordination of these phenomena for e.g. muscular movement and neuronal information processing.

Another fascinating property of (negative) polyelectrolytes could directly connect such phase-transitions to the electrical dimension. So-called diffusion or flow potentials can be generated by a flow of ionic solute through a polyanionic matrix, as first observed in 1953 by Jensen et.al. in HA solutions [223]. Later, Jensen and collaborators measured potential differences when simply exerting asymmetrical mechanical force on HA solutions, e.g. compressing it from one side [58, 73]. In the 70ies Barrett and Comper further analyzed the phenomena and speculated on potential mechanisms of biological information transfer by such mechano-electrical conversions [24, 26, 25, 80, 79]. Today these mechano-electrical phenomena are mainly studied in cartilage (bone-connective tissue and chondrocytes), where they are of immediate medical relevance and their influence on cell signaling is beginning to be unraveled [264, 1].

In the opinion of the author, both gel-sol phase-transitions and electrical dimensions of cells and organisms will be cornerstones of a putative general theory. They allow to interpret all kind of cellular and multi-cellular functions with simple physico-chemical principles. Specific protein and nucleotide based feedback modules can coordinate both, the electric dimension and morphological phase-transitions in cell-cycle and migration and can then be varied by evolution into the enormous diversity of cell-function in complex organisms and ecosystems.

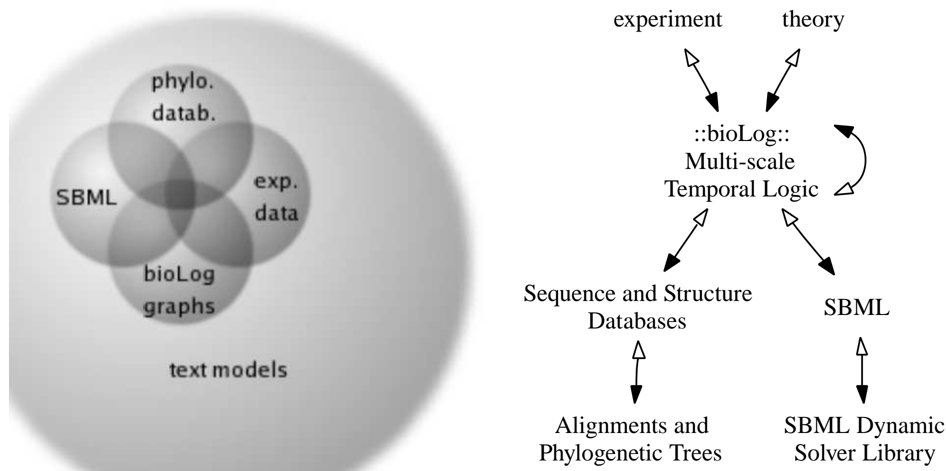


Fig. 45. Left: The relations of different knowledge representations, as used in this work. The *bioLog* graphs were employed to describe both, detailed experimental observations and comprehensive pathway interactions. The latter overlap with detailed mathematical models of underlying reaction networks, described in SBML. Phylogenetic analysis can finally provide insights on evolutionary transitions of pathways and pathway dynamics, as described in SBML and *bioLog* graphs. All of this information has been embedded into a detailed narrative, a text description that puts together the pieces and explains details, which can not be represented in either of the standardized formats alone. The text based descriptions should be transferred into a dynamic wiki/weblog system, opening such work for a collaborative community effort. Right: Such a community system could handle biological information appropriately. A formalization of the *bioLog* graphs should be realized by an abstract multi-level and temporal logic-based description framework, which could employ the various available ontologies and controlled vocabularies and data standards, cross-linked via RDF+XML based semantic web technology [512].

