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Nuclear Tyrosine Phosphorylation: The Beginning of a Map

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ABSTRACT. Tyrosine phosphorylation is usually associated with cytoplasmic events. Yet, over the years, many reports have accumulated on tyrosine phosphorylation of individual molecules in the nucleus, and several tyrosine kinases and phosphatases have been found to be at least partially nuclear. The question arises as to whether nuclear tyrosine phosphorylation represents a collection of loose ends of events originating in the cytoplasm or if there may be intranuclear signaling circuits relying on tyrosine phosphorylation to regulate specific processes. The recent discovery of a mechanism causing nuclear tyrosine phosphorylation has prompted us to review the cumulative evidence for nuclear tyrosine phosphorylation pathways and their possible role. While we found that no complex nuclear function has yet been shown to rely upon intranuclear tyrosine phosphorylation in an unambiguous fashion, we found a very high number of compelling observations on individual molecules that suggest underlying networks linking individual events. A systematic proteomics approach to nuclear tyrosine phosphorylation should help chart possible interaction pathways. BIOCHEM PHARMACOL **60**;8:1203–1215, 2000. © 2000 Elsevier Science Inc.

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In multicellular organisms, protein tyrosine phosphorylation is well known to have a pivotal role in the coordination of signal transduction after extracellular stimuli [1]. Tyrosine phosphorylation is thus involved in the control of a wide range of cellular processes such as cell adhesion, cell motility, gene transcription, immune response, differentiation, and cell proliferation. Not surprisingly, aberrant tyrosine phosphorylation is linked to a variety of human diseases, such as cancer, diabetes, immunodeficiencies, and a number of genetic disorders.

Though the yeast genome contains dual-specificity protein kinases and tyrosine phosphatases, it does not contain tyrosine kinases [2]. Rather, these appear to have coevolved with protein modules of the SH2† domain type that allow specific recognition of the phosphorylated tyrosine and to be one of the most typical biochemical hallmarks of metazoans [3]. Multicellular organisms may have needed the ability to spatially "mark" the origin of a stimulus at the cell surface, using a system that permits reversible coupling to cytoskeletal elements that form the cellular coordinates. For these reasons, and in contrast to serine/threonine phosphorylation, tyrosine phosphorylation is almost invariably associated with the induction of protein–protein interactions [4]. Prominent exceptions are represented by tyrosine phosphorylation in the activation loop of protein kinases.

There are several ways by which tyrosine phosphorylation can be envisaged to control cellular processes. First, tyrosine phosphorylation can cause cytoplasmic changes without necessarily affecting gene expression. Classical experiments performed by Thomas Graf and colleagues with temperature-sensitive alleles of v-Src, for example, showed that even enucleated fibroblasts can undergo dramatic Src-dependent morphological transformation [5]. Likewise, many other effects on membrane traffic, secretion, gap junction function, translation, proteolysis, to name just a few, are likely to occur without changes in nuclear gene expression. In the regulation of these processes, tyrosine phosphorylation commonly results in changes in phosphorylation on serine and threonine residues, as brought about by serine/threonine kinases and phosphatases. Because serine/threonine phosphorylation is the much more abundant phosphorylation form, it represents a powerful effector of tyrosine phosphorylation. In

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† *Abbreviations:* Abl, Abelson leukemia; ATM, ataxia-telangiectasia mutant; BCR, B cell receptor signaling; CARP90, Cbl-related 90-kDa protein; CDK, cyclin-dependent kinase; DNA-PK, DNA-dependent protein kinase; ERK, extracellular signal-regulated protein kinase; hnRNP, heterogeneous nuclear ribonucleoprotein; HS1, hematopoietic specific protein 1; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKK (also MEK and MKK), mitogen-activated protein kinase kinase; NF-κB, nuclear factor κB; NPM–ALK, nucleophosmin–acute leukemia kinase; PTEN, phosphatase and tensin homolog deleted on chromosome 10; pRB, retinoblastoma protein; SH2, Src homology 2; SH3, Src homology 3; SHPTP1, SH2-containing protein tyrosine phosphatase 1; Src, Rous sarcoma tyrosine kinase; STAT, signal transducer and activator of transcription; TC-PTP, T cell protein tyrosine phosphatase; TFII-I, transcription factor II-I; and TMF, TATA element modulatory factor.

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TABLE 1. Tyrosine-phosphorylated proteins in the nucleus

Protein	Name/Function	Reference
Growth control		
JAK	Cytokine signaling	[13]
ERK	Extracellular signal-regulated response kinase	[19]
JNK	Jun N-terminal kinase	[23]
p38	Extracellular signal-regulated response kinase	[23]
CDK	Cell cycle regulation	[145]
DNA repair/ Recombination/Apoptosis		
Rad51	Recombination	[50]
HS1	Apoptosis	[30]
KRC	κB-binding site and recognition component of VDJ recombination signal sequence	[110]
DNA-PK	DNA damage repair	[47]
Transcription factors/ DNA-binding proteins		
p73	p53-related protein	[44, 45]
NF-κB	Transcription factor	[119]
STAT family	Transcription factor	[146]
c-Jun	Transcription factor AP-1 family	[38]
RNA pol. II	Transcription enzyme	[40]
TMF	TATA element modulatory factor	[111]
TFII-I	Transcriptional co-factor	[116]
c-Rel	Transcription factor	[122]
Estrogen receptor	Transcription, perhaps other functions	[130]
RNA-binding proteins		
Sam68	RNA binding	[142]
hnRNPK	RNA processing, RNA transport	[135]
Adaptor proteins		
CrkL	Adaptor molecule	[98]
p95Vav	GDP/GTP exchange factor	[93]
CARP90, v-Cbl	Adaptor molecule	[103, 100]

addition, tyrosine phosphorylation is tightly linked to lipid and calcium signaling [6]. In many cases, these effectors of tyrosine phosphorylation events will control changes in gene expression by causing nuclear localization of transcription factors or serine/threonine kinases/phosphatases affecting the phosphorylation state and activation potential of transcription [7]. While this may represent the most common way by which tyrosine kinases affect nuclear events, there is another route of action for tyrosine phosphorylation that concerns direct effects on transcription factors in the cytoplasm that subsequently enter the nucleus (such as the STAT factors, see below).

