MITOGEN ACTIVATED PROTEIN KINASES

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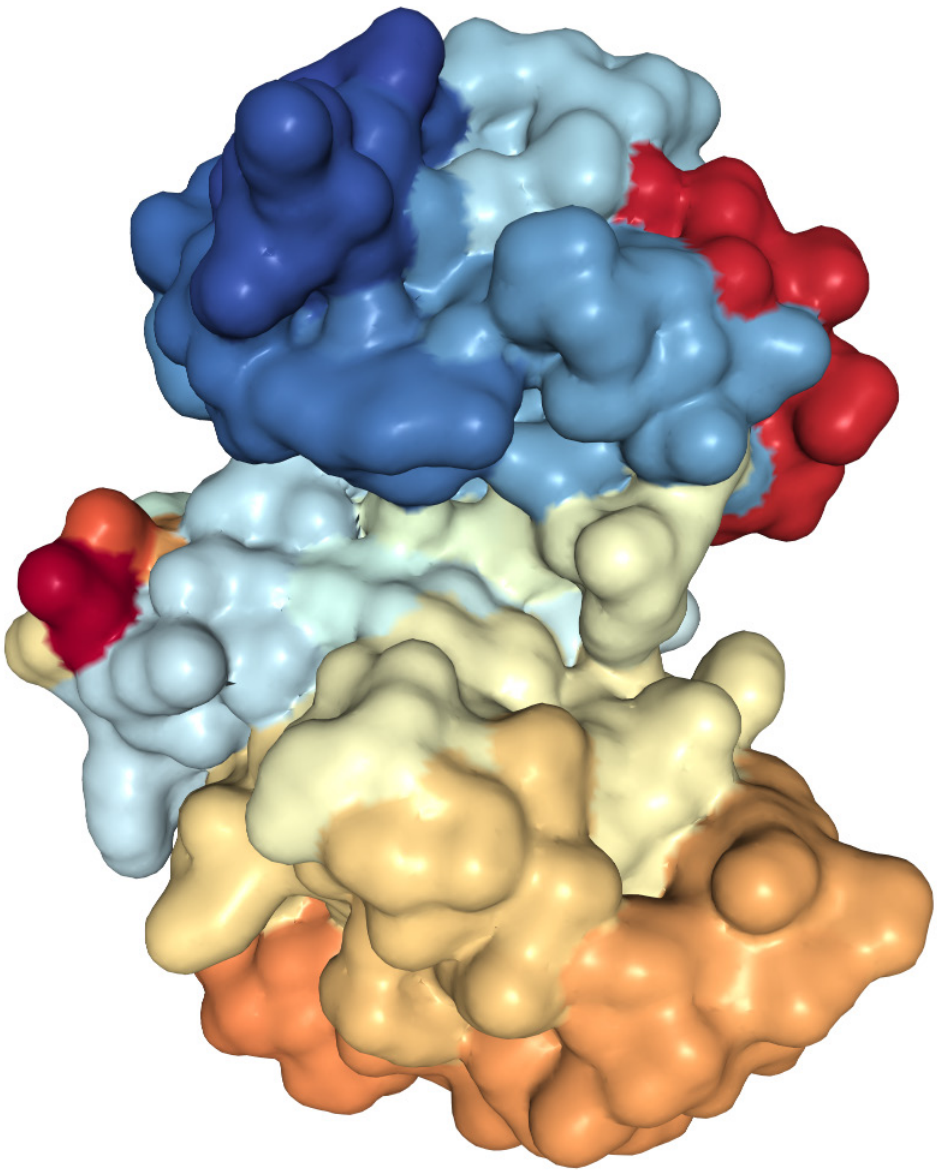

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MITOGEN ACTIVATED PROTEIN KINASES

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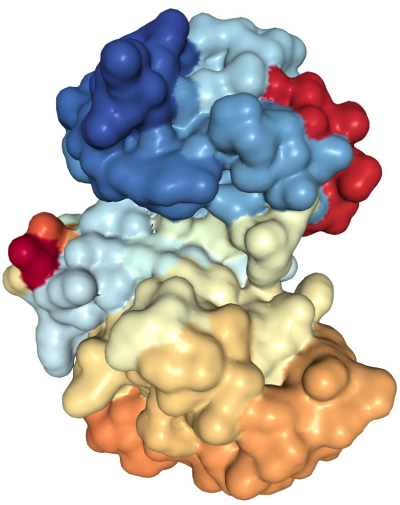

p38α structure. Colour gradation goes from the blue (N-terminal) to yellow (C-terminal).

Image: Dr. José Lozano.

Mitogen-activated protein kinase (MAPK) pathways are evolutionarily conserved in all eukaryotes and allow cells to respond to changes in the physical and chemical properties of the environment and to produce an appropriate response by altering many cellular functions. MAPKs are among the most intensively studied signal transduction systems. MAPK research is a very dynamic field in which new perspectives are continuously opening to the scientific community. Importantly, many MAPK inhibitors have been developed during the last years and are currently being tested in preclinical and clinical assays for inflammatory diseases and cancer treatment.

In this research topic, we have gathered 14 papers covering recent advances in different aspects of the MAPK research area that have provided valuable insight into the spatiotemporal dynamics, the regulation and functions of MAPK pathways, as well as their therapeutic potential. We hope that this Research Topic helps readers to have a better understanding of the progresses that have been made recently in the field of MAPK signalling. A deeper understanding of the these pathways will facilitate the development of innovative therapeutic approaches.

