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Regulation of Mitogen-Activated Protein Kinase Signaling Pathways by the Ubiquitin-Proteasome System and Its Pharmacological Potential

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ABBREVIATIONS: 17-AAG, 17-*N*-allylamino-17-demethoxygeldanamycin; ABIN-2, A20-binding inhibitor of NF-κB; APC, anaphase-promoting complex; ARAF, arapidly accelerated fibrosarcoma; ASK, apoptosis signal-regulating kinase; BCL-3, B-cell lymphoma 3; BMDM, bone marrow–derived macrophage; BRAF, B-rapidly accelerated fibrosarcoma; CDC, cell division cycle; CDH1, CDC20 homolog 1; CDK, cyclin-dependent kinase; CHIP, C terminus of Hsp70-interacting protein; cIAP1, cellular inhibitor of apoptosis protein 1; CRAF, rapidly accelerated fibrosarcoma 1; CRBN, cereblon; CRL, Cullin-RING ligase; DLK, dual leucine zipper-bearing kinase; DUB, deubiquitinating enzyme; ERK, extracellular signal–regulated kinase; FBXO31, F-box only protein 31; FBXW7, F-box and WD repeat domain containing 7; FDA, Food and Drug Administration; GSK3, glycogen synthase kinase 3; HECT, E6AP C terminus; HECTD3, HECT domain E3 ubiquitin protein ligase 3; HSP, heat shock protein; IAP, inhibitor of apoptosis protein; JNK, c-Jun N-terminal kinase; KRAS, Kirsten rat sarcoma virus; LNCaP, Lymph Node Carcinoma of the Prostate; LPS, lipopolysaccharide; LZK, leucine zipper kinase; MAP, mitogen-activated protein; MAPK, MAP kinase; MAP2K, MAP kinase kinase; MAP3K, MAP kinase kinase kinase; MCF-7, Michigan Cancer Foundation-7; MEF, mouse embryonic fibroblast; MEK, MAPK/ERK kinase; MEKK, MEK kinase; MK, MAPK-activated protein kinase; MKK, mitogen-activated protein kinase kinase; MLK, mixed lineage kinase; v-Mos Moloney murine sarcoma viral oncogene homolog; NF-κB, nuclear factor-κB; NLK, Nemo-like kinase; NSCLC, non–small cell lung cancer; PAK, p21-activated kinase; PHR, PAM/Highwire/RPM-1; PMK, piperonyl methyl ketone; PROTAC, proteolysis-targeting chimera; RAF, rapidly accelerated fibrosarcoma; RING, really interesting new gene; RPM-1, regulator of presynaptic morphology 1; SARS-CoV, severe acute respiratory syndrome coronavirus; SMURF1, SMAD specific E3 ubiquitin protein ligase 1; SOCS1, suppressor of cytokine signaling 1; Spc1, signal peptidase complex subunit 1; SRC, v-Src avian sarcoma viral oncogene homolog; TAB, TAK1-binding protein; TAK1, transforming growth factor β-activating kinase 1; TAOK, thousand-and-one kinase; TGF-β, transforming growth factor β; TNF, tumor necrosis factor; TPL2, tumor progression locus 2; β-TrCP1, β-transducin repeat-containing protein 1; TRIM, tripartite motif-containing; Trx, thioredoxin; UPS, ubiquitin-proteasome system; USP, ubiquitin-specific protease; VHL, von Hippel–Lindau; Wnd, Wallenda; XIAP, X-linked inhibitor of apoptosis protein; ZAKα, Zipper sterile-α-motif kinase α.

***Abstract*——Mitogen-activated protein kinase (MAPK) cascades are evolutionarily conserved signaling pathways that play essential roles in transducing extracellular environmental signals into diverse cellular responses to maintain homeostasis. These pathways are classically organized into an architecture of three sequentially acting protein kinases: a MAPK kinase kinase that phosphorylates and activates a MAPK kinase, which in turn phosphorylates and activates the effector MAPK. The activity of MAPKs is tightly regulated by phosphorylation of their activation loop, which can be modulated by positive and negative feedback mechanisms to control the amplitude and duration of the signal. The signaling outcomes of MAPK pathways are further regulated by interactions of MAPKs with scaffolding and regulatory proteins. Accumulating evidence indicates that, in addition to these mechanisms, MAPK signaling is commonly regulated by ubiquitin-proteasome system (UPS)-mediated control of the stability and abundance of MAPK pathway components. Notably, the biologic activity of some MAPKs appears to be regulated mainly at the level of protein turnover. Recent studies have started to explore the potential of targeted protein degradation as a powerful strategy to investigate the biologic functions of individual MAPK pathway components and as a new therapeutic approach to overcome resistance to current small-molecule kinase inhibitors. Here, we comprehensively review the mechanisms, physiologic importance, and pharmacological potential of UPS-mediated protein degradation in the control of MAPK signaling.**

***Significance Statement*——Accumulating evidence highlights the importance of targeted protein degradation by the ubiquitin-proteasome system in regulating and fine-tuning the signaling output of mitogen-activated protein kinase (MAPK) pathways. Manipulating protein levels of MAPK cascade components may provide a novel approach for the development of selective pharmacological tools and therapeutics.**

I. Introduction

A. *Mitogen-Activated Protein Kinases*

Mitogen-activated protein (MAP) kinases (MAPKs) are a family of serine/threonine kinases that play a key role in transducing chemical and physical extracellular signals into intracellular responses to maintain cellular and tissue homeostasis (Pearson et al., 2001; Cargnello and Roux, 2011; Meloche, 2018). MAPKs belong to the cyclin-dependent kinases (CDKs), MAPKs, glycogen synthase kinases, CDK-like group of protein kinases and are conserved in eukaryotic cells. The human genome encodes 14 MAPK genes that define 7 distinct MAPK signaling pathways (Fig. 1). Based on different structural and regulatory features, MAPKs can be further divided into classic MAPKs, which include the four subfamilies extracellular signal–regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNK1/2/3), p38s (p38α/β/γ/δ), and ERK5, and atypical MAPKs, consisting of

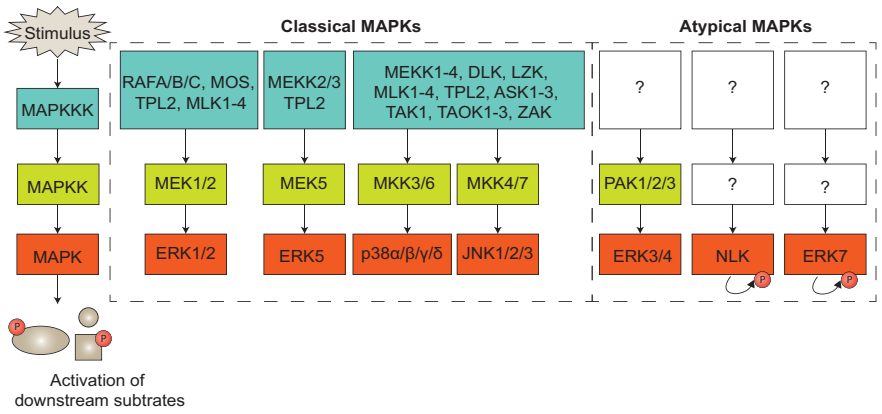

Fig. 1. The mammalian MAPK family. MAPK pathways are evolutionarily conserved signaling modules by which cells transduce extracellular environmental signals into intracellular responses to maintain homeostasis. There are 14 MAPK genes that define seven distinct MAPK pathways in humans. MAPKs can be classified into classic or atypical enzymes based on their ability to become phosphorylated and activated by members of the MAP2K family.

the ERK3/4, ERK7, and Nemo-like kinase (NLK) subfamilies (Coulombe and Meloche, 2007). The classic MAPK pathways are organized into an architecture of three sequentially activated protein kinases. Exposure to an extracellular stimulus typically leads to the activation of a cell surface receptor, which promotes the recruitment and activation of an MAPK kinase kinase (MAPKKK or MAP3K) through a multistep process. The MAP3K then phosphorylates and activates a dual-specificity MAPK kinase (MAPKK or MAP2K), which in turn activates the effector MAPK by phosphorylation of both tyrosine and threonine residues within the Thr-X-Tyr motif present in the activation loop of all classic MAPKs. Activated MAPKs then phosphorylate a large repertoire of substrates in various subcellular compartments. MAPKs are proline-directed kinases that phosphorylate substrates on the minimal consensus motif Ser/Thr-Pro. The regulation of atypical MAP kinases, which are not substrates of MAP2Ks, is less well characterized.

MAPK pathways relay information from a wide array of stimuli to regulate cellular responses such as gene expression, morphogenesis, cell division, differentiation, survival, metabolism, motility, and immune responses (Coulombe and Meloche, 2007; Weston and Davis, 2007; Rincon and Davis, 2009; Cuadrado and Nebreda, 2010; Cargnello and Roux, 2011; Kyriakis and Avruch, 2012; Nithianandarajah-Jones et al., 2012; Arthur and Ley, 2013; Lau and Xu, 2018; Daams and Massoumi, 2020; Lavoie et al., 2020). Consequently, alterations in MAPK signaling have been implicated in many human diseases, including RASopathies, cancer, inflammatory disorders, diabetes, and neurodegenerative diseases (Weston and Davis, 2007; Lawrence et al., 2008; Wagner and Nebreda, 2009; Kim and Choi, 2010; Kyriakis and Avruch, 2012; Nithianandarajah-Jones et al., 2012; Samatar and Poulikakos, 2014; Tajan et al., 2018; Asih et al., 2020; Daams and Massoumi, 2020). This has prompted the development and clinical evaluation of MAPK pathway inhibitors for different therapeutic indications. Inhibitors of the ERK1/2 MAPK pathway are now approved for the treatment of neurofibromas and for metastatic melanoma, non–small cell lung cancer, anaplastic thyroid cancer, and Erdheim-Chester disease (Roskoski, 2019; First drug approved for neurofibroma is a MEK inhibitor, 2020; Subbiah et al., 2020), whereas inhibitors of p38 and JNK MAPKs are undergoing clinical evaluation in asthma, arthritis, Alzheimer diseases, cancer, pain, and pulmonary fibrosis (www.clinicaltrials.gov).

The location, amplitude, and duration of MAPK signals must be carefully controlled to elicit the appropriate biologic response to specific environmental signals (Morrison and Davis, 2003; Ebisuya et al., 2005; Raman et al., 2007; Witzel et al., 2012). Therefore, several negative regulatory mechanisms exist to restore MAPK signaling back to a steady state. For classic MAPKs, termination of signaling is mainly achieved by dephosphorylation of the activating Thr and Tyr residues by dual-specificity MAPK phosphatases, also known as dual-specificity phosphatases (Caunt et al., 2015). Downstream kinases in the MAPK cascade can also retroinhibit the activity of upstream components either by direct phosphorylation or by transcriptional induction of specific pathway inhibitors (Avraham and Yarden, 2011; Lake et al., 2016; Zeke et al., 2016). In addition to these mechanisms, one aspect of MAPK regulation that is often overlooked is the modulation of the cellular abundance of MAPK pathway components. Accumulating evidence indicates that the signaling output of MAPK pathways is commonly regulated by controlling the stability and expression level of different cascade components via the ubiquitin-proteasome system (UPS).

Mammalian cells have evolved two major intracellular protein degradation pathways to maintain homeostasis: the UPS and autophagy (Pohl and Dikic, 2019). The UPS is mainly responsible for the degradation of short-lived proteins and misfolded soluble proteins, whereas protein aggregates and damaged organelles are degraded by the autophagy-lysosomal system. Although JNK signaling has been suggested to be involved in the activation of autophagy (Zhou et al., 2015) and p38α MAPK was described as a negative regulator of starvation-induced autophagy (Webber and Tooze, 2010), there is no study to date reporting autophagy to be a process for degrading MAPK pathway proteins. In contrast, in all organisms from yeast to mammals, proteins at each level of the MAPK cascade have been shown to be regulated and degraded by the UPS.

B. The Ubiquitin-Proteasome System

The UPS is a multistep enzymatic pathway that specifically targets ubiquitin-tagged proteins for degradation by the proteasome (Hershko and Ciechanover, 1998; Finley, 2009; Schwartz and Ciechanover, 2009). The addition of a ubiquitin chain, a signal for degradation, occurs through a three-step enzymatic mechanism. First, an ATP-dependent E1 (ubiquitin-activating enzyme) catalyzes the activation of the ubiquitin molecule. Then, the ubiquitin molecule is transferred from the E1 to the active site of an E2 (ubiquitin-conjugating enzyme). Finally, an E3 (ubiquitin ligase) promotes the transfer of the ubiquitin to the substrate protein (Fig. 2). The E3 ligases are subdivided into three families according to their domain structure and mechanism of ubiquitin transfer: really interesting new gene (RING), homology to E6AP C terminus (HECT), and RING-between-RING (Morreale and Walden, 2016). The RING E3s facilitate the direct transfer of ubiquitin from the E2 to the

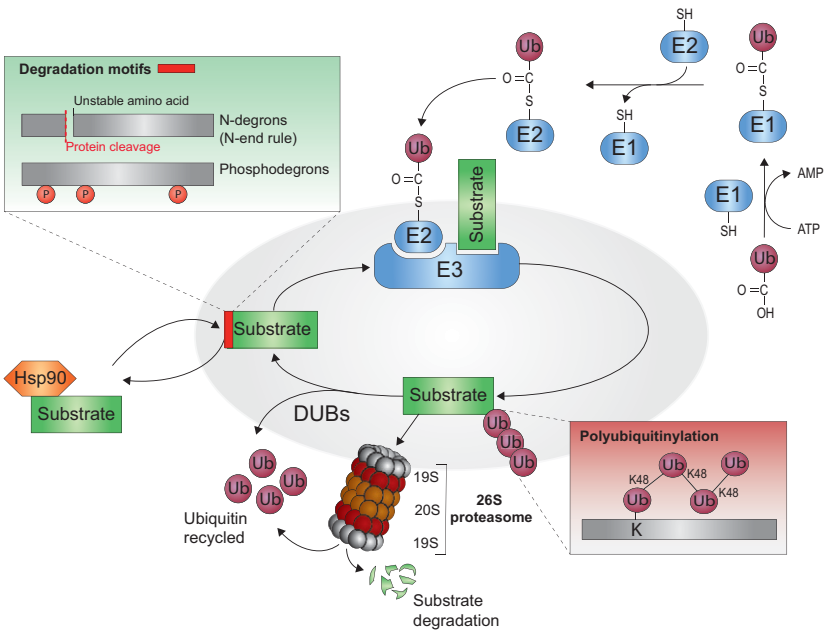

Fig. 2. The UPS. The UPS is a major proteolytic system that plays key roles in cellular regulation and protein quality control. Proteins to be degraded are tagged with ubiquitin molecules and targeted to the proteasome for destruction. Polyubiquitin K48-linked chains are the most potent signal for proteasomal degradation. Ubiquitin conjugation is carried out by an enzymatic cascade involving an E1 ubiquitin-activating, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase that promotes the transfer of ubiquitin to the substrate. E3 ligases typically recruit protein substrates by recognition of peptidic degrons, such as degrons of the N-end rule pathway and degrons modified by post-translational modifications. The ubiquitination reaction is reversed by DUBs, leading to protein stabilization. Proteins can also be protected from degradation by binding to molecular chaperones such as Hsp90. Ub, Ubiquitin.

substrate by binding to both proteins, whereas the HECT and RING-between-RING E3s ubiquitinate substrates in a two-step reaction involving transfer of ubiquitin from the E2 to the E3 and then to the substrate. E3 ligases confer substrate specificity to the ubiquitination process, and more than 600 E3s have been identified in humans (Noble et al., 2008). Ubiquitin conjugation is reversible and is opposed by a family of deubiquitinating enzymes (DUBs) that cleave ubiquitin off its substrates (Mevissen and Komander, 2017). In this review, we highlight the importance of the UPS in regulating the stability and expression level of individual MAPK pathway core components and how this impacts MAPK signaling and cellular outcome. Then, we discuss how the targeted degradation of MAPK pathway components can be pharmacologically manipulated for therapeutic purposes.