7 List of Abbreviations

Protein Names A - c-Mos

Short	Long	Category
14-3-3	14-3-3	SCAF
A-Raf	A-Raf, no long name, see c-Raf	STPK
Abl	see c-Abl	STPK
AC	adenylyl cyclase	ENZ
actin	actin	SKEL
AK	adenylate kinase	ENZ
Akt	homolog of v-akt oncogene from murine retrovirus AKT8 (thymoma in AKR mouse) also called PKB	STPK
AMPK	AMP-activated protein kinase	STPK
Arf	ADP-ribosylation factor	G
ARMS	Ankyrin-Rich Membrane Spanning Protein	coR
Arp	actin-related protein	SKEL
Ask	apoptosis signal-regulating kinase	STPK
B-Raf	B-Raf, no long name, see c-Raf	STPK
Bad	Bcl-2 associated death promoter or Bcl-2-antagonist of cell death	bcl
Bcl-2	B-cell leukemia/lymphoma 2	bcl
Bcl-xL	B-cell leukemia/lymphoma xL	bcl
BCR	B cell receptor	RcTK
BTK	Bruton agammaglobulinemia tyrosine kinase	YPK
c-Abl	cellular homolog of v-abl, Abelson murine leukemia oncogene 1	YPK
c-Cbl	Casitas B-lineage lymphoma homolog E3 ubiquitin protein ligase	E3
c-Fos	v-fos FBJ murine osteosarcoma viral oncogene homolog	TR
c-Jun	v-jun transforming gene of avian sarcoma virus 17 homolog (‘ju-nana’ japanese for 17)	TR
c-Mos	v-mos Moloney murine sarcoma viral oncogene homolog	STPK

Protein Names c-Myc - D

Short	Long	Category
c-Myc	v-myc avian myelocytomatosis viral oncogene homolog	TR
c-Raf	short for c-Raf-1, cellular homolog of the retroviral oncogenes v-raf/mil (c-raf-2 and c-raf-3 are pseudogenes)	STPK
c-Src	Cellular homolog of the transforming gene of Rous sarcoma virus (v-src) product	YPK
C3G	CRK SH3-binding guanine nucleotide release factor RapGEF1	GEF
calcineurin	protein phosphatase 2B	PP
CalDAG-GEF	Ca^{2+} and DAG-regulated GEF I-III	GEF
calmodulin	calmodulin	SCAF
CaMK	Ca^{2+}/calmodulin-dependent kinase I-IV	STPK
CaMKK	CaMK kinase	STPK
CAPRI	Ca^{2+}-promoted Ras inactivator	GAP
Cas	Crk-associated substrate	ADAP
Cbl	see c-Cbl	E3
CBP	p300/CBP, CREB-binding protein	TR
Cdc2	cell-division-cycle protein 2	CYC
Cdc25	cell-division-cycle protein 25	GEF
Cdc42	cell-division-cycle protein 42	G
Cdk	Cyclin-dependent kinase	STPK
Chs	chitin synthase	ENZ
Cip-1	cyclin/CDK inhibitor p21 WAF1/Cip1	TR
CKI	cyclin dependent kinase inhibitor	diverse
Cls	cellulose synthase	ENZ
CD44	cluster of differentiation protein 44	SKEL
CNK	connector-enhancer of KSR	SCAF
cortactin	cortactin	SKEL
CREB	cAMP responsive element binding protein	TR
Crk	homolog of the v-crk oncogene of the avian sarcoma viruse CT10	ADAP
Cx43	connexin 43, gap junction protein	GAP
cyclin D	cyclin D	CYC
DGK	DAG kinase	ENZ