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Table of Contents

05 ***Editorial: Mitogen Activated Protein Kinases***
Ana Cuenda, José M. Lizcano and José Lozano

Chapter 1: Selectivity of MAPK Pathways

08 ***MAPK-Activated Protein Kinases (MKs): Novel Insights and Challenges***
Matthias Gaestel

14 ***ERK Signals: Scaffolding Scaffolds?***
Berta Casar and Piero Crespo

Chapter 2: Functional Redundancy in the MAPK Pathways

25 ***ERK1 and ERK2 Map Kinases: Specific Roles or Functional Redundancy?***
Roser Buscà, Jacques Pouysségur and Philippe Lenormand

48 ***Redundancy in the World of MAP Kinases: All for One***
Marc K. Saba-El-Leil, Christophe Frémin and Sylvain Meloche

Chapter 3: MAPK Pathways in Differentiation

57 ***Regulation of Muscle Stem Cell Functions: A Focus on the p38 MAPK Signaling Pathway***
Jessica Segalés, Eusebio Perdiguero and Pura Muñoz-Cánoves

72 ***p38 MAPK Signaling in Osteoblast Differentiation***
Eddie Rodríguez-Carballo, Beatriz Gámez and Francesc Ventura

Chapter 4: MAPK Pathways in Inflammation

92 ***Mitogen-Activated Protein Kinases and Mitogen Kinase Phosphatase 1: A Critical Interplay in Macrophage Biology***
Jorge Lloberas, Lorena Valverde-Estrella, Juan Tur, Tania Vico and Antonio Celada

100 ***Emerging Roles of the Mitogen and Stress Activated Kinases MSK1 and MSK2***
Kathleen M. S. E. Reyskens and J. Simon C. Arthur

108 ***Induction of Macrophage Function in Human THP-1 Cells Is Associated with Rewiring of MAPK Signaling and Activation of MAP3K7 (TAK1) Protein Kinase***
Erik Richter, Katharina Ventz, Manuela Harms, Jörg Mostertz and Falko Hochgräfe

Chapter 5: MAPK Pathways in Cancer

123 ***The Complexity of the ERK/MAP-Kinase Pathway and the Treatment of Melanoma Skin Cancer***
Claudia Wellbrock and Imanol Arozarena

132 ***p38MAPK and Chemotherapy: We Always Need to Hear Both Sides of the Story***
Jesús García-Cano, Olga Roche, Francisco J. Cimas, Raquel Pascual-Serra, Marta Ortega-Muelas, Diego M. Fernández-Aroca and Ricardo Sánchez-Prieto

140 ***Gain-of-Function Mutations in the Toll-Like Receptor Pathway: TPL2-Mediated ERK1/ERK2 MAPK Activation, a Path to Tumorigenesis in Lymphoid Neoplasms?***
Simon Rousseau and Guy Martel

Chapter 6: Alternative MAPK Pathways

150 ***ERK5 and Cell Proliferation: Nuclear Localization Is What Matters***
Nestor Gomez, Tatiana Erazo and Jose M. Lizcano

157 ***p38γ and p38δ Mitogen Activated Protein Kinases (MAPKs), New Stars in the MAPK Galaxy***
Alejandra Escós, Ana Risco, Dayanira Alsina-Beauchamp and Ana Cuenda

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EDITORIAL
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Editorial: Mitogen Activated Protein Kinases

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Editorial on the Research Topic

Mitogen Activated Protein Kinases

Mitogen-activated protein kinase (MAPK) cascades are among the most intensively studied signal transduction systems. MAPK pathways are evolutionarily conserved in all eukaryotes and allow cells to respond to changes in the physical and chemical properties of the environment and to produce an appropriate response by altering many cellular functions including cell differentiation, cell death, proliferation, metabolism rate, or the interaction with other cells. Four subfamilies of MAPKs have been extensively characterized in mammalian cells: ERK1/2, JNKs, p38s and ERK5 (Schaeffer and Weber, 1999; Cuenda and Rousseau, 2007; Gaestel et al., 2009; Kyriakis and Avruch, 2012; Arthur and Ley, 2013). All MAPK cascades comprise several molecular intermediaries at sequential level, which become activated in response to a broad panel of intra- and extra-cellular stimuli. They are typically organized in a three-kinase architecture consisting of a MAPK, a MAPK activator (MEK, MKK, or MAPK kinase), and a MEK activator [MEK kinase (MEKK)]. Transmission of signals is normally achieved by sequential phosphorylation and activation of the components specific to a respective cascade (Schaeffer and Weber, 1999; Kyriakis and Avruch, 2012).

In the past decade, there has been a vast increase of new works using different approaches and technologies that have provided valuable insight into the spatiotemporal dynamics, the regulation and functions of MAPK pathways, as well as their therapeutic potential. Since MAPK research is a very dynamic field, our aim planning this topic was to generate an opportunity in which MAPK researchers could make public their latest discoveries and also review and revisit different aspects of this research area.

Several key issues in the MAPK field are discussed in this topic. One of them is how selectivity and efficiency of MAPK pathways is preserved, despite the apparent ability of their components to function in multiple pathways. Particularly, Casar and Crespo describe recent findings on the ERK1/2 scaffold proteins, which maintain pathway integrity and signaling efficiency. Scaffold proteins connect different MAPK pathway elements into multi-enzymatic complexes (Kyriakis and Avruch, 2012), which fine-tune signal amplitude and duration, and provide signal fidelity by isolating these complexes from external interferences. Also, scaffold proteins are spatial regulators of MAPK signals, and depending on the subcellular localization from which the activating signals arise, defined scaffolds determine which substrates are phosphorylated. In this respect, Gaestel describes how the MAPKs ERK1/2 and p38 signal further downstream by the activation of the so-called MAPK-activated protein kinases (MAPKAPKs). He summarizes recent findings regarding the molecular basis of signaling complexes between MAPKs and MAPKAPKs and describes the non-canonical activation of the ERK1/2 substrate RSKs by p38-MK2/3 in dendritic cells. In his mini-review Gaestel also discusses recent challenges arising from off target effects of the widely used RSK inhibitors SL0101 and BI-D1870.