II. Classic Mitogen-Activated Protein Kinase Pathways

A. The Extracellular Signal–Regulated Kinase 1/2 Signaling Pathway

The ERK1/2 MAPK pathway is activated by extracellular growth factors, cytokines, and hormones mainly through tyrosine kinase receptors. Typically, receptor stimulation leads to activation of the small GTPase rat sarcina (RAS), which recruits and promotes the activation of rapidly accelerated fibrosarcoma (RAF) isoforms. RAF, acting as an MAP3K, activates the dual-specificity MAP2Ks MEK1 and MEK2, which, in turn, phosphorylate and activate the MAPKs ERK1 and ERK2 (Fig. 1). The ERK1/2 pathway plays a key role in the regulation of cell proliferation, differentiation, survival, and various other cellular processes (Meloche and Pouyssegur, 2007; Lavoie et al., 2020).

1. Mitogen-Activated Protein Kinase Kinase Kinases. a. ARAF, BRAF, and CRAF (rapidly accelerated fibrosarcoma 1). The mechanism controlling the stability of RAF proteins is intrinsically linked to their regulation by heat shock protein 90 (Hsp90) and differs for the three members of the RAF kinase family. Unlike wild-type BRAF, the proteins ARAF and CRAF are clients of the Hsp90 chaperone machinery. CRAF was first shown to interact with Hsp90 (Schulte et al., 1995). Disruption of this interaction leads to proteasomal degradation of CRAF, leading to inhibition of the ERK1/2 pathway (Schulte et al., 1996, 1997; Piatelli et al., 2002). Since then, a plethora of studies focusing on Hsp90 inhibitors have shown that CRAF protein abundance is dependent on the Hsp90 chaperone machinery (Table 1). Mechanistically, disruption of Hsp90 binding to immature CRAF leads to misfolding of CRAF and its subsequent ubiquitination and targeting for proteasomal degradation by the quality control E3 ubiquitin ligase C terminus of Hsp70-interacting protein (CHIP) and by HECT domain E3 ubiquitin protein ligase 3 (HECTD3) (Connell et al., 2001; Demand et al., 2001; Li et al., 2017). The interaction of CRAF kinase domain with the cochaperone Cdc37 was shown to be necessary for the formation of the CRAF-Cdc37-Hsp90 ternary complex (Stancato et al., 1993; Grammatikakis et al., 1999). More recently, a large-scale quantitative analysis of Hsp90-client interactions revealed that the determinants for Hsp90 association are distributed in both lobes of the ARAF and CRAF

TABLE 1
Hsp90 inhibitors and their effects on RAF protein stability and ERK1/2 MAPK signaling

Drug	Target	Evidence of Regulation	Effect on MAPK Signaling	Model	Reference
Ansamycins and derivatives					
Geldanamycin	CRAF	Decreased protein levels	Blocks PMA-dependent ERK1/2 signaling	NIH 3T3 cells	(Schulte et al., 1997; Schulte et al., 1996)
		Decreased protein levels	CRAF-dependent induction of apoptosis	SH-SY5Y cells	(Kim et al., 2003)
		Decreased protein levels	Inhibition of ERK1/2 signaling Cells resistant to BRAF inhibition display increased sensitivity to geldanamycin	M14 cells	(Montagut et al., 2008)
Geldanamycin derivatives	CRAF	Decreased protein levels	N/A	SKBr3 cells	(An et al., 1997)
17-AAG (tanespimycin)	CRAF	Dose-dependent decrease in protein levels	Inhibition of ERK1/2 signaling	Melanoma cell lines	(Joshi et al., 2018)
		Decreased protein levels	Inhibition of ERK1/2 signaling	Uveal melanoma cell lines	(Babchia et al., 2008)
		Decreased protein levels (proteasome-dependent)	Inhibition of endothelin-1–induced ERK1/2 signaling	Neonatal rat ventricular myocytes	(Tamura et al., 2019)
		Decreased protein levels (proteasome-dependent) Effect potentiated by combination with 7-hydroxy-staurosporine	Inhibition of ERK1/2 signaling Ectopic expression of active MEK suppresses apoptosis induced by 17-AAG and staurosporine	U937 cells	(Jia et al., 2003)
		Decreased protein levels	Inhibition of myocardial infarction–induced ERK1/2 activation	Rat model of coronary artery ligation	(Tamura et al., 2019)
		Dose-dependent decrease in protein levels	Inhibition of ERK1/2 signaling	SK-Mel-31 and SK-Mel-28 cells in vitro and in vivo	(Grbovic et al., 2006)
		Dose-dependent decrease in protein levels	Inhibition of ERK1/2 signaling	HT-29, HCT 116, and HCT 15 cells	(Hostein et al., 2001)
		Decreased protein in combination with trastuzumab levels (variable between patients)	N/A	Patients with HER2+ metastatic breast cancer	(Modi et al., 2007)
		Decreased protein levels (four of six patients)	N/A	Tumor biopsies and peripheral blood leukocytes from patients with advanced cancers	(Banerji et al., 2005)
		Dose-dependent decrease in protein levels (in vitro cell models) Decreased protein levels (tumor biopsies)	N/A	Ovcar3, Ovcar5, and OV17 cells Ovarian tumor biopsies	(Hendrickson et al., 2012)
		Decreased protein levels in combination with sorafenib (four of six patients)	Inhibition of ERK1/2 signaling	PBMCs of patients with advanced cancers	(Vaishampayan et al., 2010)
	ARAF	Dose-dependent decrease in protein levels	Inhibition of ERK1/2 signaling	SK-Mel-31 and SK-Mel-28 cells in vitro and in vivo	(Grbovic et al., 2006)
		Disruption of Hsp90:ARAF interaction	N/A	LUMIER assay in 293T cells	(Taipale et al., 2012)
	BRAF (WT and V600E)	Dose-dependent decrease in BRAF V600E protein levels WT BRAF unaffected by 17-AAG, except in SK-Mel-2 cell line	Inhibition of ERK1/2 signaling 17-AAG treatment inhibits vemurafenib-induced paradoxical ERK1/2 activation in BRAF WT cells	Melanoma cell lines	(Joshi et al., 2018)
		Decreased protein levels	Inhibition of ERK1/2 signaling	HT-29, MCF-7, SK-MEL-28, and A2058 cells	(Fukuyo et al., 2008)
			Inhibition of ERK1/2 signaling		(da Rocha Dias et al., 2005)

(*continued*)

TABLE 1—*Continued*

Drug	Target	Evidence of Regulation	Effect on MAPK Signaling	Model	Reference
		Dose-dependent decrease in BRAF V600E protein levels (proteasome-dependent) BRAF WT and L597V mutant are unaffected or less sensitive to 17-AAG. Mutants V600D and G466V are sensitive to 17-AAG		Multiple cancer cell lines: Colo829, A375, MEL-501, WM266.4, and SK-MEL-2	
		Dose-dependent decrease in BRAF V600E protein levels (proteasome-dependent) Induction of ubiquitination by treatment Disruption of $BRAF^{V600E}$/Hsp90 interaction	BRAF V600E mutation confers sensitivity to 17-AAG. BRAF WT is unaffected. Inhibition of ERK1/2 signaling	SK-Mel-31 and SK-Mel-28 cells in vitro and in vivo	(Grbovic et al., 2006)
		Dose-dependent decrease in BRAF V600E protein levels Dose-dependent decrease in WT BRAF levels (less sensitive than V600E mutant)	Inhibition of ERK1/2 signaling	Uveal melanoma cell lines	(Babchia et al., 2008)
		Decreased protein levels in some patients	Decreased levels of phospho-ERK1/2 in some patients	Patients with melanoma	(Solit et al., 2008)
17DMAG	CRAF	Decreased protein levels (tumor and hepatic tissues)	N/A	MDA-MB-231 xenografts	(Eiseman et al., 2005)
		No consistent changes in protein levels between patients	N/A	Tumor biopsies and PBMCs of patients with advanced cancers	(Ramanathan et al., 2010)
	BRAF (WT and V600E)	Decreased protein levels Disruption of $BRAF^{V600E}$/Cdc37/Hsp90 complexes	Inhibition of ERK1/2 signaling Effects on BRAF V600E protein abundance and ERK1/2 signaling partially rescued by treatment with antioxidant *N*-acetylcysteine	HT-29, MCF-7, SK-MEL-28, and A2058 cells	(Fukuyo et al., 2008)
17-ABAG	CRAF	Dose-dependent decrease in protein levels	N/A	LNCaP cells	(Lin et al., 2015)
IPI-504	CRAF	Decreased protein levels	Inhibition of ERK1/2 signaling	D-54MG and U-251MG cells	(Di et al., 2014)
		Dose-dependent decrease in protein levels	N/A	H1650 cells	(Tillotson et al., 2010)
EC5	CRAF	Decreased protein levels	N/A	HNSCC cell lines	(Yin et al., 2005)
WK-88-1	CRAF	Dose-dependent decrease in protein levels	N/A	MCF-7 and MDA-MB-231 cells	(Zhao et al., 2018)
Herbimycin A	CRAF	Decreased protein levels	N/A	MCF-7 cells	(Schneider et al., 1996)
Purine-based PU-H71	CRAF	Dose-dependent decrease in protein levels	Inhibition of ERK1/2 signaling	Hep3B and HuH7 cells in vitro and in vivo	(Breinig et al., 2009)
		Dose-dependent decrease in protein levels	Inhibition of ERK1/2 signaling	MDA-MB-468 (in vitro and in vivo), MDA-MB-231, MCF-7, and SKBr3 cells	(Caldas-Lopes et al., 2009)
		Dose-dependent decrease in protein levels	N/A	MDA-MB-231 cells	(Azoitei et al., 2012)
		Dose-dependent decrease in protein levels	N/A	A673 cells	(Ambati et al., 2013)
		Dose-dependent decrease in protein levels	N/A	K562 cells	(Taldone et al., 2013)
		Decreased protein levels	N/A	K562 cells	(Moulick et al., 2011)

(*continued*)

TABLE 1—*Continued*

Drug	Target	Evidence of Regulation	Effect on MAPK Signaling	Model	Reference
	ARAF	Decreased protein levels	N/A	K562 cells	(Moulick et al., 2011)
		Disruption of Hsp90:ARAF interaction	N/A	LUMIER assay in 293T cells	(Taipale et al., 2012)
BIIB021	CRAF	Dose-dependent decrease in protein levels	N/A	MCF-7 cells and BT-474 breast tumor model	(Lundgren et al., 2009)
	ARAF	Disruption of Hsp90:ARAF interaction	N/A	LUMIER assay in 293T cells	(Taipale et al., 2012)
CUDC-305	CRAF	Decreased protein levels	Inhibition of ERK1/2 signaling	BT-474 cells H1975, A549, and U87MG xenografts	(Bao et al., 2009a; Bao et al., 2009b)
Radicicol and derivatives; resorcinol-based					
Radicicol	CRAF	Decreased protein levels	Inhibition of KRAS-induced ERK1/2 signaling	KNRK5.2, PSN-1, and SKBr3 cells	(Schulte et al., 1998; Soga et al., 1998)
	ARAF	Disruption of Hsp90:ARAF interaction	N/A	LUMIER Assay in 293T cells	(Taipale et al., 2012)
KF25706	CRAF	Decreased protein levels	Inhibition of KRAS-induced ERK1/2 signaling Impairment of xenograft tumor growth	KRAS-transformed rat NRK cells Human breast cancer xenografts	(Soga et al., 1999)
KF58333	CRAF	Decreased CRAF protein levels	Inhibition of ERK1/2 signaling	K562 cells	(Shiotsu et al., 2000)
AT13387 (onalespib)	CRAF	Dose-dependent decrease in protein levels	Inhibition of ERK1/2 signaling Overcoming of acquired resistance to BRAF and MEK1/2 inhibition	A375, SK-MEL-28, SK-MEL-2, SK-MEL-5, and WM266-4 cells	(Smyth et al., 2014)
	BRAF (WT and V600E)	Dose-dependent decrease in protein levels	Inhibition of ERK1/2 signaling Overcoming of acquired resistance to BRAF and MEK1/2 inhibition	A375, SK-MEL-28, SK-MEL-2, SK-MEL-5, and WM266-4 cells	(Smyth et al., 2014)
STA-9090 (ganetespib)	CRAF	Dose-dependent decrease in protein levels	Inhibition of ERK1/2 signaling Overcoming of acquired resistance to BRAF inhibition	Melanoma cell lines	(Wu et al., 2013b)
		Dose-dependent decrease in protein levels	Inhibition of ERK1/2 signaling	A375 cells	(Acquaviva et al., 2014)
	ARAF	Disruption of Hsp90:ARAF interaction	N/A	LUMIER assay in 293T cells	(Taipale et al., 2012)
	BRAF (WT and V600E)	Dose-dependent decrease in BRAF V600E protein levels	Inhibition of ERK1/2 signaling Overcoming of acquired resistance to BRAF inhibition	Melanoma cell lines	(Wu et al., 2013b)
		Dose-dependent decrease in BRAF V600E protein levels	Inhibition of ERK1/2 signaling Overcoming of intrinsic and acquired resistance to BRAF inhibition	A375 and SK-MEL-2 cell lines Primary melanocytes	(Acquaviva et al., 2014)
NVP-AUY922 (luminespib)	CRAF	Dose-dependent decrease in protein levels	N/A	HCT 116 cells in vitro and in vivo	(Brough et al., 2008)
		Dose-dependent decrease in protein levels	Inhibition of ERK1/2 signaling Synergy with trametinib to induce apoptosis Sensitization of trametinib-resistant cells	H647 and H1944 cells in vitro and in vivo	(Park et al., 2016a)
		Dose-dependent decrease in protein levels	Inhibition of ERK1/2 signaling	U87MG, SF268, SF188, and KNS42 cells	(Gaspar et al., 2010)
		Decreased protein levels	N/A	HCT 116	(Eccles et al., 2008)
	ARAF	Disruption of Hsp90:ARAF interaction	N/A	LUMIER assay in 293T cells	(Taipale et al., 2012)

(*continued*)

TABLE 1—*Continued*

Drug	Target	Evidence of Regulation	Effect on MAPK Signaling	Model	Reference
	BRAF (WT and V600E)	Decreased protein levels of BRAF V600E	Mild effect on ERK1/2 signaling Reactivation of ERK1/2 confers resistance to luminespib in $BRAF^{V600E}$ colon cancer cells	Lim1215, Caco-2, RKO, and WiDr cells	(Wang et al., 2016a)
KW-2478	CRAF	Dose-dependent decrease in protein levels	Inhibition of ERK1/2 signaling	OPM-2/GFP, NCI-H929, RPMI 8226, and U266 cells NCI-H929 xenografts	(Nakashima et al., 2010)
CCT018159	CRAF	Dose-dependent decrease in protein levels	Inhibition of ERK1/2 signaling	SKMEL 28 cells	(Sharp et al., 2007)
VER-49009	CRAF	Dose-dependent decrease in protein levels	N/A	HCT 116 cells	(Dymock et al., 2005)
Monocillin II	CRAF	Dose-dependent decreased protein levels Reduced stability upon treatment (CHX-chase)	Inhibition of MEK/ERK signaling	MDA-MB-231 cells	(Wei et al., 2012)
	ARAF	Dose-dependent decreased protein levels Reduced stability upon treatment (CHX-chase)	Inhibition of MEK/ERK signaling	MDA-MB-231 cells	(Wei et al., 2012)
Benzamide scaffold					
XL888	CRAF	Decreased protein levels	Inhibition of ERK1/2 signaling	BRAF inhibitor–resistant melanoma cell lines	(Paraiso et al., 2012b)
		Decreased protein levels	Inhibition of ERK1/2 signaling XL888 inhibits vemurafenib-induced paradoxical ERK1/2 activation	NRAS mutant melanoma cells and HRAS-transformed NIH 3T3 cells	(Phadke et al., 2015a)
	ARAF	Decreased protein levels	Inhibition of ERK1/2 signaling	BRAF inhibitor–resistant melanoma cell lines	(Paraiso et al., 2012b)
TAS-116	CRAF	Dose-dependent decrease in protein levels	Inhibition of ERK1/2 signaling TAS-116 in combination with dabrafenib or AZD6244 exerts synergistic cytotoxic effects	Multiple myeloma cell lines	(Suzuki et al., 2015)
	BRAF (WT and V600E)	Dose-dependent decrease in protein levels	Inhibition of ERK1/2 signaling TAS-116 treatment in combination with dabrafenib or AZD6244 exerts synergistic cytotoxic effects	Multiple myeloma cell lines	(Suzuki et al., 2015)
N-benzyl benzamide derivatives	CRAF	Dose-dependent decrease in protein levels	N/A	H1975 cells	(Park et al., 2018)
Novobiocin and derivatives					
Novobiocin, chlorobiocin, and coumermycin A1	CRAF	Dose-dependent decrease in protein levels	N/A	SKBr3 cells	(Marcu et al., 2000)
Novobiocin and derivatives	CRAF	Decreased protein levels	N/A	MCF-7 cells	(Le Bras et al., 2007)
Novobiocin, 4TCNA, and 7TCNA derivatives	CRAF	Decreased protein levels	N/A	Multiple cancer cell lines: Caco-2, HT-29, MDA-MB-231, IGROV1, ISHIKAWA, T47D	(Radanyi et al., 2009)
KU135	CRAF	Dose-dependent decrease in protein levels	Inhibition of ERK1/2 signaling	SKMEL28 cells	(Samadi et al., 2011)
	BRAF (WT and V600E)	Dose-dependent decrease in protein levels	Inhibition of ERK1/2 signaling	SKMEL28 cells	(Samadi et al., 2011)