Finally, tyrosine phosphorylation may also directly originate in the nucleus in response to intranuclear signals, like DNA damage, or extracellular signals. The logic and mode of action of nuclear tyrosine phosphorylation has not been elucidated, either in detail or systematically. On the contrary, because of its more common association with cell surface stimuli, tyrosine phosphorylation is still considered a typical cytoplasmic modification. Yet, there is an ample collection of reports of nuclear tyrosine phosphorylation. The nucleus contains both tyrosine kinases and tyrosine phosphatases, as well as tyrosine-phosphorylated proteins involved in transcription, mRNA metabolism, cell cycle, and DNA repair. It is not yet clear if nuclear tyrosine phosphorylation of these proteins is a passive phenomenon that merely represents the echo of cytoplasmic phosphorylation events or if there are indeed nuclear networks of protein interactions governed by tyrosine phosphorylation. In this review, we will attempt a brief summary of the existing evidence and venture an outlook on the possibilities. In Table 1, we list the proteins reported to be nuclear and tyrosine-phosphorylated. Table 2 lists all the tyrosine kinases and phosphatases that are known to be at least nuclear.

SYSTEMATIC STUDIES

In the 1980s, Foulkes and colleagues asked a simple question: can DNA-binding tyrosine phosphorylated proteins be found in the nuclei of cells transformed by an oncogenic tyrosine kinase? If so, would not such proteins be primary candidates for the direct effectors of these tyrosine kinase oncoproteins? The nuclei of NIH 3T3 cells transformed by the viral Abelson tyrosine kinase (v-Abl) indeed were found to contain several nuclear tyrosine-phosphorylated proteins, some of which showed high affinity for DNA [8]. In comparison, no tyrosine-phosphorylated proteins were detected in the nuclei of normal NIH 3T3 cells. Moreover, a rough ratio of 4:1 in total tyrosine phosphorylation was detected when comparing the cytoplasmic and

TABLE 2. Tyrosine kinases and phosphatases in the nucleus

Protein	Name/Function	Reference
Kinases		
c-Abl	Cell growth, DNA damage, stress response	[37]
Wee1	Cell cycle	[104]
Fes	Cell growth and differentiation	[67]
Fer	Transcription modulator of cytokine signaling pathway	[111]
Rak	Growth control?	[65]
JAK	Cytokine signaling pathway	[13, 14]
MEK	Growth factor signaling pathway	[26]
MKK7	Stress-activated pathway	[25]
DYRK	Cell growth, differentiation?	[147]
Lyn	Cell proliferation	[55]
IyK/Frk	Tumor suppressor	[66]
ALK/NPM–ALK	Kinase involved in leukemia	[80, 148]
Phosphatases		
Unidentified	Regulation STAT activity	[17]
CL100/MKP1	MAPK phosphatase	[21]
PAC-1	MAPK phosphatase (phosphatase of activated cells)	[21]
SHPTP1	JNK/SAPK phosphatase	[149]
CDC25	Cell cycle regulator	[108]
TC-PTP	Cell growth, differentiation, apoptosis?	[87]
PTEN	Tumor suppressor gene, neuronal differentiation	[83]

nuclear fractions of v-Abl-transformed cells. These results suggested to the authors that, at least potentially, nuclear tyrosine-phosphorylated proteins could represent the effectors of oncogenic tyrosine kinases for changes in gene expression. Another recent analysis of nuclear tyrosine phosphorylation showed that upon induction of differentiation of HL-60 cells, 13 nuclear phosphotyrosine proteins are dephosphorylated while 6 undergo novel tyrosine phosphorylation [9]. While these are among the few systematic analyses of nuclear tyrosine phosphorylation that we are aware of, they only relied on the detection of abundant proteins. It could be argued that many more nuclear tyrosine-phosphorylated proteins might exist that are less abundant. What are all these proteins? Do they participate in the assembly of protein complexes like their cytoplasmic counterparts? All the other evidence for nuclear tyrosine phosphorylation discussed in this commentary comes from the study of individual molecules. To investigate whether there are particular functions in which they participate preferentially, we have attempted a "functional" grouping of the cases.