Functional redundancy between MAPKs is very common since there are more than one isoform at each level of the MAPK cascades. This issue is also discusses in the topic. Buscà et al. and Saba-El-Leil et al. focus their attention on ERK1 and ERK2. Buscà et al. collect data on ERK1 vs. ERK2 gene structures, protein sequences, expression levels, structural and molecular mechanisms of activation and substrate recognition, and very nicely perform a rigorous analysis of studies regarding the individual roles of ERK1 and ERK2. They conclude that ERK1 and ERK2 exhibit functional redundancy and propose the concept of the global ERK quantity as being the essential determinant to achieve ERK function. Saba-El-Leil et al. also point out evidence supporting the ERK1 and ERK2 redundant roles in embryonic development and in physiology, and in addition discuss the redundancy of JNK (JNK1/2/3) and p38 (p38α/β/γ/δ) isoforms.

Additionally, this topic includes some latest advances on MAPK function and implication in differentiation, inflammation and cancer. Two reviews focus on p38MAPK signaling in cell differentiation; particularly, Segalés et al. nicely summarize the molecular mechanisms implicated in the transition of muscle satellite cells throughout the distinct myogenic stages and also discuss recent findings on the causes underlying satellite cell functional decline with aging. They describe the important function of p38 in myogenesis, and in building up satellite cell adaptive responses in muscle regeneration; and discuss how these responses are altered in aging. On the other hand, Rodríguez-Carballo et al., discuss the role of MAPKs—centring on p38—on the regulation of transcription factors that are essential for adipocyte, chondrocytes, osteoblasts and osteoclasts differentiation and function. They also describe how inflammatory cytokines activate MAPKs during the differentiation process. It is well established that MAPKs are not only activated in response to inflammatory cytokines, but also serve as key regulators of pro-inflammatory cytokines biosynthesis, which makes different components of these pathways potential targets for the treatment of autoimmune and inflammatory diseases (Cuenda and Rousseau, 2007; Gaestel et al., 2009; Arthur and Ley, 2013). Lloberas et al., describe how the MAPK phosphatase MKP-1 is regulated, and also explain the balancing role of MKP-1 in the control of macrophage behavior by dephosphorylating MAPKs, which in turn have a strong impact in the inflammatory response since macrophages represent the primary host response to pathogen infection and link the immediate innate defense to the adaptive immune system. Reyskens and Arthur, review the last findings on MSK1/2, which are common p38 and ERK1/2 substrates. MSK1/2 are nuclear proteins that phosphorylate multiple substrates, including CREB or Histone H3, and are highly expressed in immune and nervous systems. The anti-inflammatory role of MSKs, by regulating the production of IL-10, and their implication in neuronal proliferation and synaptic plasticity in the central nervous system are described in this review. In addition, Richter et al. present their last data on the analysis of protein kinases during macrophage differentiation by using kinomics and phosphoproteomics in the human monocytic cell line THP-1. They find that monocyte-to-macrophage differentiation is associated with major rewiring of MAPK signaling networks and demonstrate that protein kinase MAP3K7 (TAK1) is critical for bacterial killing, chemokine production and differentiation.

Other process in which MAPKs are central elements is cancer development (Wagner and Nebreda, 2009; Dorard et al., 2017). Rousseau and Martel report an analysis of non-synonymous somatic mutations found in the TLR signaling network in lymphoid neoplasms. Lymphoid neoplasms form a family of cancers affecting B-cells, T-cells, and NK cells. The authors' findings suggest that TLR-mediated ERK1/2 activation via TPL2 is a novel path to tumorigenesis, and they propose that inhibition of ERK1/2 activation would prevent tumor growth in hematologic malignancies such as Waldenstrom's Macroglobulinemia, where the majority of the cells carry the MYD88[L265P] mutation. In the skin cancer context, Wellbrock and Arozarena review the complexity of the ERK signaling pathway in melanocytes, the healthy pigment cells that give rise to melanoma. They also discuss the mechanisms of action of different ERK-pathway inhibitors and their correlation with clinical response, the mechanisms of drug-resistance that limit patient's response, and new therapeutic opportunities for melanoma treatment targeting the ERK pathway. During the last decade members of the p38 signaling pathway have joined the group of canonical signaling pathways involved in tumor development and therefore are potential target for cancer treatment (Cuenda and Rousseau, 2007; Wagner and Nebreda, 2009). To this respect, García-Cano et al. summarize the role of p38MAPK in chemotherapy as well as the advantages that p38MAPK

inhibition can bring to cancer therapy. The authors conclude that targeting p38MAPK for cancer treatment could be a double-edged sword depending on the patient's pathology and treatment.

Finally, two contributions, which shape the final outcome of the topic, address different aspects the some of the less studied MAPKs: ERK5, p38γ and p38δ. Gomez et al., review the role of ERK5 in regulating cell proliferation by mechanisms that are both dependent and independent of its kinase activity. They summarize the last findings regarding the complex regulation of ERK5 by upstream kinases and stabilizing chaperones in normal and cancer cells, and also during cell cycle. The authors describe the different mechanisms involved in the nuclear translocation of ERK5, -where mediates gene transcription- and discuss the possibility of targeting ERK5 to tackle different types of cancer. Escós et al. give a general overview of the recent advances made in defining the functions of the alternative p38, p38γ and p38δ, focusing in innate immunity and inflammation. They also discuss the potential of the pharmacological targeting of p38γ and p38δ pathways to treat autoimmune and inflammatory diseases, as well as cancer linked to inflammation.