(*continued*)

TABLE 1—*Continued*

Drug	Target	Evidence of Regulation	Effect on MAPK Signaling	Model	Reference
KU363	CRAF	Dose-dependent decrease in protein levels	N/A	MDA-1986 cells	(Cohen et al., 2012)
Others—natural products					
Tubocapsenolide A and others	CRAF	Decreased protein levels	N/A	MDA-MB-231 cells	(Chen et al., 2008; Wang et al., 2012)
Withanolides					
Scalarane Sesterterpenoids	CRAF	Decreased protein levels	N/A	Molt 4 cells	(Lai et al., 2016)
Other chemical classes					
SNX-2112	CRAF	Decreased protein levels	N/A	MCF-7 cells	(Wang et al., 2011)
		Dose-dependent decrease in protein levels	N/A	K562 cells	(Wang et al., 2015)
	ARAF	Disruption of HSP90:ARAF interaction	N/A	LUMIER assay in 293T cells	(Taipale et al., 2012)
	BRAF (WT and V600E)	Dose-dependent decrease in BRAF V600E protein levels	Inhibition of ERK1/2 signaling	A375 cells	(Liu et al., 2012b)
		Dose-dependent decrease in BRAF V600E protein levels	N/A	B16 cells	(Liu et al., 2012a)
5-Aryl-3-thiophen-2-yl-1H-pyrazoles	CRAF	Decreased protein levels	N/A	HepG2 cells	(Mohamady et al., 2020)
ITZ-1	CRAF	Dose-dependent decrease in protein levels	Inhibition of IL-1–induced ERK1/2 signaling	Human articular chondrocytes	(Kimura et al., 2010)
DCZ3112	CRAF	Dose-dependent decrease in protein levels	Cotreatment with trastuzumab inhibits ERK1/2 signaling	SK-BR-3, BT-474, and MDA-MB-231 cells in vitro and in vivo	(Chen et al., 2018)
DCZ5248	CRAF	Dose-dependent decrease in protein levels (proteasome-dependent)	N/A	HCT116 cells	(Chen et al., 2021)
BDP	CRAF	Dose-dependent decrease in protein levels	N/A	MDA-MB-231 cells	(Oh and Seo, 2017)
DPide	CRAF	Dose-dependent decrease in protein levels	N/A	MDA-MB-231 cells	(Oh et al., 2018)
FW-04-806	CRAF	Dose-dependent decrease in protein levels	N/A	SKBR3, MCF-7 in vitro and in vivo	(Huang et al., 2014)
NVP-BEP800	CRAF	Decreased protein levels	Inhibition of ERK1/2 signaling	MCF-7 and MDA-MB-231 cells	(Massey et al., 2010)
	ARAF	Disruption of Hsp90:ARAF interaction	N/A	LUMIER assay in 293T cells	(Taipale et al., 2012)
	BRAF (WT and V600E)	Dose-dependent decrease in protein levels	Inhibition of ERK1/2 signaling	A375 cells in vitro and in vivo	(Massey et al., 2010)

HER2, human epidermal growth factor receptor-2; HRAS, Harvey rat sarcoma virus; IL-1β, Interleukine 1β; LUMIER, LUminescence-based Mammalian intERactome; N/A, not applicable; NRAS, neuroblastoma rat sarcoma virus; PBMC, peripheral blood mononuclear cell; PMA, phorbol myristate acetate; WT, wild type.

kinase domain (Taipale et al., 2012). Binding to Hsp90 in association with the cochaperone Cdc37 was shown to promote CRAF autophosphorylation on Ser 621, leading to recruitment of 14-3-3 proteins and stabilization of the CRAF protein (Noble et al., 2008; Mitra et al., 2016). The non–Ser 621-phosphorylated form of CRAF is improperly folded and is thus ubiquitinated and targeted for degradation (Noble et al., 2008). Consistent with this observation, binding of CRAF to glutathione-*S*-transferase P1 induces the stabilization, dimerization, and catalytic activation of CRAF, establishing an autocrine signal loop that sustain proliferation of oncogenic *KRAS* and *BRAF* cells (Niitsu et al., 2020). Interestingly, quality control ubiquitination of CRAF seems to rely only partially on CHIP (Noble et al., 2008). Various degrees of evidence indicate that other E3 ubiquitin ligases, such as C-terminal to LisH (CTLH) complex, HECT, UBA, and WWE Domain Containing E3 Ubiquitin Protein Ligase 1 (HUWE1), HECTD3 and HECT and RLD Domain Containing E3 Ubiquitin Protein Ligase Family Member 1 (HERC1), also regulate CRAF ubiquitination and degradation (Atabakhsh and Schild-Poulter, 2012; Jang et al., 2014; Li et al., 2017; Schneider et al., 2018; McTavish et al., 2019). In addition, members of the inhibitor of apoptosis protein (IAP) family, c-IAP, XIAP, and ML-IAP, have been shown to promote the ubiquitination of CRAF, although their impact on CRAF degradation remains controversial (Dogan et al., 2008; Oberoi-Khanuja et al., 2012; Fadó et al., 2013). The interplay between these E3 ligases and the Hsp90/CHIP quality control machinery, as well as the physiologic conditions under which they are activated, remains to be fully elucidated (Fig. 3).

Although there is significantly less literature focusing on ARAF regulation by protein turnover than on CRAF regulation, ARAF was shown to be destabilized in response to Hsp90 chemical inhibition, similar to CRAF (Table 1). Moreover, large-scale quantitative analysis of Hsp90 clients showed that treatment with inhibitors of Hsp90, including 17-AAG, disrupts the interaction between ARAF and Hsp90 (Taipale et al., 2012).

In contrast to ARAF and CRAF, wild-type BRAF does not interact with Hsp90 and was shown to be less or not sensitive to Hsp90 chemical inhibition (da Rocha Dias et al., 2005; Grbovic et al., 2006). However, the $BRAF^{V600E}$ mutant, which has elevated kinase activity and accounts for approximately 80% of BRAF mutations found in cancers, has been shown to interact with Hsp90 (da Rocha Dias et al., 2005; Grbovic et al., 2006). Interestingly, $BRAF^{V600E}$ shares regulatory features with classic Hsp90 client proteins—notably, its ubiquitination and protein abundance are regulated by Cullin 5-RING ligase (CRL5) complexes (Samant et al., 2014). In addition to $BRAF^{V600E}$, mutant v-SRC, KIT proto-oncongene, and p53 share this dependence on Hsp90, which is not foreseen for the corresponding wild-type proteins (Peng et al., 2001; Bauer et al., 2006; Boczek et al., 2015). In the earlier reports describing $BRAF^{V600E}$ dependence on Hsp90, the authors reported that wild-type BRAF is stable in unperturbed cells (da Rocha Dias et al., 2005; Grbovic et al., 2006). However, various later studies demonstrated that BRAF is also regulated by its stability. The E3 ligases tumor necrosis factor (TNF) receptor associated factors 2 and RING finger 149 have been shown to target BRAF for proteasomal degradation, therefore inhibiting ERK1/2 signaling and cell survival and proliferation, respectively, in non–small cell lung cancer (NSCLC) and colorectal cancer cells (Hong et al., 2012; Wang et al., 2018a). In addition, the E3 ligase complex anaphase-promoting complex (APC)/CDC20 homolog 1 (CDH1) APC^{CDH1} was recently reported to regulate BRAF protein abundance in primary fibroblasts and melanoma cells (Wan et al., 2017). Interestingly, this pro-

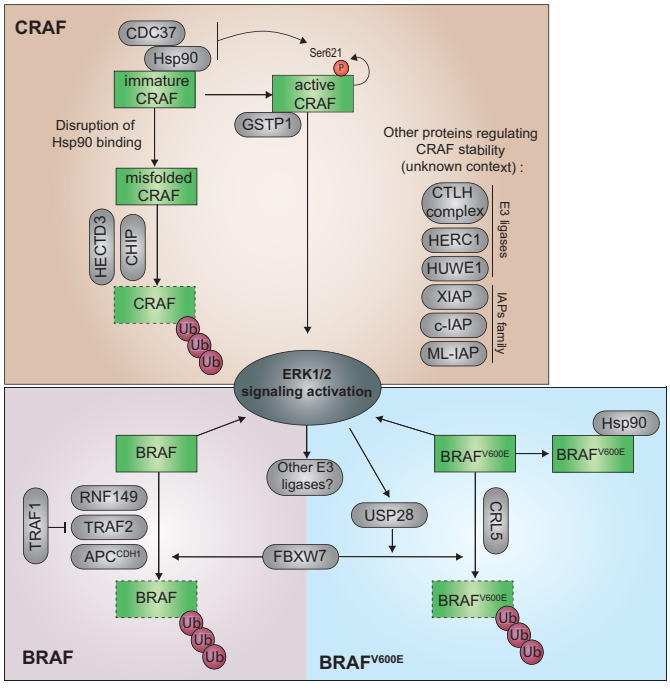

Fig. 3. Regulation of BRAF and CRAF protein stability. ERK1/2 MAPK signaling is actively regulated by the control of BRAF and CRAF stability. Interaction with the molecular chaperone Hsp90 ensures CRAF proper folding and protects CRAF from ubiquitination and subsequent proteasomal degradation. Hsp90, in association with Cdc37, also promotes CRAF autophosphorylation on Ser 621, leading to its stabilization and activation of ERK1/2 signaling. BRAF is targeted for degradation after ubiquitination by different E3 ligases such as RING finger 149 (RNF149), TRAF2, and APC^{CDH1}. $BRAF^{V600E}$ mutant is targeted for proteasomal degradation by CRL5 and $CRL1^{FBXW7}$ E3 ligase complexes. Contrary to wild-type BRAF, oncogenic $BRAF^{V600E}$ binds to Hsp90, which protects it from degradation. See text for further description. GSTP1, glutathione-*S*-transferase P1; HUWE1, HECT, UBA, and WWE Domain Containing E3 Ubiquitin Protein Ligase 1; HERC1, HECT and RLD Domain Containing E3 Ubiquitin Protein Ligase Family Member 1; Ub, Ubiquitin.

cess seems to be disrupted in cancer cells as a result of ERK1/2 and cyclin D/CDK4 hyperactivation, which leads to the suppression of APC^{CDH1} activity. Finally, the E3 ligase complex $CRL1^{FBXW7}$ [also denoted Skp, Cullin, F-box containing complex/F-box and WD repeat domain containing 7 (FBXW7) SCF^{FBXW}] promotes the ubiquitination and degradation of both wild-type BRAF and $BRAF^{V600E}$ in cancer cells (Saei et al., 2018; Yeh et al., 2020). Several FBXW7 mutations found in T-cell leukemia patients were shown to disrupt the ability of FBXW7 to induce BRAF ubiquitination and degradation. In *Caenorhabditis elegans*, abnormal cell LINeage 45 (LIN-45), an ortholog of BRAF, is a substrate for the E3 ubiquitin ligase suppressor and/or enhancer of lin-12 10 (SEL-10), which is an ortholog of FBXW7 (de la Cova and Greenwald, 2012). In melanoma cells, pharmacological inhibition of ERK1/2 activity with the BRAF inhibitor vemurafenib upregulates expression of the DUB ubiquitin-specific protease (USP) 28, a positive regulator of FBXW7 protein levels, forming a feedback loop that negatively regulates ERK1/2 signaling (Saei et al., 2018). Loss of USP28, which is observed in ~9% of patients with melanoma, stabilizes BRAF, enhances ERK1/2 activation, and confers resistance to RAF inhibitor therapy. However, another study suggested the existence of a negative feedback loop by which hyperactivation of ERK1/2 leads to destabilization of BRAF protein by a mechanism dependent on the proteasome but independent of FBXW7 (Hernandez et al., 2016). The E3 ligase involved was not identified (Fig. 3). Moreover, RAF kinases are upregulated via protein stabilization during monocyte-derived dendritic cell differentiation by unknown mechanisms (Riegel et al., 2020).

b. Tumor progression locus 2 (mitogen-activated protein kinase kinase kinase 8). The MAP3K tumor progression locus 2 (TPL2), also known as COT kinase, phosphorylates and activates MEK1/MEK2 mainly upon stimulation of receptors of the innate immune system (Patriotis et al., 1994; Salmeron et al., 1996; Chiariello et al., 2000; Dumitru et al., 2000). Activation of TPL2 can promote resistance to RAF pharmacological inhibition by reactivating ERK1/2 MAPKs through a MEK1/2-dependent mechanism (Johannessen et al., 2010). TPL2 protein stability is regulated by two opposing mechanisms. Activated TPL2 is exported to the nucleus, where it is ubiquitinated by an unidentified E3 ligase and degraded by the proteasome (Collins et al., 2019). In addition, TPL2 binds stoichiometrically to NF-κB subunit p105, which leads to the stabilization of both proteins (Belich et al., 1999; Beinke et al., 2003). The TPL2/p105 complex interacts with A20-binding inhibitor of NF-κB 2 (ABIN-2), forming a ternary complex that further stabilizes TPL2 (Lang et al., 2004). Depletion of ABIN-2 leads to a marked decrease in TPL2 protein abundance but not p105 abundance. However, in cells deficient in p105, ABIN-2 protein levels are substantially reduced since the majority of ABIN-2 is associated with p105 (Lang et al., 2004). Therefore, whether the stabilizing effect of p105 on TPL2 is due to their interactions or is a consequence of ABIN-2 recruitment to the complex is unclear. However, both p105 and ABIN-2 are required for stable expression of TPL2 and for optimal activation of ERK1/2 signaling in innate immunity (Waterfield et al., 2003; Papoutsopoulou et al., 2006). Mechanistically, the TPL2-ERK1/2 pathway is activated by two independent regulatory steps involving the inhibitor κB kinase complex: phosphorylation of NF-κB p105 to induce p105 proteolysis, releasing TPL2 from p105 and ABIN-2, and phosphorylation of TPL2 on Ser 400, which is required for MEK1/2 phosphorylation and activation (Gantke et al., 2011; Roget et al., 2012). Unbound TPL2 also interacts with B-cell lymphoma 3 (BCL-3), increasing its export to the nucleus, where it is actively degraded by the proteasome (Collins et al., 2019). Consequently, BCL-3 acts as a negative regulator of ERK1/2 signaling in innate immunity. Interestingly, overexpression of p105 counteracts BCL-3–dependent regulation of TPL2, highlighting the competitive nature of these mechanisms in controlling TPL2 levels and orchestrating temporal regulation of ERK1/2 signaling in innate immune responses (Fig. 4).

c. MOS. The MAP3K V-Mos Moloney Murine Sarcoma Viral Oncogene Homolog (MOS) controls ERK1/2 signaling during meiosis (Sun et al., 1999). MOS activates ERK1/2 MAPKs by directly phosphorylating and activating MEK1/MEK2 and by inhibiting a MAPK phosphatase (Verlhac et al., 2000). Work performed in *Xenopus laevis* oocytes revealed that the expression of MOS protein is dynamically controlled by the UPS. In the earliest stages of meiosis, MOS is a highly unstable protein with a half-life of 15–20

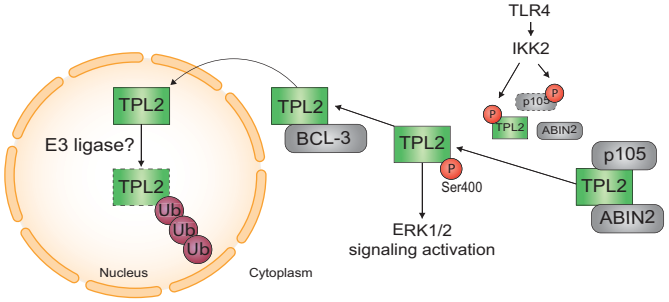

Fig. 4. Regulation of TPL2 stability. The TPL2 protein is stabilized in a ternary complex formed with the NF-κB subunit p105 and ABIN-2. After p105 and TPL2 phosphorylation by inhibitor kappa B kinase 2 (IKK2), TPL2 is released from p105 and ABIN-2, leading to ERK1/2 pathway activation. When uncomplexed from ABIN-2 and p105, TPL2 binds to BCL-3, promoting its import in the nucleus, where TPL2 is ubiquitinated and degraded. Ub, Ubiquitin.

minutes (Watanabe et al., 1989; Nishizawa et al., 1992). After germinal vesicle breakdown, MOS becomes phosphorylated and is stabilized. High protein levels of MOS induce ERK1/2 pathway activation, which is necessary to maintain meiotic metaphase arrest (Haccard et al., 1993; Colledge et al., 1994). After egg fertilization, MOS is targeted for degradation by the proteasome (Watanabe et al., 1991; Nishizawa et al., 1993).