Protein Names E - M

Short	Long	Category
EGF	Epidermal growth factor	LIG
EGFR	EGF receptor	RTK
Egr	early growth response	TR
Epac	Exchange protein directly activated by cAMP	GEF
ERK	Extracellular signal-regulated kinase	STPK
ERM	ezrin, radixin, moesin proteins	SKEL
FAK	focal adhesion kinase	YPK
FGF	fibroblast growth factor	LIG
FGFR	FGF receptor	RTK
Fos	see c-Fos	TR
FRS2	fibroblast growth factor receptor substrate 2	ADAP
Grb2	growth factor receptor-bound protein 2	ADAP
H-Ras	also called Ha-Ras, see Ras	G
Has	hyaluronan synthase	ENZ
IFN	interferon	LIG
integrin	integrin	SKEL
JAK	Janus kinase	YPK
JNK	Jun kinase	MAPK
Jun	see c-Jun	TR
K-Ras	also called Ki-Ras, see Ras	G
KDR	kinase insert domain receptor, VEGF receptor	RTK
KSR	kinase suppressor of Ras	SCAF
LKB1	serine/threonine-protein kinase LKB1	STPK
M(1)mAChR	M(1) muscarinic acetylcholine receptor	GPCR
M2K	MAPK kinase	TYPK
M3K	MAP kinase kinase	STPK
MAP kinase	see MAPK	STPK
MAPK	Mitogen-activated protein kinase	STPK
MAPKAPK	MAPK-activated protein kinase	STPK
MEK	MAPK/ERK kinase	TYPK
MKP	MAPK phosphatase	PP
MLC	myosin light chain	SKEL
MLCK	MLC kinase	STPK
Mos	see c-Mos	STPK
MP1	MEK partner 1	SCAF
MST	mammalian sterile 20-like kinase	STPK
Myc	see c-Myc	TR

Protein Names N - Ran

Short	Long	Category
NHE	Na^+/H^+ exchanger	IONT
N-Ras	homolog of K-Ras and H-Ras, see Ras	G
NGF	nerve growth factor	LIG
PAC1	GPCR type 1 PACAP-preferring receptor	GPCR
PACAP	pituitary AC activating polypeptide	LIG
PAK	p21-activated kinase	STPK
paxillin	paxillin	SKEL
PDK	3-phosphoinositide-dependent protein kinase	STPK
PI3K	phosphoinositide-3-kinase	LK
PI5K	phosphatidylinositol-5-kinase	LK
PIPK	phosphatidylinositol-5-kinase	LK
PDE	phospho-diesterase	ENZ
PDGF	platelet-derived growth factor	LIG
PDGFR	PDGF receptor	RTK
PKA	protein kinase A, cAMP-dependent protein kinase	STPK
PKB	protein kinase B, also called Akt	STPK
PKC	protein kinase C	STPK
Pkn	putative kinase	STPK
PLC	Phospholipase C	LIP
PLD	Phospholipase D	LIP
PP2A	protein phosphatase 2A	PP
PP2B	protein phosphatase 2B, calcineurin	PP
PTP	protein tyrosine phosphatase	PP
R-Ras	Ras-related protein	G
Rab	Ras-related protein Rab	G
Rac	Ras-related C3 botulinum toxin substrate	G
Ral	Ras-related GTPase	G
RalBP	Ral binding protein	GAP
RalGDS	Ral guanine nucleotide dissociation stimulator	G
	also called RalGEF	GEF
Raf	A-Raf, B-Raf and c-Raf, see there	STPK
Raf-1	short for c-Raf-1, see c-Raf	STPK
Ran	GTP-binding nuclear protein Ran	G