We hope that all the information compiled in this eBook will be useful to researchers in this exciting field, and stimulate them to continue in their efforts to increase our knowledge on MAPK cascades. We want to acknowledge the great work of the authors, co-authors, and reviewers, and to thanks the superb support received from Frontiers Team members at all times.

AUTHOR CONTRIBUTIONS

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MINI REVIEW
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MAPK-Activated Protein Kinases (MKs): Novel Insights and Challenges

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Downstream of MAPKs, such as classical/atypical ERKs and p38 MAPKs, but not of JNKs, signaling is often mediated by protein kinases which are phosphorylated and activated by MAPKs and, therefore, designated MAPK-activated protein kinases (MAPKAPKs). Recently, novel insights into the specificity of the assembly of MAPK/MAPKAPK hetero-dimeric protein kinase signaling complexes have been gained. In addition, new functional aspects of MKs have been described and established functions have been challenged. This short review will summarize recent developments including the linear motif (LM) in MKs, the ERK-independent activation of RSK, the RSK-independent effects of some RSK-inhibitors and the challenged role of MK5/PRAK in tumor suppression.

Keywords: p38 MAPK, ERK3/4, common docking motif, macrophage-specific activation, dendritic cells, mouse gene-targeting strategy, Ras-induced senescence, DMBA-induced skin tumors

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INTRODUCTION

Besides phosphorylation of other substrates, ERKs and p38 MAPKs are able to signal further downstream by the activation of so called MAPK-activated protein kinases (MAPKAPKs) (reviewed in Cargnello and Roux, 2011). These downstream kinases are the p90 ribosomal-S6-kinases (RSK1-3), the mitogen- and stress-activated protein kinases MSK1/2, the MAPK-interacting kinases MNK1/2 and the MAPKAP kinases MK2, MK3 and MK5/PRAK (Gaestel, 2006). Specific signaling complexes between MAPK and their target MAPKAPKs exist and are the structural basis for the functional downstream-extension of MAPK cascades. Canonical activation pathways have been defined for the exclusive activation of RSKs by ERK1/2, the exclusive activation of MK2/3 by p38α/β as well as the more promiscuous activation of MNKs and MSKs by both ERKs and p38 and of MK5/PRAK by p38β/ERK3/4. Here, I will discuss novel findings regarding the molecular basis of specific and productive signaling complexes between MAPKs and MAPKAPKs, the non-canonical activation of RSKs and recent challenges arising from off target effects of the widely used RSK inhibitors SL0101 and BI-D1870. Furthermore, the challenge of the anticipated tumor-suppressive function of MK5/PRAK is discussed.

NOVEL INSIGHTS

The Molecular Basis for MAPKAPK's Specific Interaction with MAPKs: Classical D Motifs and Reverse D-Motifs Constitute the Linear Motif (LM)

Specific interactions of MAPKs with their activators and substrates are established via the common docking (CD) motif of MAPKs (D-X_2-D/E) and the docking (D) motif (R/K-R/K-X_{2-6}-Ø-X-Ø) or

kinase-interacting motif (KIM) (L/V-X_2-R/K-R/K-X_5-L) of the substrate or activator (Tanoue et al., 2000; reviewed in Gaestel, 2008). However, while these interactions fully govern the recognition and phosphorylation of unstructured regions in substrates such as transcription factors, the CD-D-interaction is not completely sufficient for establishing the specificity of binding of MAPKs to important activators and other substrates. The isolated D-motifs of MKK3/6 (p38 specific MAPKK) or MKK1/2 (ERK specific MAPKK) are, for instance, not able to discriminate between p38α and ERK2, but bind to both kinases with comparable affinity (Garai et al., 2012). Further structural analyses have revealed that the CD motif of MAPKs can be divided into the negatively charged CD groove and various further hydrophobic pockets or grooves which are able to interact with the basic core of the D-motif and a pattern of further hydrophobic residues located N terminal to the D-motif designated reverse D (revD) motif (Ø-X-Ø-X_2-Ø-X_{4-6}-Ø-X_2-R/K-R/K) (Garai et al., 2012). Interestingly, this revD motif allows clear discrimination in binding affinity between p38α and ERK2. While the revD motif of RSK1 displays high affinity to ERK2, its binding affinity to p38α is 20-fold lower. Vice versa, the revD motif of MK2 shows strong affinity to p38α but only weak interaction with ERK2 (Garai et al., 2012).

Only the revD of MNK1, which is activated by both ERK2 and p38α, displays similar affinity to both kinases. Hence, a linear motif (LM) formed by the overlapping D and revD motifs is necessary and sufficient to guarantee specific interaction in the binary MAPK/MAPKAPK complexes such as ERK2/RSK1 and p38α/MK2. The following alignment shows the D-, KIM-, and revD motifs identified in MAPK substrates and activators. Together, these overlapping motifs should be regarded as the linear motif (LM). Ø stands for a hydrophobic amino acid, X_n for the number n of variable amino acids:

D:	R/K–R/K–X_{2-6}–Ø–X–Ø
KIM:	L/V–X_2–R/K–R/K–X_5–L
revD:	Ø–X–Ø–X_2–Ø–X_{4-6}–Ø–X_2–R/K–R/K
LM:	**Ø–X–Ø–X_2–Ø–X_{4-6}–Ø–X_2–R/K–R/K–X_{2-6}–Ø–X–Ø**

Although the CD motif-LM-interaction is essential for various MAPK/MAPKAPK complexes, the CD-motif of the atypical MAPKs ERK 3 and ERK4 is not sufficient for the activation of MK5/PRAK. Instead, a novel FRIEDE interaction motif in loop L16 C-terminal to the CD-motif is necessary for MK5/PRAK binding of ERK3/4 (Aberg et al., 2009). Interestingly, the L16 FRIEDE motif in ERK3/4 is activated by phosphorylation of the atypical activation loop SEG in an allosteric manner. The FRIEDE motif interacts with the C-terminus of MK5/PRAK and a mutant lacking 50 C-terminal amino acids but still containing the D-domain of MK5/PRAK is unable to bind to ERK3/4 (Aberg et al., 2006). Hence, this interaction is clearly different from the CD-LM-module.