The detailed molecular mechanism regulating MOS stability has been the subject of studies with contradictory findings. Initially, the MOS N-terminal proline (Pro 2) has been proposed to target the protein for degradation by the N-end rule pathway (Nishizawa et al., 1992, 1993). Beyond this "second-codon rule," these studies also stated that phosphorylation of Ser 3 is essential for maintaining MOS stability, probably by preventing recognition of MOS N terminus by an E3 ligase. MOS Ser 3 was later shown to be phosphorylated by cyclin B/CDK1 (Castro et al., 2001). Upon fertilization of *X. laevis* oocytes, cyclin B is degraded, and MOS is no longer phosphorylated on Ser 3, leading to its ubiquitination on Lys 34 and subsequent proteasomal degradation (Nishizawa et al., 1992; Castro et al., 2001). Thus, these studies suggest that the N-terminal degradation signal in MOS is composed of two components: the N-terminal proline residue and the unphosphorylated Ser 3 residue. However, this model conflicts with the literature on the N-end rule pathway, which shows that the N-terminal proline residue has a stabilizing effect on proteins (Bachmair and Varshavsky, 1989; Gonda et al., 1989). A subsequent study by the Varshavsky group demonstrated that Pro 2 is not a direct signal for degradation but rather indirectly induces MOS destabilization by negatively regulating the phosphorylation of Ser 3 (Sheng et al., 2002). This report was the first demonstration of a substrate of the N-end rule pathway whose degradation is dependent on the phosphorylation of its N-degron.

2. Mitogen-Activated Protein Kinase Kinases MEK1 (Mitogen-Activated Protein Kinase Kinase 1) and MEK2 (Mitogen-Activated Protein Kinase Kinase 2). MEK1 and MEK2 are the MAP2Ks of the ERK1/2 MAPK pathway. Few studies have specifically investigated the regulation of MEK1 and MEK2 protein stability. However, some reports suggest that MEK2, but not MEK1, expression is regulated by the UPS. Activation of the ERK1/2 pathway induces a feedback mechanism leading to a decrease in MEK2 protein level independent of its mRNA expression that is reversed by proteasome inhibition (Hong et al., 2015). Conversely, in the same model, MEK1 expression was found to be upregulated at both the mRNA and protein levels. More recently, the DUB USP21 has been reported to deubiquitinate and stabilize MEK2 in hepatocarcinoma cells, thereby increasing ERK1/2 activity and stimulating cell proliferation (Li et al., 2018). In addition, treatment of unstimulated LNCaP human prostate cancer cells with the proteasome inhibitor MG132 leads to an increase in MEK2 protein expression (Hong et al., 2015). A systemic search of CRL substrates by chemical inhibition and by expression of a dominant-negative form of CRL1 suggested that inactivation of CRL complexes leads to the stabilization of MEK2 (Emanuele et al., 2011). Finally, a large-scale study of protein stability suggested that MEK2 has a low protein stability index (Yen et al., 2008). However, other studies have reported that MEK2 is a stable protein. For instance, cycloheximide-chase experiments showed that MEK2 has a half-life of 12–14 hours in several in vitro cell culture models (Li et al., 2018; Ordan et al., 2018). In addition, treatment of mouse bone marrow–derived macrophages (BMDMs), mouse embryonic fibroblasts (MEFs), NIH 3T3 cells, human fibrosarcoma HT-1080 cells, and human umbilical vein endothelial cells with MG132 did not affect MEK2 protein expression (Shibata et al., 2002, 2003; Beinke et al., 2004; Cirit et al., 2012). Thus, regulation of MEK2 by the UPS appears to be dependent on the cell type and cellular context. Although no study has suggested that wild-type MEK1 is regulated by the UPS, the MEK1 Q56P and MEK2 Q60P mutants seem to be more stable than the corresponding wild-type proteins, leading to an increase in their abundances (Ordan et al., 2018).

The regulation of MEK2 protein stability and the pathophysiological relevance of this regulation in the control of ERK1/2 signaling requires additional characterization. In addition, MEK1 and MEK2 appear to be differentially regulated by the UPS. MEK1 and MEK2 share approximately 85% amino acid sequence identity, and their least similar region is a proline-rich region situated in the N terminus. The structural differences that could dictate the apparent differential regulation of MEK1 and MEK2 turnover remain to be studied.

3. Mitogen-Activated Protein Kinases Extracellular Signal–Regulated Kinase 1 (Mitogen-Activated Protein Kinase 3) and Extracellular Signal–Regulated Kinase 2 (Mitogen-Activated Protein Kinase 1). The MAPKs ERK1 and ERK2 are stable proteins that are not subject to regulation by the UPS in unstimulated cells. For example, in HEK293 cells, ERK1 protein levels are not modified after inhibition of protein synthesis with cycloheximide or after proteasome inhibition (Coulombe et al., 2003). In rat vascular smooth muscle cells, murine hematopoietic FL5.12 cells, human LNCaP cells, and glioblastoma U87 and A-172 cells, proteasome inhibition with MG132 did not affect ERK1 and ERK2 protein expression (Jiang et al., 2004; McCubrey et al., 2008; Hong et al., 2011; Ko

et al., 2011). Under conditions of hyperosmotic stress induced by sorbitol, ERK1/2 have been reported to be ubiquitinated and targeted for degradation by MEK kinase (MEKK) 1, which contains a plant homeodomain domain with E3 ubiquitin ligase activity (Lu et al., 2002). However, more recent studies reported that ERK1/2 protein levels were unchanged upon sorbitol treatment (Maruyama et al., 2010; Blessing et al., 2014; Charlaftis et al., 2014). Moreover, the stability of ERK1/2 proteins is not differentially modulated in embryonic stem cells isolated from wild-type MEKK1 or ubiquitin ligase–deficient MEKK1 mutant mice (Charlaftis et al., 2014). Other experimental conditions have been shown to impact the stability of ERK1/2 proteins. For example, ectopic expression of the severe acute respiratory syndrome coronavirus (SARS-CoV) papain-like protease appears to regulate the protein abundance of ERK1 but not ERK2 in a UPS-dependent manner (Li et al., 2011). In addition, nitration of Tyr 210 was proposed to induce the ubiquitination and degradation of ERK1 by a mechanism dependent on the quality control E3 ligase CHIP (Zhang et al., 2019). However, whether these regulatory mechanisms operate under physiologic conditions remains to be established.

Interestingly, the DUB USP47 was identified as a post-translational activator of MAPK signaling in *Drosophila* by positively regulating the stability and abundance of Rolled, the ortholog of ERK1/2 MAPKs (Ashton-Beaucage et al., 2014, 2016). Moreover, components of the N-end rule pathway (ubiquitin-conjugating enzyme E2 6, ubiquitin fusion degradation protein 4, purity of essence (POE) and the associated E3 ubiquitin ligase Potassium Channel Modulatory Factor 1 were identified as regulators of Rolled ubiquitination and proteasomal degradation, therefore counteracting the function of USP47 (Ashton-Beaucage et al., 2016). Despite this regulatory mechanism, Rolled MAPK was found to be a stable protein in unstimulated cells, with a half-life of approximately 13 hours. The existence of UPS-dependent mechanisms that actively promote the degradation of mammalian ERK1/2 MAPKs in response to specific cellular stimuli to control their activity and functions remains to be demonstrated.

B. The p38 and c-Jun N-Terminal Kinase Signaling Pathways

The p38 and JNK MAPK pathways are activated mainly by environmental stresses and proinflammatory cytokines and share many core components and upstream regulators (Fig. 1). These signaling pathways play important functions in neuronal development, innate and adaptive immunity, inflammation, and metabolism (Weston and Davis, 2007; Rincon and Davis, 2009; Cuadrado and Nebreda, 2010; Kyriakis and Avruch, 2012; Arthur and Ley, 2013).

1. Mitogen-Activated Protein Kinase Kinase Kinases. a. MEKK1 (Mitogen-Activated Protein Kinase Kinase Kinase 1), MEKK2 (Mitogen-Activated Protein Kinase Kinase Kinase 2), MEKK3 Mitogen-Activated Protein Kinase Kinase Kinase 3), and MEKK4 (Mitogen-Activated Protein Kinase Kinase Kinase 4). MEKK1–4 are a group of MAP3Ks that activate the JNK and p38 pathways via the phosphorylation and activation of MKK4/MKK7 and MKK3/MKK6 MAP2Ks, respectively (Kyriakis and Avruch, 2012). MEKK1–4 act as nonredundant signaling hubs that regulate discrete physiologic responses required for normal embryonic development and stress responses (Uhlik et al., 2004; Abell et al., 2005). Recent genomic studies have documented that *MAP3K1* and *MAP3K4* genes are frequently altered in certain cancers, suggesting a possible tumor-suppressive role in these malignancies (Pham et al., 2013; Yang et al., 2015; Kwong et al., 2020). Information on the regulation of MEKK1–4 by protein turnover is sparse. In resting T cells, the MEKK1 C-terminal domain is constitutively ubiquitinated by the E3 ligase Deltex, which promotes the degradation of MEKK1 by the proteasome (Liu and Lai, 2005). Consequently, Deltex downregulation after T-cell stimulation suppresses MEKK1 degradation and triggers the activation of p38 and JNK signaling responses.

In osteoblasts, MEKK2 is ubiquitinated by the E3 ubiquitin ligase SMAD specific E3 ubiquitin protein ligase 1 (SMURF1) and targeted for proteasomal degradation (Yamashita et al., 2005). As a result, in SMURF1-deficient mice, JNK signaling increases in osteoblasts due to MEKK2 accumulation, leading to an increase in osteoblast activity and dysregulation of bone homeostasis (Yamashita et al., 2005). More recently, the interaction of SMURF1 with nuclear DumbBell Former 2-related kinase 2 (NDR2), a kinase in the nuclear DumbBell Former 2-related/large tumor suppressor family, has been shown to promote K48-linked ubiquitination of MEKK2 and to negatively regulate interleukin-17–induced inflammation in HeLa cells (Ma et al., 2019).

To our knowledge, no data suggesting that MEKK3 or MEKK4 is regulated by protein turnover have been published in the literature. Treatment of BMDMs with MG132 did not affect the expression of MEKK3 protein (Zhou et al., 2013), and large-scale proteomic analysis of protein stability suggested that MEKK3 is a highly stable protein (Yen et al., 2008).

b. Apoptosis signal-regulating kinase 1 (mitogen-activated protein kinase kinase kinase 5), apoptosis signal-regulating kinase 2 (mitogen-activated protein kinase kinase kinase 6), and apoptosis signal-regulating kinase 3 (mitogen-activated protein kinase kinase kinase 15). Apoptosis signal-regulating kinase (ASK) 1 is an MAP3K for the MKK3/MKK6-p38 and MKK4/MKK7-JNK MAPK pathways that is activated

by oxidative stress, endoplasmic reticulum stress, and inflammatory cytokines. It is involved in death receptor–mediated apoptosis (Ichijo et al., 1997). Several mechanisms regulating ASK1 protein stability, depending on the cellular context, have been described. The redox sensing oxidoreductase thioredoxin (Trx) has been shown to form a complex with ASK1 and to inhibit its kinase activity, although the mechanism of inhibition was not determined (Saitoh et al., 1998). A subsequent study showed that Trx promotes the ubiquitination and degradation of ASK1 in endothelial cells (Liu and Min, 2002). Consistent with this finding, treatment with MG132 increases ASK1 protein expression in unstressed endothelial cells (He et al., 2006). Upon oxidative stress or treatment with TNFα, Trx dissociates from ASK1, leading to activation of ASK1-dependent JNK signaling (Liu and Min, 2002) (Fig. 5). However, treatment with AGI-1067, a small molecule that prevents lipopolysaccharide (LPS)-induced dissociation of ASK1 from Trx, has little to no impact on ASK1 protein expression (Zheng et al., 2015). In addition, treatment with the methyl carbamoylating agent 1,2-bis(methylsulfonyl)-1-[(methylamino)carbonyl]hydrazine (101MDCE) or the cyclodextrin-derived diorganyl telluride 6-(4-N,N-Dimethylaminophenyltelluro)-6-deoxy-β-cyclodextrin (DTCD) induce the dissociation of ASK1 from Trx but do not modulate ASK1 protein abundance (Ji et al., 2014). It is important to mention that the experiments in the latter studies used short-term treatments of 1 hour, and ASK1 half-life has been reported to range from 70 minutes for endogenous ASK1 to 8 hours for ectopically expressed ASK1 (Hwang et al., 2005; Nagai et al., 2009; Maruyama et al., 2014). Consequently, the chemical modulation of Trx/ASK1 interaction and the impact of this modulation on ASK1 stability and expression warrants further investigation.