STUDIES ON INDIVIDUAL MOLECULES

Cytoplasmic–Nuclear Transport

THE STATS. A wide variety of cytokines, such as interferon and several growth factors, signal through Janus kinases (JAKs) and signal transducers and activators of transcription (STATs) [3]. Seven members of the STAT family have been identified so far in mammalian cells. These are latent cytoplasmic transcription factors containing DNA-binding and transcriptional activation regions. Additionally, they possess an SH2 domain involved in the recognition of tyrosine-phosphorylated motifs present in activated receptor kinases or in JAK tyrosine kinases [3, 10]. Upon activation, a monomeric STAT phosphorylated on a single conserved tyrosine residue near amino acid 700, typically by a JAK family member, dimerizes via its SH2 domain with another STAT and the dimer translocates to the nucleus [3]. There, STATs bind specific DNA sequences to activate gene transcription. Structural data showed how the STAT1 dimer clamps around the DNA helix [11, 12]. These interactions are stabilized by reciprocal and specific interactions between the SH2 domain of one STAT molecule and the COOH terminal region, containing the phosphotyrosine, of the other [11]. Thus, the tyrosine phosphorylation event occurring in the cytoplasm is crucial for the function of STAT in the nucleus. JAK kinases have also been detected in the nuclei of some cells, so that maintenance of STAT phosphorylation by JAK action in the nucleus appears, in principle, to be possible [13, 14]. In fact, there appear to be situations, such as stimulation of growth hormone receptor by growth hormone, in which all components of the JAK pathway find themselves in the nucleus after stimulation [15, 16]. This would in some way be a case of an intranuclear tyrosine phosphorylation pathway.

Quantitative analysis of the distribution of STAT1 showed that after activation and translocation to the nucleus, the molecule returns to the cytoplasm in a dephosphorylated form, suggesting the existence of a nuclear STAT phosphatase [17]. By the action of this phosphatase, the STAT dimer is thought to dissociate, so that on top of the effects on nuclear import/export, STAT1 binding to DNA and transcription of targeted genes could also be modulated by a nuclear tyrosine phosphatase.

THE MAPKS. Mitogen-activated protein kinase (MAPK) modules are involved in all eukaryotic organisms in signal transduction to the nucleus of a wide variety of signals. These modules are composed of three protein kinases activated by successive phosphorylation to promote cellular proliferation, differentiation, development, inflammatory responses, and other processes [18]. The most upstream kinase in the module, the MAPKKK (MAPK kinase kinase) serine/threonine kinase can be activated by a variety of mechanisms that typically involve recruitment to subcellular sites by a small GTPase or by a member of the STE20/PAK (Rac p21-activated kinase) family of kinases. The MAPKKK then phosphorylates and activates the dual-specificity serine/threonine and tyrosine kinase MAPKK that in turn activates the serine/threonine kinase MAPK by dual phosphorylation of a threonine and tyrosine residue in the activation loop of the catalytic domain. The dually phosphorylated MAPK translocates to the nucleus to interact with and to phosphorylate nuclear targets. Phosphorylation in the activation loop is sufficient to cause translocation of the MAPK to the nucleus in a process involving dimerization as well as the synthesis of labile nuclear retention proteins [19, 20]. On the other hand, MAPKs are down-regulated by the action of phosphatases dephosphorylating the activation loop. Several dual-specificity phosphatases responsible for this process have been identified. Among them, CL100/MKP-1 (MAPK phosphatase) and PAC-1 (phosphatase of activated cells) are nuclear, and their transcription is induced by growth factor or stress-inducible signals [21]. Recent evidence suggests that dephosphorylation of the tyrosine in the activation loop may occur independently of dephosphorylation of the threonine residue [22]. Because dephosphorylation of the MAPK appears to result in both inhibition of its activity and export from the nucleus, MAPKs seem to resemble the STATs in this respect.

The JNK/SAPK (c-Jun N-terminal kinase/stress-activated protein kinase) MAPK module is specifically activated by cytokines and environmental stress of all sorts [23]. JNK, the MAPK of the module, is activated by dual phosphorylation within a Thr-Pro-Tyr motif located in the activation loop by MKK dual-specificity kinases (MAPKK). Phosphorylation of both threonine and tyrosine is required for their activation. JNK can localize to the nucleus after activation or may also become phosphorylated directly in the nucleus [23]. However, it appears that the activation of JNK1 needs the synergistic and probably subsequent action of two different upstream kinases. MKK4 preferentially phosphorylates the tyrosine residue, while MKK7 phosphorylates the threonine residue [24]. Moreover, recent data showed that MKK4 and MKK7 are not localized in the same subcellular compartment [25].

Nuclear localization of the MAPKK may also occur in the ERK/MAPK module. Rapid translocation of the MAPKK MEK to the nucleus in the ERK pathway, for example, may play an important role after stimulation with insulin [26]. MEK may translocate to the nucleus independently of activation and be rapidly removed from the nucleus by its NES (nuclear export signal) [27]. The regulation of this process is still largely unknown. It is possible that nuclear MEK has other substrates than MAPKs. The potential importance of nuclear accumulation of the MAPKK for cell regulation has been shown by studies on NIH 3T3 cell transformation where active and constitutively nuclear MKK was found to be very potent and to lead to high levels of active nuclear ERK in the nucleus [28].

From all these studies, it becomes apparent that differential tyrosine phosphorylation of the activation loop of kinases of the MAPK family may represent an important regulatory function of tyrosine phosphorylation in the nucleus.

HS1. The 75-kDa HS1 protein has been identified as a major substrate of the c-Src-related kinase Lyn and of the tyrosine kinase Syk in BCR signaling [29]. Mutation of critical tyrosine residues phosphorylated by Lyn/Syk impairs the translocation of HS1 in the nucleus and renders the cells insensitive to BCR-dependent apoptosis [30]. HS1 possesses motifs characteristic of transcription factors. It has been proposed, as for the STATs, that HS1 could regulate gene expression following translocation to the nucleus. How HS1 tyrosine phosphorylation may affect the localization of HS1 to the nucleus is not known. Mechanisms that include subsequent phosphorylation events and conformational changes have been proposed [30, 31]. Interestingly, HS1 is structurally related to the Abl-interacting proteins Abi 1 and Abi 2 that are targets of the Abl tyrosine kinases and localize in the nucleus as well as in the cytoplasm [32].