Primary MAPK/MAPKAPK Complexes Formed by LM-CD Motif Interaction

The binding of the LM of a MAPKAPK and the CD grooves of MAPKs (**Figures 1A,B**) is the first step of formation of specific signaling complexes but does not necessarily lead to the formation of a signaling competent complex with both MAPK and MAPKAPK activity. However, the primary "encounter" complex formation (**Figure 1B**) is already able to

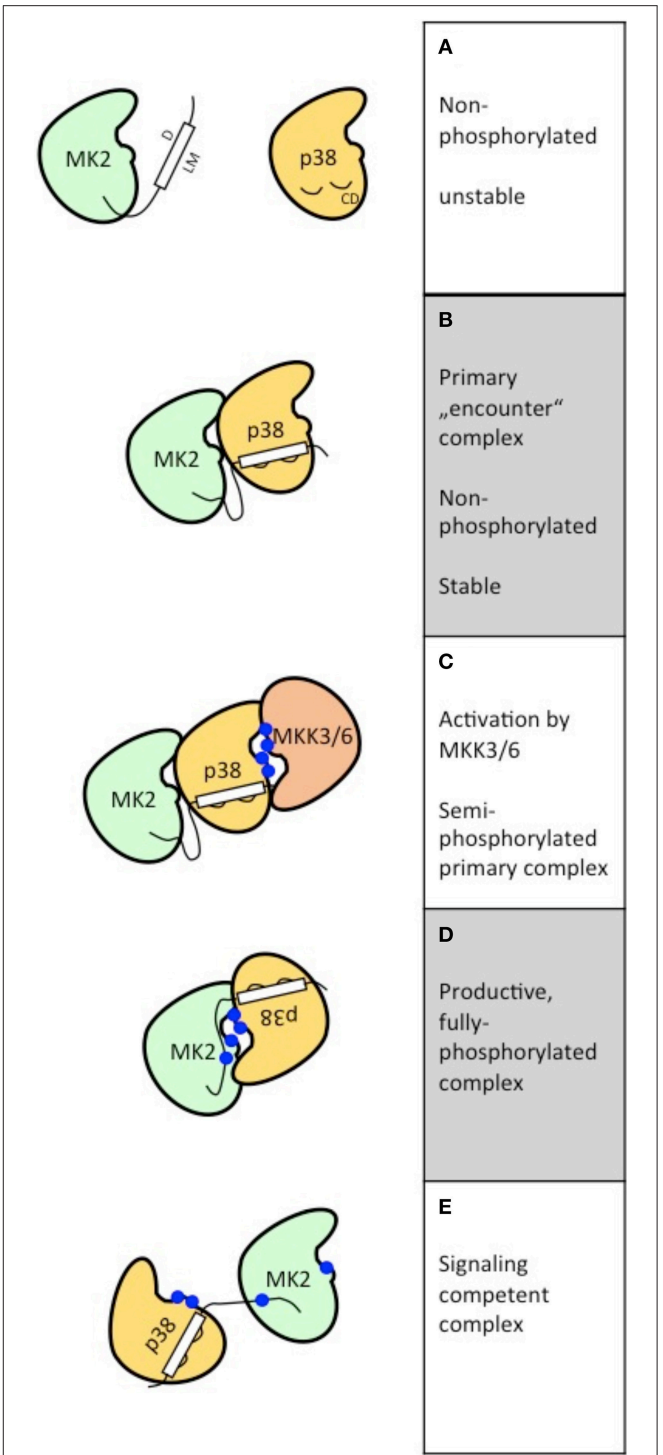

FIGURE 1 | Schematic representation of the postulated steps to reach signaling competent, fully active binary kinase complexes between MAPKs (here p38α) and MAPKAPKs (here MK2). The features of the five different states postulated **(A–E)** are depicted at the right.

cause mutual stabilization of the MAPKs/MAPKAPKs in the specific complex. *In vivo*, this stabilization is reflected by the findings that in non-stimulated MK2-deficient cells the p38α level is significantly reduced (Kotlyarov et al., 2002) and that p38α-deficient resting cells display reduced MK2 levels (Sudo et al., 2005). Furthermore, the formation of primary, non-productive kinase complexes is able to prevent binding to (and activation by) non-specific MAPKs and crosstalk with other signaling pathways. This is demonstrated *in vitro* by the fact that addition of inactive p38α strongly increases the specificity of ppERK2 toward RSK1 and blocks ppERK2's activity against MK2 (Alexa et al., 2015). This finding implies that the stoichiometry between specific MAPKs and MAPKAPKs is an important determinant to maintain the specificity of signaling also *in vivo*. Taking into account that other MAPK substrates and activators compete with MAPKAPKs in binding to the CD motif, signaling complex formation *in vivo* is likely highly sensitive to the local concentrations of these competing interactors. In this regard a high complexity of regulation will also arise due to the fact that local sub-cellular concentrations of many signaling molecules are also signal-regulated. Equal importantly, this result also indicates that artificial overexpression of a specific MAPK or MAPKAPK, which can lead to significant stoichiometric alterations between specific MAPKs and/or MAPKAPKs in the cell, could also lead to artificial activation of non-specific signaling pathways. This would explain the initial observation that MK3, a kinase downstream to p38α/β, is activated by ERKs, JNKs, and p38 in cells overexpressing these MAPKs (Ludwig et al., 1996) or that MK5/PRAK, a kinase activated by the atypical ERK3/4 (see below), also displays docking to p38α when both kinases are overexpressed (New et al., 2003).