Suppressor of cytokine signaling 1 (SOCS1) is a substrate receptor of CRL5 E3 ligases (Okumura et al., 2016). In unstimulated endothelial cells, SOCS1 forms a complex with Tyr 718–phosphorylated ASK1, leading to its ubiquitination and degradation (He et al., 2006). Similar to the dynamic regulation of the Trx/ASK1 interaction, the SOCS1/ASK1 complex is disrupted by TNFα stimulation. Mechanistically, TNFα stimulation induces dephosphorylation of ASK1 on Tyr 718 by the phosphatase Src homology 2 domain–containing protein tyrosine phosphatase-2, leading to dissociation of ASK1 from SOCS1 and reduced ubiquitination of ASK1 (Yu et al., 2009) (Fig. 5). Consequently, the ASK1-JNK signaling pathway is overactivated in SOCS1-deficient endothelial cells, both basally and in response to TNFα stimulation, leading to an increase in TNFα-induced inflammatory and apoptotic responses (He et al., 2006). Notably,

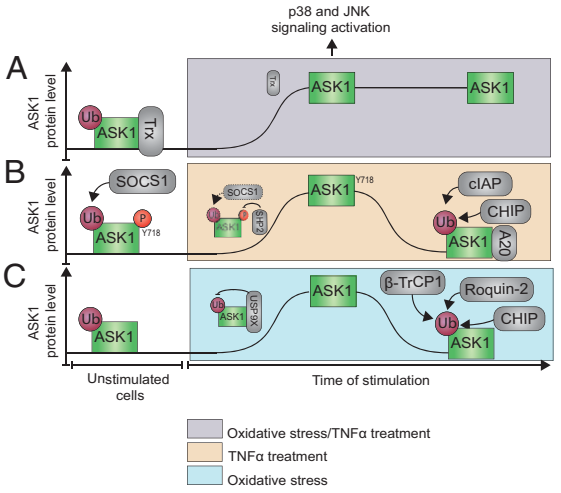

Fig. 5. Temporal regulation of p38 and JNK signaling by ASK1 protein stability. ASK1 degradation by the UPS plays an essential role in the temporal control of p38 and JNK MAPK signaling. In resting cells, ASK1 forms a complex with the redox sensor Trx, promoting its ubiquitination and degradation by the proteasome. (A) TNFα treatment or oxidative stress triggers oxidation of Trx and its dissociation from ASK1, leading to an increase in ASK1 stability and ASK1-induced activation of p38 and JNK signaling. (B) In resting endothelial cells, SOCS1 interacts with Tyr 718–phosphorylated ASK1, promoting its ubiquitination and degradation. TNFα stimulation increases ASK1 protein stability by inducing Tyr 718 dephosphorylation by Src homology 2 domain–containing protein tyrosine phosphatase-2 (SHP2), which impedes ASK1 ubiquitination by SOCS1. A negative feedback loop terminates ASK1 signaling after TNFα stimulation by inducing its ubiquitin-mediated degradation. (C) In response to oxidative stress, ASK1 is deubiquitinated by USP9X, leading to its accumulation and activation of p38 and JNK signaling. Long-term oxidative stress eventually restores the steady-state activity p38 and JNK by inducing the degradation of ASK1.

sustained TNFα stimulation causes the formation of a negative feedback loop to downregulate ASK1 signaling. Several hours after TNFα stimulation, total ASK1 protein levels are lower than that in unstimulated cells. cIAP1 was identified as an E3 ligase that binds to and ubiquitinates ASK1, and deficiency of cIAP1 prevents ASK1 downregulation and prolongs p38 and JNK MAPK signaling (Zhao et al., 2007). Overexpression of CHIP and Hsp70 was also reported to induce the ubiquitination and degradation of ASK1, resulting in a reduction in JNK activity (Hwang et al., 2005; Gao et al., 2010a). Treatment with TNFα stimulates the binding of CHIP and Hsp70 to ASK1, and increased expression of CHIP and Hsp70 inhibits TNFα-induced apoptosis (Gao et al., 2010a). Notably, overexpression of cIAP1 and CHIP promotes the ubiquitination and degradation of ASK1 even in unstimulated cells (Zhao et al., 2007; Gao et al., 2010b). TNFα stimulation also induces expression of the ubiquitin-editing enzyme A20. Overexpressed A20 was shown to interact with ASK1 and promote its ubiquitin-dependent degradation, thereby suppressing JNK signaling and apoptosis (Won et al., 2010). Thus, regulation of ASK1 stability by TNFα involves multiple mechanisms that may operate in a cell type- and context-dependent manner.

In several cell lines, induction of oxidative stress by treatment with H_2O_2 induces recruitment of the DUB USP9X to ASK1-containing protein complexes, termed the ASK1 signalosome (Nagai et al., 2009). USP9X then deubiquitinates ASK1, leading to stabilization of the protein and to oxidative stress–induced activation of the JNK and p38 MAPK pathways (Fig. 5). Thus, active deubiquitination of ASK1 cooperates with the disruption of destabilizing protein interactions to increase ASK1 protein levels in response to oxidative stress. After this initial activation step, long-term oxidative stress ultimately induces degradation of ASK1. Studies have described a role for the E3 ligases CHIP and Roquin-2 in oxidative stress–induced ubiquitination of ASK1 in various cell lines (Hwang et al., 2005; Maruyama et al., 2014). Consequently, depletion of CHIP increases JNK signaling after H_2O_2 treatment, and depletion of Roquin-2 induces sustained activation of JNK and p38 signaling under similar experimental conditions (Fig. 5). This mechanism is evolutionarily conserved, as orthologs of Roquin-2 and ASK1 in *C. elegans* show a similar epistatic relationship in response to bacterial infection (Maruyama et al., 2014). The molecular basis of the oxidative stress–dependent ubiquitination of ASK1 by CHIP and Roquin-2 remains to be defined. One study reported that β-arrestins bind to ASK1 in response to H_2O_2 and act as a scaffold to recruit CHIP and thus promote ASK1 ubiquitination and degradation (Zhang et al., 2009). Notably, Roquin-2 and CHIP also promote the degradation of ASK1 in unstimulated cells when overexpressed (Hwang et al., 2005; Maruyama et al., 2014). Consistent with this observation, ASK1 protein levels are elevated in hepatocytes from CHIP-deficient mice (Kim et al., 2016). However, little is known about the UPS components regulating ASK1 protein abundance under steady-state conditions. Immunoprecipitation-mass spectrometry analysis of ASK1 interactome allowed the identification of β-transducin repeat-containing protein 1 (β-TrCP1) as a novel partner of the ASK1 signalosome. Interestingly, depletion of β-TrCP1 in unstimulated HeLa and HEK293 cells increased ASK1 protein levels, suggesting that $CRL1^{\beta TrCP}$ acts as an E3 ligase to controls ASK1 levels under steady-state conditions (Cheng et al., 2018). The same study showed that, under oxidative stress, ASK1 is polyubiquitinated by β-TrCP1 and degraded by the proteasome, resulting in a reduction of caspase 3–dependent cell death. Whether the transient accumulation of ASK1 during oxidative stress is due to competition between prodegradation signals from CHIP, Roquin-2, and β-TrCP1 and counteracting stabilization by USP9X remains to be determined.

In addition to these well studied mechanisms of ASK1 regulation by oxidative stress and TNFα signaling, other studies have suggested additional modes of ASK1 regulation. For example, ectopic expression of the active form of the G protein Gα13 was reported to stabilize and increase the level of ASK1 protein by disrupting the interaction between ASK1 and CHIP (Kutuzov et al., 2007). This observation is consistent with previous studies reporting that exogenous expression of Gα12 and Gα13 stimulates ASK1-dependent JNK signaling (Berestetskaya et al., 1998). Also, treatment of differentiated U937 cells with the antibiotic minocycline reduces the intracellular levels of ASK1, resulting in suppression of p38 and JNK MAPK activation (Follstaedt et al., 2008). However, it is not known if this effect occurs through direct regulation of ASK1 stability, and ASK1 is not upregulated after treatment with minocycline in vivo.

ASK2 and ASK3 are two additional members of the ASK family that are closely related to ASK1 but are less well studied (Wang et al., 1998; Kaji et al., 2010). ASK2 heterodimerizes with ASK1 to activate the JNK pathway. ASK2 levels are controlled by proteasomal degradation, and interaction with ASK1 has been demonstrated to stabilize ASK2 expression (Takeda et al., 2007). However, there is no information on the regulation of ASK2 by cellular stresses that modulate ASK1 stability and expression. There is no report about the regulation of ASK3 protein stability.

c. Mixed lineage kinase 1 (mitogen-activated protein kinase kinase kinase 9), mixed lineage kinase 2 (mitogen-activated protein kinase kinase kinase 10), mixed lineage kinase 3 (mitogen-activated protein kinase kinase kinase 11), and mixed lineage kinase 4 (mitogen-activated protein kinase kinase kinase 21). Mixed lineage kinases (MLKs) are MAP3Ks that potently activate JNK and p38 MAPKs and that have been associated to multiple diseases, including inflammatory, neurologic, and metabolic disorders (Craige et al., 2016). MLK1–4 can also directly phosphorylate MEK1/2 to activate the ERK1/2 pathway independently of RAF and mediate resistance to RAF inhibitors (Marusiak et al., 2014). Very few studies have investigated the regulation of MLK1 and MLK2 protein turnover. However, one study characterizing p38 and JNK MAPK signaling during *C. elegans* axonal regeneration reported that the MLK1 ortholog is targeted for degradation by the E3 ubiquitin ligase regulator of presynaptic morphology 1 (RPM-1) (Nix et al., 2011).

MLK3 protein levels are upregulated in breast and ovarian cancer cells (Chen et al., 2010; Zhan et al., 2012). MLK3 is a client protein for Hsp90/Cdc37 in breast and colorectal cancer cells. Pharmacological inhibition of Hsp90 leads to a decrease in MLK3 protein abundance (Zhang et al., 2004; Haupt et al., 2012; Blessing et al., 2014). Mechanistically, dissociation of MLK3/Hsp90 complex triggers the association

of MLK3 with Hsp70, the E3 ligase CHIP, and members of the UBC4/5 homolog 5 E2 family, resulting in proteasomal degradation of MLK3 (Blessing et al., 2014). In addition to Hsp90 inhibition, heat shock and hyperosmotic stress induce a decrease in MLK3 protein abundance that is abrogated by genetic depletion of CHIP (Blessing et al., 2014). In resting HEK293 cells, ectopically expressed MLK3 undergoes K48-linked polyubiquitination, suggesting that MLK3 levels are regulated by proteasomal degradation in unstimulated cells (Korchnak et al., 2009). The functional consequences of these regulatory mechanisms on the functions of MLK3 have not been extensively studied. However, CHIP appears to inhibit JNK signaling in response to heat shock and osmotic stress in HEK293 cells (Blessing et al., 2014).

MLK4 is a less well studied member of the MLK family that primarily activates the MKK4/7-JNK and MEK1/2-ERK1/2 MAPK pathways, with little or no impact on p38 (Marusiak et al., 2016). Interestingly, MLK4 is also regulated by Hsp90 and the E3 ligase CHIP (Blessing et al., 2017). Cellular stresses such as heat shock or hyperosmotic environments result in dissociation of the MLK3/MLK4β complex, leading to the activation of MLK3 and the subsequent degradation of MLK3 and MLK4β.

*d. Transforming growth factor-*β*–activating kinase 1 (mitogen-activated protein kinase kinase kinase 7).* Transforming growth factor-β–activating kinase 1 (TAK1) is an MAP3K that activates p38 and JNK MAPK signaling, notably during inflammation, stress, and DNA damage (Xu and Lei, 2021). TAK1-dependent signaling can be activated by TNFα or genotoxic stress induced by doxorubicin. Interestingly, in both cellular contexts, TAK1 is degraded during the late stage of its activation (Fan et al., 2010, 2012; Liang et al., 2013). In the mouse brain, TAK1 protein expression is reduced after ischemia, independent of its mRNA expression (Naito et al., 2020). TAK1 protein abundance is also decreased upon serum deprivation of MEFs in a proteasome-dependent manner. Pharmacological inhibition of glycogen synthase kinase 3 (GSK3) in cultured pancreatic cancer cells induced a proteasome-dependent decrease in TAK1 protein level (Bang et al., 2013; Santoro et al., 2020). However, neither genetic inactivation of GSK3 in MEFs nor RNA interference-mediated knockdown or pharmacological inhibition of GSK3 in murine macrophages appeared to affect TAK1 protein levels (Ko et al., 2015). In addition to these findings in different cellular contexts, the results of proteomic studies suggest that TAK1 has a short half-life in cultured cells (Yu et al., 2014). Moreover, TAK1 was identified as a putative substrate for CRL4 complexes in a large-scale study exploiting both chemical inhibition of CRL complexes with MLN4924 and ectopic expression of a dominant-negative mutant of Cullin 4 (Emanuele et al., 2011).

Mechanistically, several E3 ubiquitin ligases have been reported to ubiquitinate TAK1. XIAP positively regulates TAK1 ubiquitination and proteasomal degradation in response to transforming growth factor-β treatment in mouse hepatocytes (Kaur et al., 2005). However, whether XIAP directly ubiquitinates TAK1 remains to be determined. The E3 ligase Itch promotes TAK1 ubiquitination and degradation, thereby terminating TAK1-dependent signaling and inflammatory response (Ahmed et al., 2011). Consequently, in response to TNFα stimulation, temporal regulation of p38 and JNK signaling is deregulated in MEFs or BMDMs deficient in Itch (Ahmed et al., 2011; Fan et al., 2012). Other mechanisms have been reported to regulate TAK1 stability via protein-protein interactions. The adaptors TAK1-binding proteins (TABs) form a complex with TAK1, regulating TAK1 activity (Hirata et al., 2017). TAB1 overexpression stabilizes ectopically expressed TAK1. However, whether this interaction is essential to the control of endogenous TAK1 protein stability has not been clearly demonstrated (Bertelsen and Sanfridson, 2007). On the other hand, modulation of TAB2 and TAB3 expression by USP15 has no effect on endogenous TAK1 protein levels (Zhou et al., 2020). Moreover, galectin-3-binding protein interacts with TAK1 and promotes its degradation in response to LPS stimulation (Hong et al., 2019). Notably, binding to galectin-3-binding protein also appears to inhibit the interaction of TAK1 with the adaptor proteins TAB1/2/3, leading to a decrease in TAK1 activation. TAK1 protein abundance is also regulated by the chaperones Hsp70 and Hsp90. TAK1 has been shown to be a client of the Hsp90/Cdc37 chaperone system (Liu et al., 2008; Shi et al., 2009). Interestingly, Hsp90 seems to compete with TAB1 for interaction with TAK1 (Liu et al., 2008). Since interaction with TAB1 is necessary for TAK1 activity, Hsp90-bound TAK1 is likely an inactive pool of the protein. Therefore, only prolonged inhibition of Hsp90 downregulates TAK1 and affects TAK1-dependent proinflammatory signaling. In addition, inducible Hsp70 expression in response to interleukin-1β was reported to destabilize TAK1 (Cao et al., 2012). A possible mechanistic explanation for this effect is that Hsp70 interferes with formation of the Hsp90-TAK1 complex, although the exact mechanism remains to be determined. Notably, the latter study suggests that TAK1 is stable in the absence of the Hsp70 interaction. This observation is consistent with the results of cycloheximide-chase experiments indicating that TAK1 half-life is longer than 12 hours in unstimulated cells (Shi et al., 2009; Liang et al., 2013; Hong et al., 2019). In *Drosophila*, the E3 ubiquitin ligase Plenty of SH3s was shown to ubiquitinate TAK1

ortholog and promote its degradation (Tsuda et al., 2005). Similar to mammals, ubiquitination of TAK1 terminates JNK signaling in the innate immune response. Cdc37 also acts as a molecular chaperone for TAK1 in *Drosophila*, as knockdown of Cdc37 in larvae induces a decrease in TAK1 protein abundance (Lee et al., 2019).

e. Dual leucine zipper-bearing kinase (mitogen-activated protein kinase kinase kinase 12) and leucine zipper kinase (mitogen-activated protein kinase kinase kinase 13). Dual leucine zipper-bearing kinase (DLK) and leucine zipper kinase (LZK) are related MAP3Ks acting upstream of the MKK4/7-JNK and MKK3/6-p38 MAPK pathways that play a pivotal role in the neuronal response to stress and injuries (Jin and Zheng, 2019). In invertebrates, the single ortholog DLK-1 in *C. elegans* and Wallenda (Wnd) in *Drosophila* are involved in synapse and cilia morphogenesis (Nakata et al., 2005; Collins et al., 2006; van der Vaart et al., 2015). Activation of DLK and LZK is absolutely dependent on their homodimerization via the leucine zipper domain, which leads to autophosphorylation of the kinase domain (Nihalani et al., 2000; Ikeda et al., 2001). Consequently, the regulation of DLK and LZK protein abundance is thought to be a critical mechanism controlling their biologic activity. The PAM/Highwire/RPM-1 (PHR) family of proteins, comprising PAM in human, Highwire in *Drosophila*, and RPM-1 in *C. elegans*, are conserved RING E3 ubiquitin ligases that regulate DLK abundance and signaling across different organisms (Jin and Zheng, 2019).

The most conclusive evidence that DLK is regulated by the UPS comes from studies in *C. elegans*. In this model organism, the ortholog DLK-1 is involved the regulation of synapse morphogenesis through its role as an MAP3K for the p38 MAPK PMK-3 (Nakata et al., 2005). Genetic studies have shown that inactivation of the DLK-1-PMK-3 pathway suppresses *rpm-1* loss-of-function phenotypes, whereas constitutive activation of the pathway mimics the synaptic defects observed in *rpm-1* mutants (Nakata et al., 2005). Expression of DLK-1, but not MKK-4 or PMK-3, is elevated in *rpm-1* mutants compared with wild-type animals, and overexpression of RPM-1 partially reverses the gain-of-function effects of DLK-1. At the biochemical level, cotransfection experiments in HEK293 cells showed that RPM-1 promotes the ubiquitination of DLK-1. A subsequent study has identified a recombinant peptide that disrupts the interaction between RPM-1 and the F-box protein F-box/SPRY domain-containing protein 1 (FSN-1) and showed that transgenic expression of this peptide inhibits the formation of a functional RPM-1/FSN-1 E3 ligase and causes defects in axon termination. These defects were suppressed by loss of function of *dlk-1* (Sharma et al., 2014). However, the effect of the peptide on DLK-1 stability was not assessed in this study. Interestingly, another study reported that RPM-1 has an additional role in regulating the phosphorylation state of DLK-1 via recruitment of the phosphatase PPM-2 (Baker et al., 2014). The relative importance of these E3 ligase and phosphatase-based mechanisms in controlling DLK-1 signaling remains to be determined.