Growth Control

ABL. The cellular form of c-Abl tyrosine kinase has been cloned from human, mouse, fruit fly, and the worm [33, 34]. c-Abl is a non-receptor tyrosine kinase and is composed of several individual domains: an SH3 domain, an SH2 domain, a catalytic domain, and a long C-terminal region containing functional motifs that provide unique properties to c-Abl, including three nuclear localization signals, a putative DNA-binding domain, a putative actin-binding domain, and a nuclear export signal [33]. c-Abl localizes both in the cytoplasm and nucleus of cells. The proportion of nuclear c-Abl can vary dramatically and depends on the cell type and the physiological state of the cell. The transformation and antiapoptotic properties of several mutant forms of Abl correlate with its cytoplasmic localization, while the cytostatic or proapoptotic properties require nuclear localization [35]. Cell adhesion seems to favor the transient relocalization of the nuclear form of c-Abl to sites of focal adhesions. The reasons why c-Abl has nucleocytoplasmic shuttling properties are not known [36]. Clearly, c-Abl has distinct cytoplasmic and nuclear targets. Nuclear partners and targets are proteins involved in

growth control, transcription, and DNA damage-activated pathways [37, 38].

The activity of nuclear c-Abl has been reported to be regulated in the cell cycle through interaction with pRB [39]. Hyperphosphorylation of pRB by the cyclin-dependent kinase complexes CDK6/cyclin D or CDK4/cyclin D disrupts the interaction with the nuclear c-Abl form and correlates with an increase in the kinase activity of c-Abl, specifically during S phase [39]. These data suggest that c-Abl could phosphorylate nuclear targets in a cell cycle-dependent manner. c-Abl can interact with and phosphorylate the COOH-terminal domain of RNA polymerase II, enhancing the transcriptional activity of specific reporter genes, possibly by affecting elongation [40]. Moreover, c-Abl phosphorylated on tyrosine and serine/threonine binds to DNA, although it is not clear if *in vivo* this occurs directly or through the interaction of DNA-binding proteins [33]. One DNA-binding partner of c-Abl is the RFX1 protein, a protein that binds promoters/enhancers of several viral and cell cycle-regulated genes. *In vitro* activation of c-Abl by interaction with RFX1 suggests that RFX1 may recruit active c-Abl to defined DNA *cis*-elements [41].

c-Abl is activated in response to DNA-damaging agents, such as ionizing radiation and radiomimetic compounds [42, 43]. Following DNA damage, c-Abl may interact with the tumor suppressor gene p53. No tyrosine phosphorylation of p53 has been identified. In contrast, p73, a p53-related protein, binds and is phosphorylated on tyrosine by c-Abl in response to DNA damage [44, 45]. This phosphorylation stabilizes p73 and is essential to trigger apoptosis in irradiated cells [44–46].

Double-strand breaks in DNA damage activate the DNA-PK and the related ATM (the product of the gene mutated in ataxia-telangiectasia) which are recruited to the DNA strand breaks and play a major role in the DNA repair signaling process [47]. DNA-PK is a trimeric nuclear serine/threonine kinase consisting of a large catalytic subunit and the Ku heterodimer. Ionizing radiation stimulates the binding of c-Abl and DNA-PK and induces its association with Ku. c-Abl has been reported to become activated by phosphorylation by DNA-PK and ATM. In turn, c-Abl may phosphorylate these enzymes and inhibit their activity and their ability to bind the DNA in a potential feedback loop mechanism [48]. The Rad51 protein, a homologue of bacterial RecA, is involved in DNA double-strand break repair and genetic recombination [49]. In response to ionizing radiation, c-Abl phosphorylates Rad51. However, it is not yet clear whether this phosphorylation inhibits Rad51 DNA binding and its function in ATP-dependent DNA strand exchange reactions [50] or if it stimulates its association with Rad52 to form repair protein complexes [51]. These results suggest a mechanism by which c-Abl may be involved in the regulation of repair in response to DNA damage. Indeed, c-Abl-deficient cells failed to undergo cell cycle arrest after DNA damage [43]. However, recent genetic analysis showed that cells lacking c-Abl (or even cells lacking c-Abl as well as the Abl-related gene Arg) do not show obvious defects in DNA damage-induced cell cycle checkpoints [52, 53]. On the other hand, c-Abl-defective chicken DT40 cells showed resistance to radiation-induced apoptosis, confirming a role for c-Abl in cell fate decision following DNA damage [53].

Recent findings showed an alternative way, different from interaction and phosphorylation by ATM and DNA-PK, by which nuclear c-Abl may be activated [38]. Accumulation of the c-Jun transcription factor causes a strong increase in c-Abl activity. Moreover, activated c-Abl efficiently phosphorylates c-Jun on a specific tyrosine in the middle of the c-Jun protein. Activation of c-Abl also leads to activation of the JNK serine/threonine kinase. In murine fibroblasts, phorbol ester treatment leads to an increase in JNK activity that depends entirely on c-Jun accumulation. The consequences of this tyrosine phosphorylation event on c-Jun are not known, but tyrosine-phosphorylated c-Jun enters a complex with c-Abl that depends on the c-Abl SH2 domain for stability [38]. The mechanism by which c-Jun turns on c-Abl and JNK activity is not known, but is currently under investigation in the authors' laboratory. Presumably, c-Jun accumulation in the absence of not-yet-identified concomitant signals may specifically activate the nuclear pool of JNK. All three proteins, i.e. c-Abl, c-Jun, and JNK, have been involved both in growth-stimulatory and-inhibitory processes as well as in the regulation of apoptosis. It is tempting to speculate that the c-Jun/c-Abl/JNK network may integrate growth- and stress-related stimuli for cellular fate decision. Interestingly, the SHPTP1 tyrosine phosphatase, capable of down-regulating the induction of JNK activity following certain stimuli, has been found to interact with c-Abl in the nucleus after ionizing radiation of monocytic cells. Induction of SHPTP1 tyrosine phosphorylation by c-Abl could play an important role in the modulation of JNK activity in the nucleus in these phosphorylation circuits [48].