The three-dimensional structure of a primary MAPK-MAPKAPK-complex between non-phosphorylated p38α/MK2 has been established (White et al., 2007). In this complex the LM of MK2 is bound to the CD motif of p38α. Both kinases bind in a parallel "head to head" orientation (**Figure 1B**), but catalytic and substrate regions are distantly located at different sides of the kinase heterodimer making it unlikely that this is a signaling competent complex. However, this orientation would enable upstream activators, such as MKK3 or MKK6, to phosphorylate the activation loop of p38α leading to a semi-phosphorylated primary complex (**Figure 1C**).

Productive Dimerization Leading to Active Signaling Complexes

The three-dimensional structure of another non-phosphorylated MAPK/MAPKAPK complex consisting of ERK2 and RSK1 has recently been determined revealing a structure for a pre-catalytic state of anti-parallel "head to tail" orientation where both kinases face each other and the activation loop of RSK2 is located close to the catalytic center of ERK2 (Alexa et al., 2015). After phosphorylation of ERK2 by the upstream activator MEK1/2 only minor readjustments of the orientation of the binary complex seem necessary to activate RSK1 by phosphorylation of the CTD leading to a productive signaling module (Alexa et al., 2015).

In the case of p38α/MK2 more complex changes in orientation of the molecules in the complex seem necessary to enable p38 to phosphorylate the regulatory sites of MK2 (**Figure 1D**). It could be assumed that these changes are allosterically induced by phosphorylation of p38α at the activation loop. After phosphorylation of the regulatory sites of MK2 at the activation loop and in the hinge region between catalytic core and C-terminal extension, MK2 itself undergoes a structural transition involving a major conformational change of the atypically structured APE motif of MK2 (Alexa et al., 2015). As a result of this process a fully active signaling complex is formed (**Figure 1E**). The transition from the primary "encounter" complex to the fully active p38α/MK2 signaling complex is accompanied by a reduction of the affinity of interaction reflected by a increase of the K_d-value from 2.5 nM for non-phosphorylated MK2 and p38α to about 60 nM for phosphorylated MK2 and p38α (Lukas et al., 2004). Interestingly, a number of proteins and cellular structures, such as LIMK1 (Kobayashi et al., 2006), keratin K8/K20, or K8/K18 complexes (Menon et al., 2010) and the neighboring immediate early promoter binding factors CREB/SRF (Heidenreich et al., 1999; Ronkina et al., 2011) are substrates for both p38α and MK2 indicating that the fully active p38α/MK2 complex might act cooperatively to phosphorylate these proteins and structures.

Non-Canonical Activation of RSK in Dendritic Cells

Although there is a specific interaction between ERKs and RSKs via the CD-LM-interaction in many cell types, an ERK-independent but p38α-dependent activation of RSK by MK2 and MK3 has been described in dendritic cells. In these cells MK2/3 bypass phosphorylation of the C-terminal kinase domain (KD) by ERKs by directly phosphorylating the auto-phosphorylation site S386 between N- and C-terminal KD, a prerequisite for the activation of the N-terminal KD by PDK1 (Zaru et al., 2007). Recently, the structural and functional basis for the cell type-specific operation of this alternative activation mechanism of RSKs has been characterized further (Zaru et al., 2014). It has turned out that the non-canonical activation of RSKs is specific for hematopoietic cells, such as dendritic cells and macrophages, and that the C-terminal KD of RSK is dispensable for this activation. Furthermore, the existence of the non-canonical activation mechanism is accompanied by an increased constitutive cytoplasmic localization of p38α/MK2/3 in these cells and a very low activation of ERKs by inflammatory stimuli, such as LPS. Hence, in these cells a certain plasticity of MAPK signaling guarantees the LPS-induced TLR-mediated interferon-β induction via the p38α/MK2/3-RSK-pathway. The interaction between MK2/3 and RSK in these cells seems rather transient (Zaru et al., 2014) and it is not clear whether further cell type-specific protein partners facilitate this interaction in macrophages and dendritic cells.

ESTABLISHED FUNCTIONS CHALLENGED

Challenged Specificity of the Compounds BI-D1870 and SL0101 and mTORC1-Related Function of RSKs

In tests against a panel of recombinant protein kinases the compounds BI-D1870 and SL0101 appeared as relatively specific inhibitors for RSK1 and RSK2 (Bain et al., 2007). However, said panel did not contain mTOR or mTORC1 and a recent study demonstrated that BI-D1870 and SL0101 also modulate mTORC1-p70S6K signaling in different directions (Roffé et al., 2015). Since SL0101 clearly also inhibits mTORC1-p70S6K signaling, the demonstration that RSK phosphorylates ribosomal protein S6, a substrate of p70S6K, using this inhibitor is challenged. Interestingly, BI-D1870 increased p70S6K activation in an ERK1/2- and RSK-independent manner by a mechanism unknown to date. In the light of these findings, the interpretation of the results presented in nearly 100 publications describing effects of these inhibitors without confirming these effects by further experiments, such as knockdown or overexpression of active kinase, should be reassessed. Meanwhile, novel and more specific RSK-inhibitors have also been identified (Jain et al., 2015) enabling us to better define the *in vivo* function of these kinases.