In *Drosophila*, Wnd protein abundance is regulated by the PHR family member Highwire, as loss of *Hiw* increases Wnd expression (Collins et al., 2006). In addition, changes in Wnd and Highwire expression levels in response to axonal injury are inversely correlated in *Drosophila* larvae (Xiong et al., 2010). Highwire has been assumed to promote the degradation of Wnd by direct ubiquitination (Wu et al., 2007; Brace et al., 2014; Borgen et al., 2017; Asghari Adib et al., 2018). However, a recent study suggested that the regulation of Wnd by Highwire is indirect. The increase in Wnd protein levels observed upon loss of Highwire is abrogated in the absence of fragile X mental retardation protein (dFMRP), a regulator of Wnd mRNA translation (Russo and DiAntonio, 2019). In mice, loss of function of Phr1 in the *Magellan* mutant increases DLK protein levels in distal axons and induces axon morphology defects that can be reversed by treatment with the p38 MAPK inhibitor SB203580 (Lewcock et al., 2007). However, conflicting results have been reported on the impact of Phr1 on DLK protein levels. For example, no difference in DLK expression was measured in the brain of Phr1-deficient mouse embryos (Bloom et al., 2007). Similarly, no difference in LZK expression was found by immunoblot analysis of embryonic heads between wild-type and *Phr1* mutants. On the other hand, cultures of dorsal root ganglia deficient for Phr1 or expressing a loss-of-function allele of Phr1 exhibit an increase in DLK protein abundance (Babetto et al., 2013; Huntwork-Rodriguez et al., 2013). In addition, Phr1 inactivation decreases the level of ubiquitinated DLK (Huntwork-Rodriguez et al., 2013). These discrepant findings may reflect differences in assay methodologies. Interestingly, phosphorylation of DLK by JNKs reduces the ubiquitination of DLK and increases its stability, generating a positive feedback loop that may serve to amplify JNK signaling in axons (Huntwork-Rodriguez et al., 2013). Thus, the regulation of DLK stability and activity by PHR E3 ligases is an evolutionarily conserved mechanism.

In addition to the PHR protein family, the mammalian DUB USP9X, called Fat facets in *Drosophila*, has been described as a positive regulator of DLK protein abundance in murine dorsal root ganglia and *Drosophila* larvae (Collins et al., 2006; Huntwork-Rodriguez et al., 2013). DLK is also a client of the Hsp90 molecular chaperone system, as revealed by its

interaction with Hsp90 and by a decrease of DLK protein abundance upon chemical inhibition of Hsp90 (Karney-Grobe et al., 2018) . There is no report describing the regulation of LZK by the UPS.

f. Zipper sterile-α-motif kinase (mitogen-activated protein kinase kinase kinase 20). Zipper sterile-α-motif kinase α (ZAKα) and its splice variant ZAKβ, also known as MLK-like mitogen-activated protein triple kinase α and β, is an MAP3K for the p38 and JNK MAPK pathways (Gallo and Johnson, 2002; Vind et al., 2020). ZAK is activated by cellular stresses and plays a key role in the ribotoxic stress response (Gotoh et al., 2001; Vind et al., 2020). No study has specifically addressed the regulation of ZAK by the UPS. However, it has been reported that ZAK protein abundance can be modulated by treatment with high concentrations of doxorubicin or by expression of estrogen receptor β in a proteasome-independent manner (Sauter et al., 2010; Pai et al., 2018). In these studies, pharmacological inhibition of the proteasome with MG132 showed no effect on ZAK protein levels.

g. Thousand-and-one kinase 1 (mitogen-activated protein kinase kinase kinase 16), thousand-and-one kinase 2 (mitogen-activated protein kinase kinase kinase 17), and thousand-and-one kinase 3 (mitogen-activated protein kinase kinase kinase 18). Thousand-and-one kinases (TAOK) are less well characterized MAP3Ks that activate p38 and JNK MAPKs with different specificity (Tassi et al., 1999; Zhang et al., 2000; Kyriakis and Avruch, 2012). The regulation and functions of these kinases remain largely unexplored. TAOK1 was identified from a functional genomic screen as a kinase regulating microtubule dynamics and spindle checkpoint signaling (Draviam et al., 2007). These authors reported that TAOK1 protein abundance decreases quickly in HeLa cells after mitotic exit, suggesting a possible regulation by protein degradation similar to other mitotic regulators. In contrast, another study failed to show any regulation of TAOK1 levels throughout mitosis and challenged the role of TAOK1 in regulating the spindle checkpoint (Westhorpe et al., 2010). Additional work suggested a role for TAOK1 and TAOK2 in mitosis rounding and spindle positioning but did not observe changes in TAOK1 or TAOK3 protein levels during cell cycle progression (Wojtala et al., 2011).

2. The p38 Signaling Pathway. a. Mitogen-activated protein kinase kinases MKK3 (mitogen-activated protein kinase kinase 3) and MKK6 (mitogen-activated protein kinase kinase 6). MKK3 and MKK6 are the MAP2Ks of the p38 MAPK pathway. There is no publication describing the regulation of MKK3 at the level of protein stability, although it has been reported that MKK3 is ubiquitinated in response to hyperosmotic stress and that inhibition of the proteasome induces accumulation of ubiquitinated MKK3 in resting cells (Ahn and Kurie, 2009; Pedrazza et al., 2020). Cycloheximide-chase experiments indicate that MKK3 is a relatively stable protein with a half-life of more than 12 hours (Pedrazza et al., 2020). The MKK6 protein is ubiquitinated in response to osmotic and genotoxic stresses (Ahn and Kurie, 2009; Liu et al., 2014).

In the context of genotoxic stress, activated MKK6 is ubiquitinated by the E3 ligase $CRL1^{FBXO31}$ and degraded by the proteasome to terminate p38 signaling (Liu et al., 2014). Thus, F-box only protein 31 (FBXO31) acts as a negative regulator of p38-induced apoptosis under genotoxic stress. The short isoform of the E3 ligase TRIM9 has also been shown to regulate MKK6 protein levels by protecting it against degradation by the UPS (Liu et al., 2018). Interestingly, TRIM9 appears to stabilize MKK6 by promoting the transition of K48-linked to K63-linked polyubiquitination and counteracting FBXO31-mediated degradation. Moreover, p38 phosphorylates TRIM9 to stabilize the protein, establishing a positive feedback loop that potentiates p38 signaling and suppresses glioblastoma progression.

b. Mitogen-activated protein kinases p38α (mitogen-activated protein kinase 14), p38β (mitogen-activated protein kinase 11), p38γ (mitogen-activated protein kinase 12), and p38δ (mitogen-activated protein kinase 13). LPS stimulation of BMDMs promotes K48-linked and K63-linked polyubiquitination of p38α by the E3 ligase neural precursor cell expressed developmentally down-regulated protein 4 (Nedd4) (Liu et al., 2017a). Polyubiquitination through K48 linkage triggers proteasomal degradation of p38α. Interestingly, the phosphorylation status of p38α was found to modulate its ubiquitination and stability. Expression of MKK6, which increases phosphorylation of p38α, favors K48-linked ubiquitination and proteasomal degradation, contributing to a decrease in p38α signaling. Depletion of Nedd4 by RNA interference increases LPS-stimulated TNFα in BMDMs. Overexpression of heme oxygenase-1 was also reported to trigger the proteasomal degradation of p38α in endothelial cells (Silva et al., 2006). There is little information about the regulation of other p38 isoforms by the UPS. However, all p38 isoforms are described as proteins with a short half-life based on results from proteomic studies (Yen et al., 2008; Yu et al., 2014). Intriguingly, overexpression of MKK6 and p38α in HEK293T cells was shown to decrease p38γ abundance by a proteasome-dependent mechanism (Qi et al., 2007). Whether endogenous p38γ is regulated by a similar mechanism is not known. Finally, p38 isoforms associate with their substrate MAPK-activated protein kinase 2 (MK2), resulting in the stabilization of p38 proteins (Kotlyarov et al., 2002). As a result, p38 levels are decreased in MK2-

deficient cells and tissues. The molecular basis of this regulation is unknown.

3. The c-Jun N-Terminal Kinase Signaling Pathway. a. Mitogen-activated protein kinase kinases MKK4 (mitogen-activated protein kinase kinase 4) and MKK7 (mitogen-activated protein kinase kinase 7). MKK4 and MKK7 are the MAP2Ks of the JNK MAPK pathway. MKK4 is ubiquitinated by the E3 ligase Itch and degraded by the proteasome in response to osmotic stress in HEK293T cells (Ahn and Kurie, 2009). MKK4 ubiquitination is mediated by a negative feedback loop dependent on JNK activation, and only a low level of MKK4 ubiquitination is observed in unstimulated cells. Consistent with this result, MKK4 appears to be a remarkably stable protein in unstimulated prostate cancer cell lines, since neither treatment with cycloheximide for up to 24 hours nor inhibition of the proteasome affected MKK4 protein abundance (Robinson et al., 2008). The C terminus of MKK4 seems to be a critical component of its stability, as several deletion mutants found in lung adenocarcinoma are unstable and appear to have a higher ubiquitination level than the full-length protein (Ahn et al., 2011). MKK4 exert tumor suppressor functions in various cancers and accelerated degradation of *MAP2K4* mutants may contribute to its loss of function.

Treatment with sorbitol also induces the polyubiquitination and degradation of MKK7 in different cell types (Ahn and Kurie, 2009; Sakai et al., 2014). In glioma cells, genetic or pharmacological inhibition of histone deacetylase 6 leads to a decrease in JNK activity by causing downregulation of MKK7 via a proteasome-dependent mechanism (Huang et al., 2020). The inhibition of the MKK7-JNK-c-Jun signaling pathway was associated with glioma tumor growth inhibition. The molecular mechanisms underlying MKK7 regulation by the UPS remain to be investigated.

b. Mitogen-activated protein kinases c-Jun N-terminal kinase 1 (mitogen-activated protein kinase 8), c-Jun N-terminal kinase 2 (mitogen-activated protein kinase 9), and c-Jun N-terminal kinase 3 (mitogen-activated protein kinase 10). There are no data suggesting that any of the three JNK isoforms is regulated by protein degradation. However, in the yeast *Schizosaccharomyces pombe*, the JNK ortholog Spc1 is stabilized after binding to the chaperone protein Cdc37 (Tatebe and Shiozaki, 2003). Thus, cells with a *cdc37* mutation have lower expression levels of genes commonly induced by Spc1 signaling. Contrary to most Cdc37 substrates, Spc1 regulation appears to be independent of Hsp90.

C. The Extracellular Signal–Regulated Kinase 5 Signaling Pathway

The ERK5 MAPK pathway is activated by extracellular growth factors and cellular stresses and plays important roles in cellular growth, proliferation, and differentiation. Deregulation of ERK5 activity has been associated with pathologic conditions such as heart diseases and cancer (Nithianandarajah-Jones et al., 2012; Gallo et al., 2019; Stecca and Rovida, 2019). Activation of the ERK5 pathway is initiated by the MAP3Ks MEKK2, MEKK3, and TPL2, which phosphorylate and activate the dual-specificity MAP2K MEK5, which in turn phosphorylates and activates the MAPK ERK5. The regulation of MEKK2, MEKK3, and TPL2 has been described in previous sections.

1. Mitogen-Activated Protein Kinase Kinase MEK5 (Mitogen-Activated Protein Kinase Kinase 5). The MAP2K MEK5 was identified as a substrate of CRL4 complexes by a large-scale proteomics approach (Emanuele et al., 2011). Ectopic expression of dominant-negative forms of Cullin 4A and Cullin 4B led to an increase in the global protein stability index of MEK5 in response to UV-like DNA damage (Yen et al., 2008; Emanuele et al., 2011). Subsequent validation by immunoblot analysis confirmed that MEK5 is stabilized by the expression of inactive Cullin 4 mutants. These initial observations suggested that MEK5 activity is controlled in part by protein turnover in the context of genotoxic stress. Interestingly, MEK5 signaling has recently been reported to play a role in the DNA damage response (Broustas et al., 2020). In addition, c-Myc promoter-binding protein 1 was shown to physically interact with MEK5 and to induce its degradation by the proteasome, resulting in downregulation of ERK5 target genes and inhibition of prostate cancer cell growth (Ghosh et al., 2005). Although the regulation of MEK5 protein stability has not been extensively investigated, several studies have reported that high MEK5 protein expression is associated with cancer development and poor outcome, notably in colorectal and prostate cancer (Mehta et al., 2003; Hu et al., 2012; Diao et al., 2016). The exact contribution of the UPS to MEK5 overexpression in cancer warrants further investigation.

2. Mitogen-Activated Protein Kinase Extracellular Signal–Regulated Kinase 5 (Mitogen-Activated Protein Kinase 7). Interestingly, a recent report suggested that ERK5 catalytic activity is dispensable for immune responses and cell proliferation (Lin et al., 2016). Therefore, controlling the protein abundance of ERK5 offers a potential mechanism to modulate its activity. ERK5 was identified as a substrate of the CRL2/Von Hippel–Lindau protein (VHL) $CRL2^{VHL}$ E3 ligase complex in several human cell lines (Arias-Gonzalez et al., 2013). The ubiquitination and degradation of ERK5 are dependent on proline hydroxylation, similar to other substrates of VHL, such as hypoxia-inducible factor 1α (Arias-Gonzalez et al., 2013).

ERK5 is also a substrate of the Hsp90/Cdc37 molecular chaperone system, as inhibition of either Hsp90 or Cdc37 promotes the ubiquitination and subsequent degradation of ERK5 (Erazo et al., 2013). Notably, activating phosphorylation of ERK5 by MEK5 induces the dissociation of Hsp90, and ERK5 is no longer sensitive to Hsp90 inhibitors. A recent study also reported that sumoylation of ERK5 induces the dissociation of Hsp90 from the ERK5-Cdc37 complex, allowing nuclear translocation of ERK5 and activation of gene transcription (Erazo et al., 2020). The interplay between different aspects of Hsp90-mediated ERK5 regulation needs to be further characterized to assess the relative importance of ERK5 degradation for its biologic activity. In addition to these mechanisms, ERK5 is degraded by calpain-1 in a UPS-independent manner in response to oxidative stress (Liu et al., 2017b).

III. Atypical Mitogen-Activated Protein Kinase Pathways

A. The Extracellular Signal–Regulated Kinase 3/4 Signaling Pathway

Little is known about the regulation and physiologic functions of the atypical MAPKs ERK3 and ERK4. Cellular studies suggest potential roles in transcriptional control, cytoskeleton dynamics and cell migration, cell differentiation, and metabolism (Mathien et al., 2018; Bogucka et al., 2020; El-Merahbi et al., 2020). ERK3 and ERK4, which are not substrates of MAP2Ks, are phosphorylated and activated by the group I p21-activated kinases (PAKs) PAK1, PAK2, and PAK3 (De la Mota-Peynado et al., 2011; Déléris et al., 2011).

1. p21-Activated Kinase 1, p21-Activated Kinase 2, and p21-Activated Kinase 3. Very few studies have examined the regulation of PAKs protein stability. However, PAK1 and PAK2 were reported to be ubiquitinated and degraded by the proteasome (Jakobi et al., 2003; Weisz Hubsman et al., 2007). The proteasomal degradation of PAK1 is dependent on its kinase activity, establishing a feedback loop to terminate PAK1 signaling. The UPS machinery responsible for the ubiquitination of PAK1 and PAK2 remains to be identified.