Altogether, c-Abl appears to have nuclear substrates and binding partners that are prominent players in growth control and cell fate decisions. It seems a plausible hypothesis that the role of Abl-controlled nuclear tyrosine phosphorylation may be to link different and often opposing signaling pathways and contribute to the coordination of the cellular response to these stimuli. Certainly, c-Abl represents a case for which intranuclear tyrosine phosphorylation has a *raison d'être* independent of tyrosine phosphorylation as triggered by membrane or cytoplasmic tyrosine kinases.

LYN. The c-Src family kinase member Lyn is expressed in a wide range of tissues and cell types and has been shown to physically and functionally associate with various receptor molecules [54]. Surprisingly, using cell fractionation and immunostaining experiments, Lyn has been reported to be present in the nuclear matrix [55]. Moreover, tyrosine phosphorylation in the nuclear matrix is cell cycle-dependent and reaches a maximum at the G1/S transition. This activation coincides with the start of DNA synthesis, at

which time DNA replication forks are known to associate with the nuclear matrix. Lyn activity is increased by DNA damage [56]. Following DNA damage, Lyn binds and phosphorylates CDK2, DNA-PK, and SHPTP1, affecting their activity [57–59]. How Lyn activation relates to activation of c-Abl is not known. However, at least in the cytoplasm, the activity of Src family kinases can lead to activation of c-Abl [60]. This raises the possibility of Lyn being upstream of c-Abl in certain nuclear signaling pathways.

RAK/FRK. The Rak/Frk tyrosine kinase has been identified from breast cancer cells and human B lymphoma cells [61, 62]. Subsequently, members of the same family called lyk and Bsk (brain-specific kinase) have also been cloned [63, 64]. These kinases, approximately 50% identical to Src family members, possess SH3 and SH2 domains and may be regulated by an Src-like mechanism. Unlike Src family members, Rak/Frk kinases lack a myristoylation signal (though Bsk may contain one) and reside both in the nucleus and the cytoplasm [61]. Indeed, these kinases possess an NLS (nuclear localization sequence) in their SH2 domains. It has been shown that Rak binds to the pRB in the A/B pocket, a region frequently mutated in cancer. This interaction may be involved in the growth-suppressing activity of the kinase [65]. In addition, Iyk mutants that had increased activity and a correlating increased nuclear localization have been reported to inhibit cell proliferation [66]. Elucidation of the nuclear substrates of the Rak/Frk family of kinases is expected to be particularly interesting.

FES/FER. The Fes/Fer non-receptor tyrosine kinase family possesses a catalytic domain at the COOH-terminal, a central SH2 domain, and an N-terminal region which contains three predicted coiled-coil forming regions involved in oligomerization [67]. Non-receptor tyrosine kinases with a similar organization form the most abundant class in the genome of *Caenorhabditis elegans* [68]. Oligomerization may be essential for autophosphorylation and activation of c-Fes, whereas trimerization of c-Fer is apparently not necessary for its kinase activity [69, 70]. c-Fes is highly expressed in myeloid hematopoietic cells, and while it is dispensable for hematopoiesis in general, it is involved in GM-CSF (granulocyte-macrophage colony-stimulating factor) and ERK signaling in macrophages [71]. Fes has been detected in the nucleus and in the granular and plasma membrane [72]. Several cytoplasmic, but no nuclear, substrates of Fes are known [73]. The c-Fer protein exists as two splice variants. The smaller of the two is mostly expressed in meiotic cells and is constitutively nuclear, while the larger ubiquitous form localizes to the nucleus in a cell cycle-dependent manner [74]. The only known substrate of nuclear Fer is the TATA element modulatory factor (TMF, see below), but many more can be expected.

DYRK. The human *DYRK1A* gene is localized on chromosome 21, in the Down Syndrome critical region. Six related kinases have so far been identified in mammals. The DYRK kinases represent a new family of dual-specificity kinases which are able to catalyze tyrosine-directed autophosphorylation, needed for their activation and phosphorylation of serine/threonine residues in exogenous substrates [75]. DYRKs contain bipartite nuclear localization motives. DYRK1A, a ubiquitous family member, and DYRK1B, a form specially expressed in muscle and testis, were found in the nucleus of transfected cells [75, 76]. The function and possible substrates of these proteins remain to be elucidated. However, the activity of the *Saccharomyces cerevisiae* homologue Yak1 is induced by cell cycle arrest, and mutation in the gene compensates for growth defects of mutants lacking the cyclic AMP-dependent protein kinase (PKA), suggesting that Yak1 acts in an opposing manner to the PKA to negatively regulate cell proliferation [77, 78]. Moreover, DYRKs are the vertebrate homologues of the *Drosophila minibrain* (MNB) gene, involved in the proliferation of neuronal precursors [79]. A role in cell proliferation can be envisaged for the vertebrate counterpart.