Challenged Function of MK5/PRAK as Tumor Suppressor

Controversial discussions regarding the activation mechanism and function of MK5/PRAK have been published. As seen from the LM alignment below, the sequence of the LM present in this protein kinase bears similarity to both the LM of RSK and MK2, indicating possible interaction with ERKs or p38 MAPKs:

```
RSK1:       721-PQLKPIESSILAQRRVRKLPS-741
                  ::::.       .. .:    .
MK5/PRAK:   348-VSLKPLHSVNNPILRKRKLLGTK-364
                . .:  .   . ::.: ::.   .
MK2:        372-IKIKKIEDASNPLLLKRRKKARA-392

LM:               ØXØXXØXXXXXXØXXRRXXXØXØ
                                  KK
```

In line with this similarity, activation of MK5/PRAK has been observed by p38 MAPKs and by ERKs when these kinases were overexpressed in mammalian cells (New et al., 1998; Ni et al., 1998). Furthermore, there is the FRIEDE-binding region (see above) in the C-terminal stretch of 50 amino acids, which enables interaction of this kinase with ERK3/4 (Aberg et al., 2009). Overexpression of both p38α and ERK3/4 leads to phosphorylation of MK5/PRAK at its regulatory site T182 and its activation as measured by phosphorylation of the peptide PRAKtide. While several publications describe a p38-dependent activation of MK5/PRAK (New et al., 1998), others could not detect activation of MK5/PRAK by stimuli, which activate p38 MAPKs, such as arsenite or high osmolarity (sorbitol) treatment (Shi et al., 2003). ERK3/4 activity and binding of the FRIEDE motif to MK5/PRAK can be stimulated by phosphorylation by p21-activated kinase 1 (PAK1) in the SEG motif in the activation loop (De la Mota-Peynado et al., 2011; Déléris et al., 2011) connecting MK5 to signaling of the small GTP-ase Rac. Furthermore, acetylation of MK5/PRAK at lysine K364 in the putative LM has also been described to increase its activity, although it should interfere with binding of the appropriate MAPK (Zheng et al., 2013), and various substrates of MK5/PRAK, such as p53 (Sun et al., 2007), HSP27 (Kostenko et al., 2009), FoxO3a (Kress et al., 2011), Foxo1 (Chow et al., 2013), and Rheb (Zheng et al., 2011) have been proposed.

TABLE 1 | Comparison of the results of the targeting approaches for MK5/PRAK.

	"MK5 knockout" (Shi et al., 2003)	**"PRAK knockout" (Sun et al., 2007)**
MK5/PRAK targeting strategy	Deletion of exon 6 (Δex6)	Deletion of exon 8 (Δex8)
Protein	Truncated, deletion of 30 amino acids (131–160)	Truncated, deletion of 27 amino acids (194–220)
Stability	Instable	Stable (similar to WT)
Localization	Cytoplasmic	Nuclear (similar to WT)
Kinase domain	Subdomains VIa, VIb missing	Stretch between subdomains VIII and IX shortened
Kinase activity	Not-detectable	Residual autophosphorylation
Reduction of H-Ras-G12V-induced $p21^{WAF}$ expression in targeted MEFs	–	+
Ras-induced tumorigenity/growth of targeted MEFs in soft agar	–	+
Increased skin tumor formation in the one step DMBA model in the targeted mouse strain	–	+

The function of MK5/PRAK has been mainly characterized by two different mouse knockout approaches targeting exon 6 and exon 8, respectively (Shi et al., 2003; Sun et al., 2007). Surprisingly, it has recently turned out that both knockout approaches for MK5/PRAK failed to delete the entire protein (Ronkina et al., 2015). Instead, two different truncated MK5/PRAK forms are still present in the knockout mice (**Table 1**). Since these mutants display different biochemical and cellular properties (**Table 1**) it is not surprising that the effects of expression of these mutants in cellular systems differ: While MEFs from the Δex6-targeted mice did not show altered $p21^{WAF}$ level in response to H-Ras-G12V expression and did not grow in soft agar, Δex8-targeted MEFs displayed reduced levels of the key marker of tumor suppression $p21^{WAF}$ and growth in soft agar (Sun et al., 2007; Ronkina et al., 2015). It is highly probable that the properties of the different MK5/PRAK deletion mutants also contribute to the phenotype of the targeted mice in the established DMBA one-step skin tumor model. Hence, it is not surprising that Δex6-targeted mice did not display increased skin tumor formation in this

model, while Δex8-targeted did (Sun et al., 2007; Ronkina et al., 2015). Since the results obtained using the Δex8-targeted mice formed the basis for the formulation of the tumor suppressive role of MK5/PRAK (Sun et al., 2007) as well as for the identification of MK5/PRAK as a tumor-promoting angiogenic factor (Yoshizuka et al., 2012), these roles are challenged and should be revisited by MK5 targeting approaches deleting the entire protein kinase. Once the phenotype of the real MK5/PRAK knockout mouse has been described, these results should serve to clarify the physiological function of MK5/PRAK.

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ERK Signals: Scaffolding Scaffolds?