Protein Names Rap - Y

Short	Long	Category
Rap	Ras-related proteins 1 and 2b	G
Ras	Ras p21 protooncogene products: homologs of the Harvey (H-Ras) and Kirsten (K-Ras) murine sarcoma viruses and N-ras	G
RASAL	Ras protein activator like	GEF
RasGRP	Ras guanyl releasing protein, CalDAG-GEF	GEF
RHAMM	receptor for hyaluronan mediated motility	SKEL
Rheb	Ras-homolog enriched in brain	G
Rho	Rho	G
Rok	Rho-associated/dependent kinase, ROCK	STPK
Rsk	Ribosomal S6 kinase	STPK
SAPK	Stress-activated protein kinase	MAPK
Sef	similar expression to FGF genes	SCAF
Shc	Src homology 2 domain containing protein	ADAP
SHP2	Src homology-2 domain-containing protein-tyrosine phosphatase	PP
SOS	Son-of-sevenless	GEF
Sprouty	Sprouty	SCAF
Spry	Sprouty	SCAF
Src	short for c-Src, see c-Src	YPK
Ste	sterile genes, yeast mating	diverse
SUR-8	suppressor of ras 8	SCAF
Syk	spleen tyrosine kinase	YPK
Syn	src/yes-related novel gene	YPK
TCR	T cell receptor	RcTK
TAK	TGFβ-activated kinase	STPK
TGF	transforming growth factor	LIG
TNF	tumour-necrosis factor	LIG
TrkA	tyrosine kinase proto-oncogene product A NGF receptor	RTK
v-Src	see c-Src	YPK
VEGF	vascular endothelial growth factor	LIG
WAF	p21 Cip-1/WAF1, wound fluid angiogenesis factor	TR
WASP	Wiskott Aldrich syndrome protein	SKEL
Yes	homolog of the Yamaguchi sarcoma viral oncogenes v-yes-1	YPK

Protein Categories

ADAP	adaptor protein
coR	co-receptor
bcl	Bcl-2 family of apoptosis regulators
E3	E3 ubiquitin ligase
ENZ	enzyme
G	small G protein
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GPCR	trimeric G-protein coupled receptor
LIG	extracellular ligand
LIP	lipase
LK	lipid kinase
PP	protein phosphatase
RTK	receptor tyrosine kinase
RcTK	receptor coupled to cytosolic tyrosine kinase
SCAF	scaffold protein
SKEL	structural/cytoskeleton protein
STPK	serine/threonine protein kinase
TR	transcription regulator
TYPK	threonine/tyrosine protein kinase
YPK	non-receptor tyrosine protein kinase

Protein Domains

CR	cysteine-rich domain
DEF	docking site for ERK, FXFP
NES	nuclear export sequence
NLS	nuclear location sequence
SH2/3	Src homology domain 2 or 3
PASTA	penicillin-binding protein and STPK associated domain
PH	pleckstrin homology domain
RBD	Ras binding domain

Metabolites

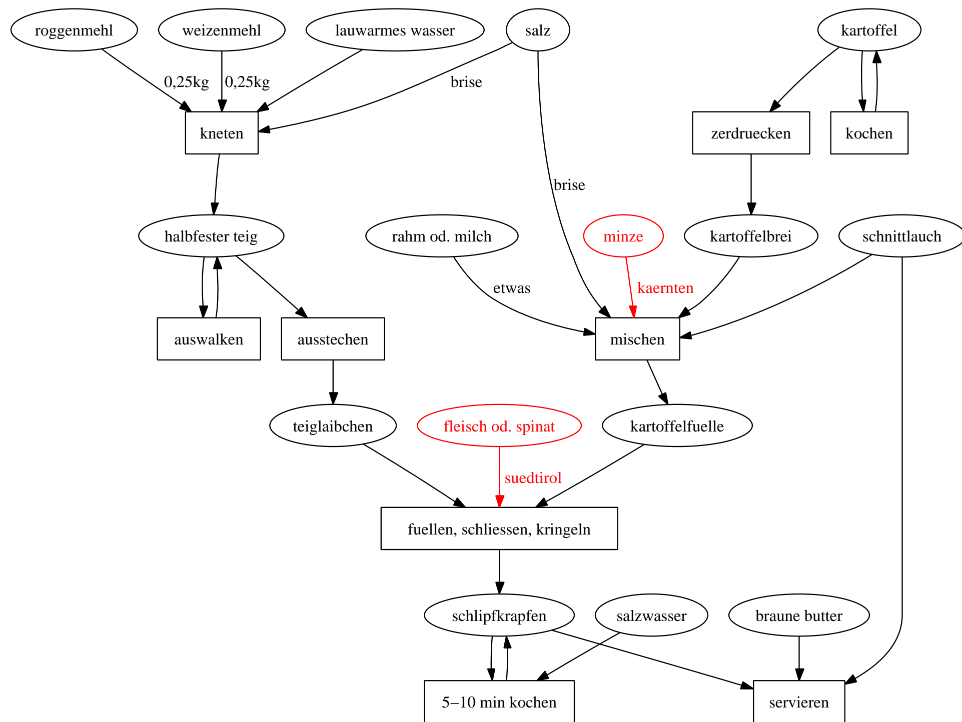
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
cAMP	adenosine 3', 5'- cyclic monophosphate
DAG	diacylglycerol
GTP	guanosine 5'-triphosphate
HA	hyaluronan, hyaluronic acid, hyaluronate
IP3	inositol 1,4,5-triphosphate
LPA	lysophosphatidic acid
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
o-HA	HA oligo-saccharides
PIP2	PI(4,5)P2, 1-phosphatidyl-inositol-4,5-biphosphate
PIP3	PI(3,4,5)P3, 1-phosphatidyl-inositol-3,4,5-triphosphate
ROS	reactive oxygen species