ALK, NPM–ALK. The fusion of the nucleolar protein NPM to the tyrosine kinase receptor ALK, resulting from a chromosomic translocation, has oncogenic properties and is associated with anaplastic large cell lymphoma [80]. This translocation produces a constitutive kinase activity and a cytosolic, nuclear, and nucleolar localization of the NPM–ALK oncoprotein. It is possible that NPM–ALK generates growth regulatory signals within the nucleus, possibly interfering with the function of normal counterparts. While NPM–ALK is not a "natural" protein, its substrates are, so that elucidation of potential nuclear substrates of NPM–ALK will be very interesting.

PTEN. PTEN encodes a dual-specificity protein phosphatase and a phosphatidylinositol phosphate (PIP) phosphatase and represents a tumor suppressor gene product of primary importance [81, 82]. During neuronal differentiation, PTEN is detected both in the nucleus and the cytoplasm [83]. PTEN plays multiple biological roles: inhibition of PIP levels and consequent inhibition of cell proliferation and survival, cell migration by its action in focal adhesion, and inactivation of the MAP kinase pathway [81]. Because the majority of biological functions can be ascribed to the lipid phosphatase activity of PTEN, it is not yet clear whether the tyrosine phosphatase potential plays any physiological role [82].

TC-PTP. The T cell protein tyrosine phosphatase TC-PTP, a ubiquitous phosphatase abundant in hematopoietic cells, was originally cloned from T cells and represents a prototypic non-receptor tyrosine phosphatase [84]. Deletion of the gene in mouse leads to death 3–5 weeks after birth and coincides with specific defects in bone marrow microenvironment erythropoiesis as well as impaired T and B cell functions [85]. An alternative splicing of the TC-PTP message gives rise to a 48-kDa and 45-kDa protein

[86]. Whereas the 48-kDa protein is localized to the endoplasmic reticulum, the smaller one, lacking a COOH-terminus part, is located in the nucleus [86]. A nuclear localization signal in TC-PTP interacts with the nuclear import factor p97 [87]. In *Drosophila*, the DPTP61F tyrosine phosphatase message is also spliced to produce two phosphatases located in the cytoplasm or in the nucleus [88]. These data show that alternative splicing is a mechanism commonly used to target tyrosine phosphatase isoforms to distinct subcellular sites and contributes to give differential substrate specificity. The function and substrates of these nuclear forms of the phosphatases are still unknown. The development of substrate-trapping mutants of the nuclear forms of these phosphatases may lead to the identification of their nuclear substrates [89].

VAV. The Vav proto-oncogene is a large signaling adaptor and docking protein highly expressed in hematopoietic cells. Vav contains multiple structural motifs commonly found in signaling molecules, such as the SH2 and SH3 domains, a pleckstrin homology domain, and a guanine nucleotide exchange domain necessary for GTPase activity on Rho-like proteins [90]. While Vav is known to have cytoplasmic functions, it also possesses two nuclear localization sequences. In fact, interferon treatment induces translocation of Vav to the nucleus of megakaryocytic cells [91]. The GTPase activity of Vav is increased by tyrosine phosphorylation mediated by Lck during T cell activation [90]. Vav induces NF-AT (transcription nuclear factor activated in T cells) during T cell activation, independently of Vav's GTP exchange factor activity [92]. Vav has been found to be tyrosine-phosphorylated and to interact with phospholipase C-γ1 as well as phosphoinositol 3-kinase (PI3K) in the nucleus of HL-60 cells [93]. Vav has also been found to be associated with hnRNP C (heterogeneous nuclear ribonucleoprotein) in an RNA-dependent manner [94]. Even if its nuclear function is not fully understood, these data suggest that Vav is an adaptor protein involved in RNA metabolism and transcription during differentiation and may act by recruiting signaling molecules to the nucleus.

CRKL. CrkL, a member of the Crk family of adaptor proteins, is expressed in hematopoietic cells and is involved in T cell signaling [95]. CrkL is composed of a single SH2 domain flanked by two SH3 domains. This adaptor is a target of the Bcr–Abl fusion protein kinase and has been implicated in the pathogenesis of chronic myelogenous leukemia [96, 97]. Phosphorylated CrkL is present in the nucleus and has been detected in STAT5/DNA complexes [98]. Moreover, CrkL increases transcriptional activation from a Stat-responsive reporter construct [98]. This suggests a function for CrkL in bridging cytokine activation and gene expression in hematopoietic cells.

CBL AND CARP90. c-Cbl is a common component of many signaling pathways and was originally characterized as the cellular homologue of a retroviral oncogene inducing B cell lymphoma [95]. Whereas c-Cbl is localized in the cytoplasm, the viral form, v-Cbl, which corresponds to a COOH-terminus truncation of c-Cbl fused to the viral protein Cas N1, presents both a cytoplasmic and nuclear localization, binds DNA, and induces acute leukemia [99, 100]. It has been suggested that retention of c-Cbl in the cytoplasm could inhibit its ability to affect cell growth. Recently, a ubiquitin–ligase activity in the RING finger of c-Cbl has been characterized [101, 102] as being responsible for the down-regulation of tyrosine kinase receptors. c-Cbl appears to recognize tyrosine-phosphorylated proteins via its SH2 domain and targeting them for degradation. A nuclear protein related to c-Cbl protein, CARP90, has recently been identified in medullary and cortical thymocytes [103]. There exists the exciting possibility that CARP90 may be involved in the degradation of tyrosine-phosphorylated signaling proteins in the nucleus.