2. Mitogen-Activated Protein Kinases Extracellular Signal–Regulated Kinase 3 (Mitogen-Activated Protein Kinase 6) and Extracellular Signal–Regulated Kinase 4 (Mitogen-Activated Protein Kinase 4). ERK3 and ERK4 differ from classic MAPKs by the presence of a single phospho-acceptor site in their activation loop (Coulombe and Meloche, 2007). ERK3 was the first MAPK reported to be actively regulated by the UPS. Contrary to most protein kinases, ERK3 is a highly unstable protein that is constitutively ubiquitinated and degraded by the proteasome in proliferating cells (Coulombe et al., 2003). The half-life of ERK3 ranges from 30 to 60 minutes in different cell lines (Coulombe et al., 2003; Mikalsen et al., 2005; Mathien et al., 2017; Bogucka et al., 2020). Notably, ERK3 is one of the few known substrates of the UPS to be ubiquitinated at the N-terminal amino group (Coulombe et al., 2004). Although the different UPS enzymes that control the ubiquitination state of ERK3 remain to be identified, recent work has shown that USP20 deubiquitinates ERK3 and increases its stability and protein abundance (Mathien et al., 2017). Hydroxylation of ERK3 on Pro 25 by the prolyl hydroxylase prolyl hydroxylase 3 also protects ERK3 from proteasomal degradation under normoxic conditions (Rodriguez et al., 2016). Interestingly, recent findings suggest a possible cross-talk between the classic ERK1/2 MAPK pathway and ERK3, where ERK1/2 signaling upregulates ERK3 expression. It has been initially reported that ectopic expression of oncogenic BRAF V600E induces the accumulation of ERK3 mRNA and protein in NIH 3T3 cells, whereas genetic inhibition of BRAF or treatment with MEK1/2 inhibitor downregulates ERK3 expression in melanoma cells (Hoeflich et al., 2006). Treatment with the RAF inhibitor sorafenib did not alter the degradation rate of ERK3 in that study. A more recent study confirmed that BRAF regulates ERK3 levels in melanoma cells and further suggested that BRAF signaling stabilizes ERK3 protein in addition to inducing accumulation of ERK3 mRNA (Chen et al., 2019b). Intriguingly, these authors suggested that BRAF increases ERK3 stability by a kinase-independent mechanism. Treatment with the potent MEK1/2 inhibitor trametinib also markedly decreased ERK3 protein levels in HT-29 cells, with no significant effect on ERK3 mRNA expression (Bogucka et al., 2020). Similar to the effect of MK2 on p38 levels, physical association of ERK3 with its substrate MK5 increases ERK3 protein stability and abundance, suggesting a chaperone function of MK5 for ERK3 (Schumacher et al., 2004; Seternes et al., 2004; Aberg et al., 2009).

The regulation of ERK3 protein stability is believed to play a major role in controlling its biologic activity, since ERK3 is constitutively phosphorylated in its activation loop (Déléris et al., 2008). Consistent with this idea, regulation of ERK3 by the UPS is a highly controlled process modulated by physiologic and pathologic stimuli (Fig. 6). Differentiation of neurogenic and myoblast precursors induces the stabilization of ERK3, leading to accumulation of the protein (Coulombe et al., 2003). Upon mitosis entry in HeLa cells, ERK3 is phosphorylated in its C-terminal extension by cyclin B-CDK1, resulting in its stabilization (Tanguay et al., 2010). Dephosphorylation of ERK3 by the phosphatases CDC14A/B at mitotic exit is associated

with a decrease in ERK3 protein levels. ERK3 stability is also regulated by the cellular environment. In cell culture models, acidification of the medium or treatment with hypoxia mimetic agents results in stabilization of ERK3 protein (Mathien et al., 2017). A recent study showed that β-adrenergic stimulation induces the stabilization and accumulation of ERK3 in adipocytes, which is necessary for the induction of lipolysis by ERK3 signaling (El-Merahbi et al., 2020). The proposed mechanism involves an increase in the formation of stabilizing ERK3/MK5 complexes that requires protein kinase A–dependent phosphorylation of MK5. Consequently, fasting of mice, which increases catecholamine levels, induces the accumulation of ERK3 in epigonadal white adipose tissue, highlighting the physiologic relevance of this regulatory mechanism. As discussed above, pathologic insults such as oncogenic BRAF expression can also modulate ERK3 stability and expression (Chen et al., 2019b).

Unlike its paralog, ERK4 is a stable protein, and its cellular abundance is not modulated by MK5 expression (Åberg et al., 2006; Kant et al., 2006). ERK3 and ERK4 share 73% amino acid identity in the kinase domain, but ERK3 has a long C-terminal extension. The molecular basis of their different stability remains to be understood.

B. The Extracellular Signal–Regulated Kinase 7 (Mitogen-Activated Protein Kinase 15) Signaling Pathway

ERK7 is a poorly characterized atypical MAPK with potential roles in cell proliferation, genome stability maintenance, and autophagy (Lau and Xu, 2018). Unlike classic MAPKs, ERK7 appears to be mainly activated by autophosphorylation, and no upstream regulatory kinase has been identified. ERK7 is an unstable protein, with a half-life of ∼2 hours, that is constitutively degraded by the UPS in proliferating cells (Kuo et al., 2004). The N-terminal 20 amino acids are both necessary and sufficient to target ERK7 for degradation, as revealed by expression of chimeric constructs between ERK7 and the stable ERK2 protein (Kuo et al., 2004). Although the exact molecular mechanisms controlling ERK7 ubiquitination remain to be delineated, overexpression of a dominant-negative form of Cullin 1 was found to stabilize ectopically expressed ERK7 in HEK293T cells, suggesting the possible involvement of CRL1 complexes in regulating ERK7 turnover (Kuo et al., 2004). Interestingly, regulation of ERK7 stability is a conserved process, as amino acid starvation stabilizes the ERK7 ortholog CG32703 in *Drosophila* S2 cells, leading to ERK7 protein accumulation (Zacharogianni et al., 2011). In contrast, induction of genotoxic stress by methyl methanesulfonate results in a decrease of ERK7 expression that is dependent on proteasome activity (Klevernic et al., 2009).

The atypical MAPKs ERK3 and ERK7 share several regulatory features (Fig. 7): they are unstable protein kinases that are constitutively degraded by the UPS in proliferating cells; they are targeted for proteasomal degradation through recognition of their N-terminal domain; their regulation by the UPS is a dynamic process modulated by several pathophysiological stimuli; and they are constitutively phosphorylated in their activation loop. This suggests that the UPS plays a major role in controlling their biologic activity and functions.

C. The Nemo-Like Kinase Signaling Pathway

NLK is a distantly related member of the MAPK family that shares 45% amino acid identity with ERK2 in the kinase domain (Coulombe and Meloche, 2007). NLK lacks a tyrosine phosphorylation site in the activation loop and is therefore classified as an atypical MAPK. It is activated by multiple growth factors and developmental cues, and regulates cellular processes involved in embryonic development, neuronal survival,

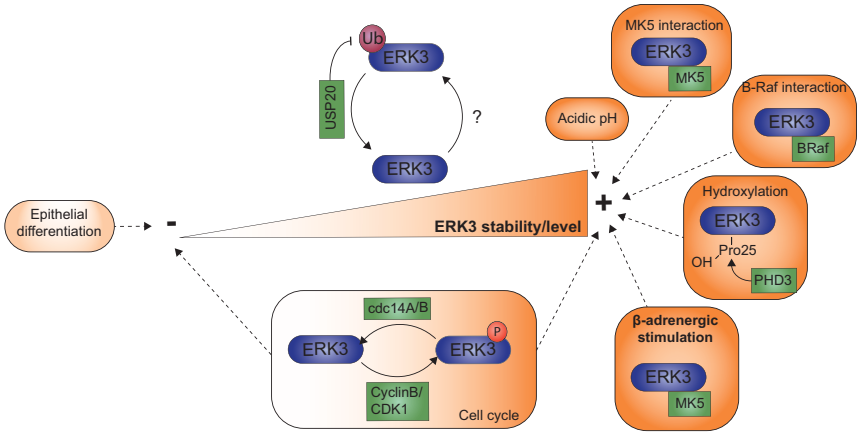

Fig. 6. ERK3 biologic activity is regulated by protein turnover. ERK3 is a very unstable kinase that is constitutively ubiquitinated and degraded by the proteasome in proliferating cells. Various physiologic and pathologic stimuli lead to the stabilization and accumulation of ERK3, suggesting that the biologic activity of ERK3 is mainly controlled by protein turnover. USP20 has been identified as a DUB that controls ERK3 ubiquitination and protein levels. MK5 binds to ERK3 and acts as a chaperone to stabilize ERK3. PHD3, prolyl hydroxylase 3.

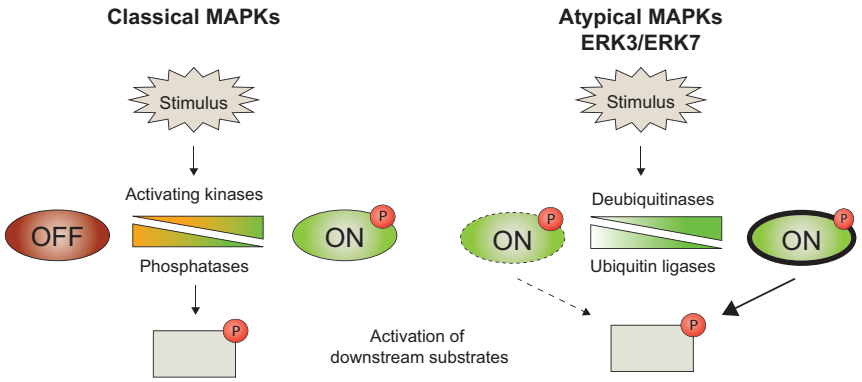

Fig. 7. Comparative regulation of classic and atypical MAPKs. The activation state of classic MAPKs is controlled in large part by phosphorylation/dephosphorylation of their activation loop regulated by a balance between the activity of MAP2Ks and MAPK phosphatases (MKPs) in response to diverse stimuli. In contrast, the atypical MAPKs ERK3 and ERK7 are constitutively phosphorylated in the activation loop. Their biologic activity appears to be mainly controlled by protein abundance, which is regulated by a balance between the activity of specific E3 ligases and DUBs. PHD3, prolyl hydroxylase 3.

and hematopoiesis (Ishitani and Ishitani, 2013; Daams and Massoumi, 2020). A study has shown that NLK is activated by intermolecular autophosphorylation of activation loop Thr 286 after ligand-induced dimerization (Ishitani et al., 2011). There is no report on the regulation of NLK by the UPS.

IV. Targeting Mitogen-Activated Protein Kinase Stability as a New Pharmacological Strategy

A. Proteolysis-Targeting Chimeras

Small-molecule kinase inhibitors are powerful reagents that have been instrumental in enabling the study of the cellular functions of protein kinases and their roles in human disease. Many of these molecules have been developed into drug candidates and clinically approved for cancer and other indications (Roskoski, 2021). Specifically, several inhibitors of MAPK pathways have been approved or are undergoing clinical development (Table 2). However, kinase inhibitors have limitations. The duration of the response is limited, requiring exposure to high concentrations of inhibitor for a sustained period of time. This can lead to a rewiring of the kinome that restores signaling via alternative pathways. Kinase often lacks selectivity over protein family members. In the clinic, treatment with kinase inhibitors is invariably associated with the rapid acquisition of drug resistance. These limitations have led research groups from the academia and industry to explore alternative strategies that aim to inhibit kinase function by eliminating the protein rather than blocking enzymatic activity (Salami and Crews, 2017; Chamberlain and Hamann, 2019; Verma et al., 2020). One such modality of targeted protein degradation, termed proteolysis-targeting chimera (PROTAC), uses a heterobifunctional molecule containing an E3 ligase ligand fused via a chemical linker to a target-binding ligand to induce the ubiquitination and proteasomal degradation of the target (Burslem and Crews, 2020). PROTACs may offer several advantages over kinase inhibitors, such as more durable response, decreased susceptibility to kinase rewiring, inhibition of noncatalytic functions inhibitors, and higher selectivity because of their transient "event-driven" mechanism of action. The advantages of PROTACs have been highlighted in a proof-of-concept study comparing the effect of receptor tyrosine kinases PROTACs and kinase inhibitors (Burslem et al., 2018). The clinical potential of PROTACs has been validated with the discovery that the immunomodulatory drug lenalidomide works through a PROTAC-like mechanism of action in multiple myeloma (Kronke et al., 2014; Lu et al., 2014). In 2019, the molecules ARV-110 and ARV-471, developed by Arvinas and targeting, respectively, the androgen receptor and estrogen receptor, became the first PROTACs to enter the clinic (NCT03888612 and NCT04072952). Since then, the number of clinical trials with PROTACs has consistently increased, and it is expected that at least 15 different protein degraders will reach the clinic by the end of 2021 (Mullard, 2021). Multiple protein kinases have been targeted with PROTACs in recent years, including components of MAPK pathways.

1. BRAF. BRAF has been identified as an oncogenic driver in multiple solid cancers (Davies et al., 2002). Three BRAF inhibitors have been approved by the Food and Drug Administration (FDA) as single or combination therapies for advanced melanoma, non–small cell lung cancer, anaplastic thyroid cancer, and Erdheim-Chester disease. Despite initial responses to BRAF inhibitor treatment, patients inevitably develop resistance within 1 year through multiple mechanisms typically involving reactivation of ERK1/2 signaling (Holderfield et al., 2014; Proietti et al., 2020). In addition, BRAF inhibitors can paradoxically activate the ERK1/2 MAPK pathway by inducing RAF dimerization and allowing the inhibitor-bound RAF molecule to transactivate the drug-free RAF dimeric partner (Hatzivassiliou et al., 2010; Lavoie et al., 2013; Poulikakos et al., 2010). These observations have provided a rationale for the development of BRAF PROTACs. Wang and coworkers selected the BRAF inhibitor vemurafenib and the pan-RAF inhibitor BI882370 as warheads and coupled them to thalidomide, a ligand of the CRL4 substrate receptor cereblon (CRBN). Linker length

TABLE 2
FDA-approved and investigational MAP3K, MAP2K, and MAPK inhibitors

	Drug	Status	Indication
MAP3Ks			
BRAF	Vemurafenib	Approved	Melanoma, Erdheim-Chester disease
	Dabrafenib	Approved	Monotherapy: melanoma In combination with trametinib: melanoma, NSCLC, anaplastic thyroid cancer
	Encorafenib	Approved	In combination with: - Binimetinib: melanoma - Cetuximab: colorectal cancer
	PLX8394	I/II	Unresectable solid tumors
BRAF/CRAF	LHX254	II	Melanoma and NSCLC, in combination with LTT462, trametinib or ribociclib
pan-RAF	TAK580	I	Gliomas and other tumors
	Lifirafenib	I/II	Advanced or refractory solid tumors, in combination with mirdametinib
	DAY101	II	Relapsed or progressive low-grade glioma
dualRAF/MEK	VERSUS-6766	II	Low-grade serous ovarian cancer, NSCLC: monotherapy and in combination with defactinib
	RO5126766	I	RAS-RAF-MEK pathway mutant solid tumors: in combination with everolimus Advanced solid tumors: in combination with FAK inhibitor VERSUS-6063
MAP2Ks			
MEK1/2	Trametinib	Approved	Monotherapy: melanoma In combination with dabrafenib: melanoma, NSCLC, anaplastic thyroid cancer
	Cobimetinib	Approved	In combination with vemurafenib: melanoma
	Selumetinib	Approved	Pediatric neurofibromatosis type 1
	Binimetinib	Approved	In combination with encorafenib: melanoma
	Pimasertib	I/II	Cancer with brain metastases
	Mirdametinib	I/II	Advanced or refractory solid tumors, in combination with lifirafenib
	E6201	I	Metastatic melanoma central nervous system metastases
	RO5126766	I	Solid tumors, NSCLC, multiple myeloma: monotherapy or in combination with everolimus
	HL-085	I/II	Melanoma, solid tumors, NSCLC: monotherapy or in combination with vemurafenib
	SHR7390	II	Metastatic castration-resistant prostate cancer
	CS-3006	I	Advanced or metastatic solid tumors
	FCN-159	I	Melanoma
MAPKs			
ERK1/2	Ulixertinib	I/II	Several active studies in solid tumors and melanoma, in monotherapies or in combination
	MK-8353	I	Colorectal cancer, in combination with pembrolizumab
	LTT462	I/II	Melanoma and NSCLC, in combination with LXH254 Myelofibrosis, in combination with JAK inhibitor ruxolitinib
	LY3214996	I/II	Several active studies in solid tumors and AML, in monotherapies or in combination
	HH2710	I/II	Advanced tumors
	JSI-1187	I	Monotherapy and in combination with dabrafenib: solid tumors with MAPK pathway mutations
p38α	Neflamapimod	II	Dementia and Alzheimer
p38α/β	Losmapimod (GW856553X)	III	SARS-CoV-2

AML, Acute myelogenous leukemia; JAK, Janus Kinase; SARS-CoV, severe acute respiratory syndrome coronavirus.

optimization indicated that degraders with short linkers have a higher potency with vemurafenib as warhead, whereas BI882370-based degraders require longer linker lengths to induce degradation. Both vemurafenib and BI882370-based PROTACs induce BRAF degradation and ERK1/2 inhibition at low nanomolar concentrations, impairing the proliferation of $BRAF^{V600E}$-expressing cell lines A375 and HT-29 (Han et al., 2020). Another study used the CRBN ligand pomalidomide to synthesize a series of PROTACs with the RAF-binding molecule rigosertib. Compound 2 effectively induced the degradation of BRAF and inhibited the proliferation of MCF-7 breast cancer cells with an IC_{50} of 2.7 μM (Chen et al., 2019a). A study by Sicheri and coworkers tested the activity of 16 different PROTACs designed by fusing the BRAF binders dabrafenib or BI882370 to one of three E3 ligase binders, namely the CRBN ligands pomalidomide and thalidomide or the $CRL2^{VHL}$ ligand VH032. Detailed functional characterization revealed that all BI882370-based PROTACs were effective to some extent at reducing BRAF levels and ERK1/2 signaling, whereas only one dabrafenib-based PROTAC displayed some activity. The most effective PROTAC, named P4B and made from a fusion between BI882370 and pomalidomide, decreased BRAF V600E protein levels by 70% at 100 nM, translating into potent inhibition of ERK1/2 signaling and proliferation of $BRAF^{V600E}$-expressing cell lines (Posternak et al., 2020). Interestingly, P4B was shown to be effective in cells harboring alternative *BRAF* mutations. SJF-0628, a vemurafenib-based PROTAC molecule developed by Crews and colleagues, is also effective to degrade BRAF mutants, with a higher selectivity for BRAF mutants than wild-type RAF family members (Alabi et al., 2021).