Cell lines

Name	**Description**	**Type**
COS-7	african green monkey CV-1 cell line transformation with a replication defective mutant of SV40 virus	fibroblast-like growing in monolayers
MEF	mouse embryonic fibroblasts	fibroblast
NIH-3T3	mouse cell line, NIH Swiss	fibroblast
PC12	rat phaeochromocytoma cell line 12 neural crest of the adrenal medulla	chromaffin
UT-7	human acute myeloid leukemia cell line	megakaryoblast

Structures and Processes

Short	**Long**
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition, also see MET
ER	endoplasmatic reticulum
IEG	immediate early genes
SR	sarcoplasmatic reticulum, ER of muscle cells
FA	focal adhesion
FAC	focal adhesion complex
MET	mesenchymal-epithelial transition, also see EMT
RDF	Resource Description Framework
SBML	Systems Biology Markup Language
XML	eXtensible Markup Language



Schlipfkrapfen: Man nimmt ½ kg Mehl, halb Roggenmehl, halb Weizenmehl.
Mit lauwarmem Wasser und etwas Salz einen nicht zu festen Teig machen.
Der Teig wird ausgewalkt und runde Laibchen ausgestochen.
Die Laibchen werden mit Kartoffelfülle gefüllt und am Rand fest zusammengedrückt.
Jetzt werden sie in kochendem Salzwasser 5 – 10 Minuten gekocht,
dann herausnehmen, mit Butter abschmelzen und servieren.
Kartoffelfülle: Man nimmt gekochte Kartoffeln, zerdrückt sie,
gibt etwas Rahm oder Milch dazu, Salz, Zwiebel oder Schnittlauch, macht eine Fülle,
die dann in die ausgestochenen Laibchen gefüllt wird.

Fig. 46. Reaction network of a delicious recipe from southern Austria called Schlipfkrapfen, and its phylogenetic relation to Kärtner Kasnudel and Südtiroler Schlutzkrapfen, all belonging to the great family of the samosa-likes.

.