Cell Cycle

The Wee1 tyrosine kinase was first identified in the fission yeast *Schizosaccharomyces pombe* as a negative regulator of the entry into mitosis that is conserved in all organisms [104]. Wee1 is localized in the nucleus and exported to the cytoplasm prior to mitosis [105]. This kinase inhibits by phosphorylating the CDKs when bound to cyclins [106]. To prevent premature entry into mitosis, Wee1 phosphorylates the CDC2/cyclin B complex on tyrosine 15 of CDC2, within the ATP-binding site [104]. After ultraviolet treatment, the CDK4/cyclin D complex is inactivated and is correlated with an increase in the phosphorylation of the CDK on the equivalent of tyrosine 15 [107]. These data suggest that the Wee1 kinase could be involved in the regulation of the other CDK/cyclin complexes and is activated to stop cell cycle progression after DNA damage.

If Wee1 inhibits CDK/cyclin complexes, the CDC25 dual-specificity phosphatases, shuttling between the cytoplasm and the nucleus in a cell cycle-regulated manner, dephosphorylate and activate these complexes at different key points of the cell cycle. In humans, three different phosphatases have been identified so far: CDC25A, CDC25B, and CDC25C [108]. They present different CDK/cyclin specificity contributing to the sequential activity of the kinases, which is required for cell cycle progression [108].

DNA Repair/Recombination

The cellular responses to DNA damage are primary candidates for processes that may rely on nuclear tyrosine phosphorylation. As we have seen above, the activity of the c-Abl tyrosine kinase is induced by DNA damage, and several Abl substrates, such as Rad51, p73, and DNA-PK, have been implicated in DNA repair processes. For all three proteins, phosphorylation by Abl causes changes in their

activity, suggesting a functional "coordinating" role for tyrosine phosphorylation in this process [37, 48].

KRC. The DNA-binding protein KRC (for κB binding and recognition component of the V(D)J recombination sequence) is a member of a large zinc finger protein family. It possesses two separate DNA-binding domains, one binding the κB DNA motif (recognized by the transcription factor NF-κB) and the other the V(D)J recombination recognition sequence [109]. Phosphorylation of KRC on tyrosine and serine residues by nuclear extracts increases its ability to bind DNA. Moreover, EGF (epidermal growth factor) receptor tyrosine kinase as well as CDC2 can phosphorylate KRC and enhance its DNA binding *in vitro*. The presence of phosphorylation sites for tyrosine kinases in KRC family members could suggest that nuclear-activated tyrosine kinases may activate KRC to promote transcription [110].

Transcription

C-ABL. With regards to nuclear tyrosine phosphorylation and transcription, the prototypical nuclear tyrosine kinase c-Abl has been a primary suspect for many years. On top of the high affinity of Abl for DNA and chromatin, c-Abl appears to bind a number of transcription factors (RFX1, EP, c-Jun, p73, CREB (cAMP response binding protein)) and to phosphorylate RNA polymerase II (see above) [37]. The precise role of c-Abl on transcription, however, is not yet clear. Presumably, a systematic analysis using DNA chips of transcripts induced by c-Abl activity, as well as a systematic analysis of c-Abl substrates in the nucleus, will help define the mechanism by which c-Abl affects gene expression.

TMF. The c-Fer protein and p51FerT, a testis-specific form of c-Fer, bind and phosphorylate TMF [111]. TMF phosphorylated by c-Fer interacts with the TATA element in the RNA polymerase II promoters and inhibits transcription *in vitro*.

TFII-I. TFII-I is a multifunctional transcription factor, initially isolated as a factor that can bind TATA and TATA-less promoters [112]. TFII-I promotes formation of serum response factor complexes on the c-*fos* promoter [113]. In response to a wide range of growth factors, TFII-I is phosphorylated on both serine/threonine and tyrosine residues. Phosphorylated TFII-I binds to STAT1 and STAT3 proteins to enhance the transcription of the c-*fos* gene [114]. Mutation of the tyrosine phosphorylation site in TFII-I affects the transcription of a viral promoter *in vivo* [115]. Btk (Bruton's tyrosine kinase) binds TFII-I in the cytoplasm, forming an inactive complex. Upon activation, Btk phosphorylates TFII-I, thereby promoting the disruption of the complex and translocation of TFII-I to the nucleus [116]. Tyrosine phosphorylation might not be necessary for nuclear translocation, but might be required for maximal transcriptional activity [116].

C-REL. The c-Rel proto-oncogene belongs to the NF-κB family of ubiquitous transcription factors that exist as homo- or heterodimers in the cytoplasm of most eukaryotic cells [117]. They share 300 amino acids called the REL homology domain. This motif is responsible for dimerization, DNA binding, and interaction with the IκB proteins (inhibitor of NF-κB), as well as containing the nuclear localization signal. The involvement of serine/threonine phosphorylation in NF-κB is well established. In most unstimulated cells, NF-κB is retained in the cytoplasm through its association with a group of inhibitory proteins known as IκBs. After stimulation, IκBs are serine-phosphorylated, leading to their proteolysis in a ubiquitin/proteasome-dependent pathway allowing the translocation of NF-κB protein to the nucleus. It has been shown that tyrosine phosphorylation of IκB might also participate in NF-κB regulation independently of IκB proteolysis [118]. Oxidative stress induces nuclear translocation of NF-κB without IκB degradation and increases its tyrosine phosphorylation in endothelial cells [119]. p80 c-Rel is also rapidly tyrosine-phosphorylated upon G-CSF (granulocyte colony-stimulating factor) treatment in neutrophils or in T cells, increasing its DNA-binding activity [120, 121]. Moreover, in myeloid cells expressing p210 Bcr–Abl, tyrosine phosphorylation of p80 c-Rel is increased, resulting in a mild activation of its DNA-binding activity [122].