2. MEK1 (Mitogen-Activated Protein Kinase Kinase 1) and MEK2 (Mitogen-Activated Protein Kinase Kinase 2). Four MEK1/2 inhibitors are approved by the FDA as single or combination therapies for the

treatment of advanced melanoma, non–small cell lung cancer, anaplastic thyroid cancer, and neurofibroma. Similar to other targeted agents, the efficacy of MEK1/2 inhibitors is compromised by acquired resistance mainly through reactivation of the ERK1/2 pathway (Caunt et al., 2015; Kozar et al., 2019). Two groups reported the synthesis and initial biologic evaluation of MEK1/2 PROTACs designed by linking the MEK1/2 inhibitor PD0325901 (Wei et al., 2019) or refametinib (Vollmer et al., 2020) to a ligand of VHL or CRBN. Treatment with the VHL-recruiting PROTAC MS432 induced the degradation of MEK1 and MEK2 proteins, suppressed ERK1/2 phosphorylation and inhibited the proliferation of $BRAF^{V600E}$-expressing melanoma and colorectal carcinoma cell lines with potencies ranging from 30 to 200 nM (Wei et al., 2019). Proteomic analysis revealed that MS432 is a highly selective degrader of MEK1 and MEK2. Preliminary in vivo evaluation showed that MS432 displays good plasma exposure and is well tolerated, paving the way for future efficacy studies. In a more recent study, the Jin group tested additional PD0325901-derived MEK1/2 degraders fused to VHL or CRBN ligands by a variety of linkers. These extensive structure-activity relationship studies led to the discovery of two improved VHL-recruiting MEK1/2 PROTACs that show higher potency and plasma exposure than MS432, and a first CRBN-recruiting MEK1/2 PROTAC (Hu et al., 2020).

3. Extracellular Signal–Regulated Kinase 1 and Extracellular Signal–Regulated Kinase 2. As an approach to circumvent the limitations of solubility and cell permeability associated with the high molecular weight of PROTACs, Lebraud et al. (2016) devised a click chemistry strategy to generate the heterobifunctional PROTAC intracellularly from two smaller precursor molecules. As a proof of concept to in-cell click-formed PROTAC (CLIPTAC), the authors treated A375 melanoma cells with a covalent *trans*-cyclo-octene–tagged ERK1/2 inhibitor followed by tetrazine-tagged thalidomide and showed that the ERK1/2 CLIPTAC elicits a time-dependent and complete degradation of ERK1 and ERK2 proteins.

4. p38. The development of isoform-selective kinase inhibitors is a major challenge in medicinal chemistry. Many potent small-molecule inhibitors of p38α and p38β have been developed over the years, but the p38δ isoform has remained chemically intractable. In a recent study, the Crew group reported the development of p38α- and p38δ-selective PROTACs based on the nonselective p38 family inhibitor foretinib and a VHL-recruiting ligand (Smith et al., 2019). The PROTAC SJFα degraded p38α with an IC_{50} of 7 nM while degrading p38δ with an IC_{50} of 299 nM and being inactive against p38β and p38γ. PROTAC SJFδ selectively degraded p38δ with an IC_{50} of 46 nM. Isoform selectivity was obtained by varying the length and orientation of the linker. Of note, time course experiments with protein degraders can be used to determine the resynthesis rate of the target proteins and evaluate the durability of the response. Targets with a slow resynthesis rate offer the best opportunities for PROTAC development, as degradation will be superior to enzymatic inhibition. Interestingly, treatment with SJFα leads to a sustained degradation of p38α for 72 hours postwashout, whereas SJFδ maintains p38δ degradation for 24 hours, suggesting that PROTACs can be used at low doses during a short period to achieve a durable decrease of p38 protein levels. This elegant study also illustrates the power of the PROTAC technology to selectivity target the degradation of closely related protein kinase isoforms. Two other PROTACs, NR-7h and NR-6a, were shown to be effective at nanomolar concentrations to selectively degrade p38α and p38β isoforms in malignant and nonmalignant cell lines (Donoghue et al., 2020).

B. Small-Molecule Modulators of the Ubiquitin-Proteasome System

1. Heat Shock Protein 90 Inhibitors. Since numerous oncoproteins are clients of the molecular chaperone Hsp90, the development of pharmacological inhibitors of Hsp90 has been subject to intense efforts by the academic and industry sectors. To date, close to 20 different Hsp90 inhibitors have been evaluated in more than 170 clinical trials (Sanchez et al., 2020). Single-agent studies with Hsp90 inhibitors have shown limited efficacy, mainly due to toxicity issues. However, several combination therapies with Hsp90 inhibitors are now tested in the clinic (Kryeziu et al., 2019). Interestingly, melanoma, in which oncogenic BRAF mutants act as major oncogenic drivers, is one therapeutic area of focus for the clinical development of Hsp90 inhibitors (Mielczarek-Lewandowska et al., 2020).

The Hsp90 molecular chaperone is an important regulator of the ERK1/2 MAPK pathway via its role in stabilizing RAF proteins. A large number of Hsp90 pharmacological inhibitors can disrupt Hsp90-RAF complexes, resulting in RAF degradation and inhibition of ERK1/2 signaling in preclinical models and clinical samples (Table 1). Preclinical studies have shown that Hsp90 inhibition by ganetespib decreases ERK1/2 MAPK pathway signaling in melanoma and colorectal cancer cells harboring oncogenic $BRAF^{V600E}$ mutant (Acquaviva et al., 2014; He et al., 2014). Notably, dual targeting of Hsp90 and $BRAF^{V600E}$ or MEK1/2 was found to provide combinatorial benefit in melanoma, colorectal cancer, NSCLC, and triple-negative breast cancer models (Paraiso et al., 2012; Acquaviva et al., 2014; Park et al., 2016; Chen et al., 2017). Inhibition of Hsp90 overcomes acquired BRAF or MEK1/2 inhibitor resistance in these models

(Paraiso et al., 2012; Wu et al., 2013; Acquaviva et al., 2014; Park et al., 2016). One of the potential mechanisms of resistance to BRAF inhibitors is the upregulation of CRAF protein expression. It has been reported that elevated CRAF protein levels contribute to the acquired resistance of melanoma cells to the BRAF inhibitor AZ628, rendering these cells exquisitely sensitive to the Hsp90 inhibitor geldanamycin (Montagut et al., 2008). A recent study also showed that the Hsp90 inhibitor XL888 prevents vemurafenib-induced paradoxical ERK1/2 activation by decreasing CRAF expression in Neuroblastoma RAS viral oncogene homolog mutant melanoma cells and limits hyperproliferative skin lesions in patients (Phadke et al., 2015). However, it is important to note that the molecular mechanism by which Hsp90 inhibition synergizes with BRAF inhibitors may not rely entirely on its role as a chaperone for BRAF or CRAF but on the additional deregulation of other Hsp90 clients (Paraiso et al., 2012; Smyth et al., 2014; Mielczarek-Lewandowska et al., 2019). Another study reported that Hsp70, a component of the Hsp70-Hsp90 chaperone cascade, is overexpressed in a significant proportion of melanomas and that BRAF V600E is an Hsp70 client protein (Budina-Kolomets et al., 2016). These authors further showed that the Hsp70 inhibitor PET-16 downregulates mutant BRAF and synergizes with vemurafenib to repress the growth of melanoma tumor xenografts.

The clinical potential of combining small-molecule inhibitors of BRAF and Hsp90 has been evaluated in a small number of clinical trials. A phase I study tested the combination of the multikinase inhibitor sorafenib with increasing doses of tanespimycin (17-AAG) in 27 patients with various solid tumors of unknown BRAF mutation status (Vaishampayan et al., 2010). Interestingly, four of six patients evaluated for CRAF protein levels showed a decrease expression after treatment. A second phase I study conducted in 21 patients with unresectable $BRAF^{V600E}$ mutant melanoma investigated the combination of vemurafenib with escalating doses of XL888 (Eroglu et al., 2018). An objective response rate of 75% was observed with a tolerable side-effect profile, warranting further evaluation of XL888 with standard-of-care vemurafenib plus cobimetinib combination in these patients (NCT02721459). Another phase I study is exploring the combination of the Hsp90 inhibitor onalespib with dual inhibition of BRAF and MEK1/2 with dabrafenib and trametinib, respectively, in patients with BRAF mutant metastatic or unresectable solid tumors (NCT02097225).

2. Statins. Statins are 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors that have long been used as cholesterol-lowering drugs for the prevention of cardiovascular disease. In addition, statins have recently gained traction as potential anticancer agents in the context of combination therapies (Matusewicz et al., 2020). Treatment with simvastatin induces a dose-dependent decrease in CRAF protein levels concomitant with inhibition of ERK1/2 phosphorylation in breast cancer cell lines (Wang et al., 2016b). In addition, treatment with simvastatin markedly increased the turnover rate of BRAF in colorectal carcinoma cell lines (Lee et al., 2011). Simvastatin was shown to synergize with BRAF and MEK1/2 inhibitors to inhibit the proliferation of drug-resistant melanoma, colorectal, and lung cancer cell lines (Theodosakis et al., 2019). The mechanism of action of statins and their potential utility as modulators of the ERK1/2 MAPK pathway in cancer remain to be investigated.

3. Inhibitor of Apoptosis Protein Inhibitors. Studies have reported that c-IAP, XIAP, and ML-IAP promote the ubiquitination and degradation of CRAF (Dogan et al., 2008; Oberoi-Khanuja et al., 2012) (Fig. 3). The small molecules birinapant and AT-406 are antagonists of IAP proteins currently evaluated in clinical trials. Interestingly, pharmacological inhibition of IAPs with birinapant has been reported to synergize with BRAF inhibition to induce apoptosis of $BRAF^{V600E}$ colorectal adenocarcinoma cells (Perimenis et al., 2016).

4. Deubiquitinating Enzyme Inhibitors. DUBs are emerging as promising drug targets for a variety of clinical indications (Harrigan et al., 2018). Small-molecule inhibitors of DUBs known to regulate MAPK pathways have been developed in recent years. For example, WP1130 was described as a nonselective inhibitor of USP9X, USP5, USP14, and UCH37 (Kapuria et al., 2010). USP9X positively regulates ASK1 protein stability and downstream p38 and JNK signaling in response to oxidative stress (Nagai et al., 2009) (Fig. 5). A recent study reported the efficacy of the USP20 inhibitor GSK2643943A in abrogating 3-hydroxy-3-methylglutaryl coenzyme A reductase stabilization by USP20, resulting in reduced cholesterol biosynthesis and decreased serum lipid contents (Lu et al., 2020). The atypical MAPK ERK3 is another substrate of USP20 (Mathien et al., 2017). The DUB USP28, by deubiquitinating the $CRL1^{FBXW7}$ substrate receptor FBXW7, promotes the proteasomal degradation of RAF proteins (Saei et al., 2018) (Fig. 3). Several small molecules have recently been reported to inhibit the enzymatic activity of USP28 (Wrigley et al., 2017; Liu et al., 2020; Wang et al., 2021). USP28 has attracted attention as a potential therapeutic target for cancer (Wang et al., 2018b). However, genetic depletion of USP28 in models of melanoma leads to resistance to BRAF inhibitors (Saei et al., 2018), raising potential concerns about the therapeutic use of USP28 inhibitors.

V. Concluding Remarks

The amplitude and duration of MAPK pathway signaling is a major determinant of cell fate (Marshall, 1995). The signaling output of MAPK pathways is negatively regulated by reversible phosphorylation/dephosphorylation events operating directly at the level of effector MAPKs or through establishment of negative feedback loops suppressing the activity of upstream regulators. However, it is becoming increasingly apparent that irreversible inactivation of MAPK pathway components by proteasomal degradation plays a significant role in fine-tuning MAPK signaling. All MAPK pathways, with the possible exception of the distantly related NLK pathway, are subject to regulation by the UPS, and several of these mechanisms are evolutionarily conserved. The prevalence and importance of this regulation is underappreciated in the literature.

A striking feature of the UPS-mediated control of MAPK signaling is the predominance of regulatory mechanisms operating at the MAP3K level. Although MAP3Ks are more numerous than MAP2Ks and MAPKs, these enzymes are a common target of E3 ligases and are often targeted by multiple degradation mechanisms. MAP3Ks integrate various upstream signals and confer specificity to classic MAPK pathways (Cuevas et al., 2007). Thus, targeted degradation of MAP3Ks by adding an additional layer of regulation may sharpen the signaling output of MAPK pathways and dictate specific cellular outcomes. Another intriguing observation is that the biologic activity of the atypical MAPKs ERK3 and ERK7 appears to be regulated mainly by protein turnover. Contrary to classic MAPKs, these kinases are constitutively phosphorylated in their activation loop and continuously degraded by the UPS in proliferating cells. Indeed, ERK3 is among the mammalian proteins with the shortest half-lives (Toyama and Hetzer, 2013; Chen et al., 2016). The size of a protein is positively correlated with its half-life (Yen et al., 2008). The human ERK3 protein consists of 721 amino acids. Based on different predictive models including not only the size but also the amino acid composition and N-terminal structure of proteins, ERK3 should be classified in the top 25% of the most stable proteins (Yen et al., 2008; Patrick et al., 2012). Why active protein turnover has evolved as a major, if not the main, mechanism of regulation of ERK3 and ERK7, and how this relates to their physiologic functions, is a question of interest.

Proof-of-concept studies have shown that modulating the stability of MAPK pathway components is a viable alternative to small-molecule kinase inhibitors. This strategy finds applications in both biologic discovery and drug development. PROTACs can be used to selectively and acutely inhibit the activity of closely related kinase isoforms to get insights into their pathophysiological functions. The combination of Hsp90 inhibitors with BRAF and MEK1/2 kinase inhibitors has already reached the stage of clinical evaluation. Future studies will tell whether targeted protein degradation can address the current limitation of kinase inhibitors, most notably the acquisition of drug resistance.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Mathien, Tesnière, Meloche.

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