abstraKt

Die Mitogen-aktivierte Kinasekaskade des Proteins (DIAGRAMM) ist ein allgemein verwendetes Modul in den zellularen Zeichengabenetzen von Eukaryotes. Sie besteht aus zwei serine/threonine Kinasen, die durch eine Zwischenthreonine/tyrosine Kinase verbunden werden und hat Rollen in der osmotischer und Zelle Zyklusregelung konserviert. Zugehörige Eigenschaften dieser Kaskade — seine verstärken, ultrasensitive und bistabile Antwort zu aktivierenden Signalen — sind durch mathematische Methoden von Enzymkinetik studiert worden. Hier sind zwei ergänzende Annäherungen gefolgt worden, um weiter auszuarbeitn solche theoretische Modelle und ihre Resultate zu deuten:
(1) folgt ein *Begriffsbericht* der Integration der Raf/MEK/ERK Kaskade in globale zellulare Zeichengabenetze. Eine Diagramm-gegründete Beschreibung Sprache (*bioLog*) wurde, um experimentelle Einblicke zu verfolgen entwickelt und vom Static und von den lediglich graphischen Interaktion Diagrammen zu standardisierten Formaten für das Beschreiben der grundlegenden biochemischen Reaktion Netze, wie die Systeme Biologie-Preisaufschlag-Sprache (SBML) zu überbrücken. SBML Modelle erlauben weitere Analyse der komplizierten dynamischen Eigenschaften, die in den Reaktion Netzen mit Rückgespräch, nämlich Pendelbewegungen und Multistabilität entstehen können.
(2) ist die *SBML ODE Löser-Bibliothek* ein Werkzeug für Ziffernwertung solcher Reaktion Netze. Es wird bedeutet, um Teil einer Reihe Werkzeuge zu werden, die gut begründete theoretische Methoden für einfache Integration in hochgradige biologische Anwendungen der Analyse Werkzeuge und der Systeme zur Verfügung stellen.

Die Ras/Raf Schnittstelle liefert die meisten Informationen über die globale Verdrahtung der Kaskaden, während ERK Modulation der aufwärts gerichteten Bestandteile Rückgesprächzyklen schließt, die schließlich die Antwort einer einzelnen Zelle definieren. Einige interessante Anwärter solcher Rückgesprächmodule sind im Begriffsbericht gekennzeichnet worden und konnten in 4 (Sätze von) eindeutig aber in zusammenhängend Hypothesen kondensiert werden (sehen Sie Zusammenfassung unten). Der vielversprechendste Anwärter für weitere theoretische Studien wurde in den beobachteten Rückgesprächinteraktionen mit der Ähnlichkeit — häufig Schwingungs — Kalziumsignalisieren gefunden. Während solche ausführliche Beobachtungen von der Sorte – und von der Zelle-Art –

spezifische Ausdruck Muster abhängen, lädt Natur des Kalziums als zweiwertiges Kation für einen allgemeinen Vergleich mit großräumigen phosphorylations ein, gedeutet als Immobilisierung der negativen Aufladungen von Atp zu den großen Multiprotein Komplexen, häufig an bereits negativ Gebührenstrukturen wie Membranen und Actinheizfäden. Von solch einer biophysikalischer Perspektive kann die konservierte Doppelrolle der DIAGRAMM-Kinasen in der osmotischer und Zelle Zyklusregelung versöhnt werden gründete auf der lokalen osmotischen Regelung des Besonderen und globale morphologische bergänge während der Migration, Zelle Wachstum und cytokinesis integrieren. Einige mögliche Drehbücher für den Ursprung der Kaskade an den Wurzeln des eukaryotic Lebens werden vorgeschlagen. Als Beweis des Konzeptes, werden die mutmaßlich ergänzenden Rollen der Kalzium- und Phosphorylierungkaskaden in der morphologischen Korrdination zu den mehrzelligen Interaktionen im vertebrate somitogenesis vorgetragen.

Solche biophysikalische Betrachtungen sollten in eine *allgemeine Theorie der Zelle*, die weiter ausgearbeitt werden würden vereinfachen groß das Systeme biologische Ziel eines system-level Verständnisses des Lebens.

... as translated by Altavista's babelfish.

A Appendix

Table 1: Usage and command-line options for the *SBML ODE Solver*

USAGE: odeSolver <sbmlfile.xml> [OPTION(s)]

Options		Argument	Description
GENERAL OPTIONS			
-h	–help		Print usage information
-i	–interactive		Start the interactive mode
	–gvformat	string	Output format for graphviz module
SBML **FILE PARSING**			
-v	–validate		Validate *SBML* file
	–model	string	*SBML* file name 'sbmlfile.xml'
	–mpath	path	Set Model file path
	–spath	path	Set Schema file path (default: mpath)
(1) PRINT REACTIONS AND DERIVED ODEs			
-e	–equations		Print model and derived *ODE* system
-o	–printsbml		Construct *ODE*s and print as *SBML*
-g	–modelgraph		Draw graph of reaction network