ESTROGEN RECEPTOR. The estrogen receptor belongs to the superfamily of nuclear receptors that regulates the expression of specific genes controlling the development, maintenance of homeostasis, cell proliferation, differentiation, and death upon steroid stimulation [123]. This family of receptors is composed of multiple structural domains: the ligand-binding domain, including a dimerization interface, and transcriptional transactivation domains [124]. Crystal structure analysis has shown that different classes of ligands confer distinct orientations of the α-helix 12 on the receptor and generate different binding surfaces for transcription intermediary coactivator proteins [125, 126]. Several reports have pointed out that the tyrosine located in this α-helix is phosphorylated after estradiol treatment, possibly by an Src family kinase [127–129]. Mutation of this tyrosine residue to different amino acids has different effects. It can either reduce receptor stability, alter hormone binding, slightly decrease transcriptional activation, or constitutively activate receptor activity [130, 131]. Moreover, tyrosine phosphorylation of the receptor appears to be required for its DNA binding and its dimerization [132]. It has been hypothesized that phosphorylation of this residue is an important component of the intricate structure–function mechanism of the estrogen receptor, presumably by changing the conformation in α-helix 12. Although

this complex mechanism is not yet known, the regulation of the interactions or "cross-talk" between the α-helix and the other domains appears to be important for the regulation of the receptor [130].

RNA Metabolism

Heterogeneous nuclear ribonucleoproteins (hnRNPs) contain KH domains that interact with pyrimidine-rich binding sequences in target RNA or DNA double strands. These proteins are involved in the processing of mRNA, in the export of the mRNA from the nucleus to translation sites in the cytoplasm, as well as in transcription [133]. hnRNP K shuttles between the cytoplasm and the nucleus, suggesting that it may not only be involved in nuclear RNA processing but possibly in nucleocytoplasmic transport of RNA as well. hnRNP K is phosphorylated on tyrosine and serine/threonine residues in response to interleukin-1 [134]. Tyrosine phosphorylation of hnRNP K augments its interaction with Src family kinases, Lyn, PKC (protein kinase C), and Vav [135]. The increase in interaction with many partners decreases the ability of hnRNP K to bind RNA [134, 135]. hnRNPK could act as a platform, a docking protein for both serine/threonine and tyrosine kinases and their substrates, leading to their interdependent activation and sequential phosphorylation. However, the function of tyrosine phosphorylation in RNA processing or in transcription remains unclear. The tyrosine phosphorylation of hnRNP K by Lyn could take place in the nucleus to regulate its import/export in the nucleus for RNA processing and/or transcription in response to extracellular signals.

The Src substrate associated in mitosis, protein Sam68, could also be involved in RNA-related processes [136, 137]. Sam68 has been reported to be predominantly nuclear [138]. Sam68 possesses a KH domain mediating interaction with RNA *in vitro*, and tyrosine phosphorylation decreases its ability to bind RNA [137, 139, 140]. Sam68 is not only a c-Src substrate, but also interacts with signaling molecules such as growth factor receptor-bound protein-2 (Grb2) and phospholipase C-γ [141]. A pool of Sam68 in the cytoplasm may act as an adaptor protein downstream of signals activating Src kinases. Recent data showed that Sam68 is tyrosine-phosphorylated in a manner that requires Fyn localization to the cell membrane compartment and thereafter can accumulate in the nucleus [142]. The tyrosine phosphorylation of Sam68 does not affect its localization. However, the phosphorylation of Sam68 disrupts its interaction with the splicing-associated factor YT521-B in the nucleus [143]. Together, these data strongly suggest that Sam68 may influence RNA metabolism not only by its RNA binding but also by its interaction with other nuclear proteins. Interestingly, the SH3 and SH2 domain-containing adaptor Nck is also found in the nucleus, where it binds to Sam68 [144]. Sam68 could be a link between extracellular-activated pathways and posttranscriptional regulation of gene expression.

OUTLOOK

In our survey, we found several "areas" that seem to point to a role for nuclear tyrosine phosphorylation. Besides cases of nuclear–cytoplasmic transport and the cell cycle in general, which rely mainly on tyrosine phosphorylation as generated by dual-specificity kinases, cellular responses to DNA damage, transcription, and RNA metabolism seem to be "hotspots". Moreover, many nuclear tyrosine kinases, such as Fer and Rak/Frk, have only a few known substrates. Many more can be envisaged. The tyrosine kinases that localize in the nucleus are likely to be embedded in tyrosine phosphorylation circuits that remain to be discovered. Moreover, tyrosine-phosphorylated proteins with other docking sites and protein–protein interaction domains, such as the SH2 or SH3 domains, suggest the existence of phosphotyrosine-dependent nuclear complexes.

Whole-genome sequences may help reveal all the potential players of nuclear tyrosine phosphorylation pathways, such as homologues of nuclear tyrosine kinases and phosphatases or, in general, tyrosine phosphorylation-related signaling molecules with potential nuclear localization signals. However, only the systematic identification of nuclear tyrosine-phosphorylated proteins by a proteomics approach involving purification and identification using mass spectrometry will show which tyrosine-phosphorylated proteins really tread on the nuclear stage. However, to establish links between proteins, interaction data will be required. The systematic analysis of tyrosine phosphorylation-dependent protein complexes will be instrumental in charting the underlying networks. Because nuclear events often represent the functional culmination of signaling pathways and because tyrosine phosphorylation almost invariably implies the creation of a molecular association, the study of tyrosine phosphorylation-dependent nuclear complexes may well represent a promising source of drug targets.

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