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Table 2: Usage and command-line options for the *SBML ODE Solver*, continued

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(2) INTEGRATION PARAMETERS			
-f	–onthefly		Print results during integration
-j	–jacobian		Toggle use of the jacobian matrix
-s	–steadyState		Abort integration at steady state
-n	–event		Detect and evaluate events (do not abort). ACCURACY DEPENDS ON STEP SIZE!!
	–param	string	Choose parameter for batch integration, from 0 to value in 50 steps
	–printstep	integer	Time steps of output (default: 10^3)
	–time	float	Integration end time (default: 10^3)
	–error	float	Absolute error tolerance (default: 10^{-18})
	–rerror	float	Relative error tolerance (default: 10^{-14})
	–mxstep	integer	Maximum step number (default: 10^5)
(3) INTEGRATION RESULTS			
-a	–all		Print all available results
-y	–jacobianTime		Print time course of Jacobian matrix
-k	–reactions		Print time course of the reactions
-r	–rates		Print time course of the *ODE*s
-w	–write		Write results to file or save XMGrace file
-x	–xmgrace		Print results to XMGrace
-m	–matrixgraph		Draw Jacobian matrix graph

Table 3: Final output: *CVODE* integration parameters and statistics

Short	**Meaning**
CVODE	**integration parameters:**
mxstep	maximum number of steps *CVODE* used at each internal time step |h|
rel.err	relative error tolerance at each internal time step |h|
abs.err.	absolute error tolerance at each internal time step |h|
CVODE	**integration statistics:**
nst	cumulative number of internal steps taken by the solver
nfe	number of calls to the ODE evaluation function ‘f’
nsetups	number of calls to the linear solver’s setup routine
nje	number of Jacobian evaluations, i.e. either calls to the function that evaluates the automatically generated Jacobian matrix expressions or the internal approximation CVDenseDQJac.
nni	number of NEWTON iterations performed.
ncfn	number of nonlinear convergence failures that have occurred
netf	number of local error test failures that have occurred

Table 4: *CVODE* **failure messages**

Flag	**Message**	**Description**
0	SUCCESS	*CVODE* completed integration.
-1	CVODE_NO_MEM	The cvode_mem argument passed to *CVODE* was null. This error should not appear in the *SBML ODE Solver*.
-2	ILL_INPUT	One of the inputs to *CVODE* was illegal, including the situation when one of the error vectors becomes ¡ 0 during *CVODE*'s internal time stepping. The printed error message will give specific information. In the *SBML ODE Solver*, this failure occurs when e.g. the out-time passed was '0'.
-3	TOO_MUCH_WORK	The solver took a maximum of internal steps but could not reach the next print-step. The default step number is 100000; it can be set with command-line option '–mxstep'.
-4	TOO_MUCH_ACC	The solver could not satisfy the accuracy demanded (via options –error and –rerror) for an internal time step.
-5	ERR_FAILURE	Error test failures occurred too many times during one internal time step or occurred with $\|h\| = h_{min}$, i.e. the necessary internal time step became too small.

continued on next page ...

Table 5: *CVODE* **failure messages, continued**

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Flag	Message	Description
-6	CONV_FAILURE	Convergence test failures occurred too many times during one internal time step or occurred with |h| = hmin. This can sometimes happen either with or without using the automatically generated Jacobian matrix. That is why the the *SBML ODE Solver* tries to integrate again upon this error, but now without or with use of the Jacobian matrix (resetting option '-j'). In other cases this error can be avoided by allowing a bigger error tolerances
-7	SETUP_FAILURE	The linear solver's setup routine failed in an unrecoverable manner. This error has not occurred (during test runs).
-8	SOLVE_FAILURE	The linear solver's solve routine failed in an unrecoverable manner. This error has not occurred (during test runs).

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