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ERK1/2 MAP Kinases become activated in response to multiple intra- and extra-cellular stimuli through a signaling module composed of sequential tiers of cytoplasmic kinases. Scaffold proteins regulate ERK signals by connecting the different components of the module into a multi-enzymatic complex by which signal amplitude and duration are fine-tuned, and also provide signal fidelity by isolating this complex from external interferences. In addition, scaffold proteins play a central role as spatial regulators of ERKs signals. In this respect, depending on the subcellular localization from which the activating signals emanate, defined scaffolds specify which substrates are amenable to be phosphorylated. Recent evidence has unveiled direct interactions among different scaffold protein species. These scaffold-scaffold macro-complexes could constitute an additional level of regulation for ERK signals and may serve as nodes for the integration of incoming signals and the subsequent diversification of the outgoing signals with respect to substrate engagement.

Keywords: MAP kinase, ERK, scaffold protein, protein-protein interactions, signaling pathways

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Exhaustive research efforts undertaken during the past decades, have positioned the signaling module mediated by Extracellular signal-Regulated Kinases 1 and 2 (ERKs) Mitogen-Activated Protein Kinases (MAPKs), among the best known signal transduction processes ever studied. ERKs signaling cascade encompasses sequential tiers, composed of sundry types of molecular intermediaries, which become activated in response to a broad panel of intra- and extra-cellular stimuli. The ERKs cascade is generally activated at its origin by GTPases of the RAS family that subsequently switch-on, by not fully understood mechanisms, an upstream echelon constituted by MAPKKKs of the RAF family. These, at their turn, convey signals downstream by phosphorylating/activating dual-specificity MAPKKs MEK 1 and 2, ultimately responsible for the phosphorylation and the unleashing of ERKs activity (Roskoski, 2012). It is also well-known that this signaling pathway is involved in the regulation of prime physiological processes, such as cellular proliferation, differentiation, cell cycle control, development and survival, in addition to hundreds of cell- and tissue-specific events. Consequently, unregulated or aberrant ERK signaling results in multiple pathological conditions ranging from psoriasis to cancer (Robinson and Cobb, 1997; Raman et al., 2007; Shaul and Seger, 2007).

SCAFFOLD PROTEINS: ORCHESTRATORS OF ERK SIGNALS

ERKs pathway signal output is not solely the result of the diverse phospho-transfer reactions that occur among the constituents of the echelons that build up the route. In addition to the main players, the kinases, past research has unveiled the existence of several types of regulatory and ancillary proteins that participate at different stages of the cascade, and provide further levels of control to the signal flux. Scaffold proteins represent the most abundant, diverse and widespread category (Dhanasekaran et al., 2007). Among the regulatory proteins that

associate to the constituents of a signaling cascade, the accepted requisite for considering a protein a "scaffold," is its capacity to simultaneously bind to at least two members of such cascade, forming a functionally stable complex. The primeval evidence for a protein serving a scaffolding role in a MAPK cascade came from studies in the budding yeast *S. pombe*, in which the protein *Ste5* was found to stabilize the complex formed by *Fus3* (a MAPK), *Ste7* (MAPKK), and *Ste11* (MAPKKK) and to increase their local concentration at the tips of mating projections, in response to mating pheromones (Choi et al., 1994). The identification of mammalian scaffold proteins involved in ERKs signaling followed soon after, with the characterization of KSR1 (Kinase Suppressor of Ras) as a protein binding to CRAF, MEK1/2, and ERK1/2, forming a high-molecular weight macrocomplex (Therrien et al., 1996) whereby signaling flux through the RAS-ERK cascade was regulated. The identification of MP1 (MEK Partner 1) was next. Such scaffold was found to bind MEK1 and ERK1 but nor CRAF, and it exhibited isoform specificity as it potentiated the activation of ERK1 but not ERK2 (Schaeffer et al., 1998). Since then, the list of mammalian proteins that qualify as scaffolds for the RAS-ERK pathway has steadily expanded up to 15-odd members (**Table 1**). Intriguingly, none of these proteins share significant sequence homology, neither among themselves nor with Ste5 of which no mammalian homolog has been identified yet (Kolch, 2005; Dhanasekaran et al., 2007).

Our knowledge on the MAPKs scaffold proteins has grown significantly in recent years. We currently know that their functions extend beyond their central role as hubs for the assembly of the kinases signaling module, whereby MAPKs signals amplitude and duration are fine-tuned (Witzel et al., 2012). In this respect, several notions have gained a solid foothold in the literature. Whilst in some cases not fully demonstrated, an in others subject to vivid controversy, the concepts that follow constitute the bedrock on which our understanding of scaffold proteins has been built upon (for extensive reviews see Kolch, 2005; Dhanasekaran et al., 2007; Good et al., 2011; Witzel et al., 2012; Smith and Scott, 2013; Garbett and Bretscher, 2014), though, as usual in science, they may be far from covering all that there is to be learned about these proteins.

From the structural aspect, the prevailing model is that scaffold proteins would optimize signaling: on one hand, by tethering, thereby increasing the effective concentrations of enzymes and substrates. And on the other hand, by orienting these proteins relative to each other in order to facilitate the phospho-transfer reactions, in such a way that MAPKs will be optimally phosphorylated by the overlaying MAPKKs in a processive fashion (Scott et al., 1995; Levchenko et al., 2000). In addition, scaffolds can also enhance signal flux by acting as allosteric stimulators. For example, it has been shown that overexpression of KSR potentiates RAF activation. This is achieved via the kinase-homology domain of KSR directly binding to RAF and allosterically inducing its kinase activity (Rajakulendran et al., 2009). Furthermore, RAF interaction with KSR in *cis*, triggers a conformational switch on MEK in such a way that its activation loop is exposed and amenable for phosphorylation by RAF in trans (Brennan et al., 2011; **Figure 1**). These allosteric mechanisms represent additional modes of optimizing signal flux, beyond the simple tethering of the different constituents of the cascade together.

Another deep-rooted concept is that scaffold proteins somewhat shield MAPKs from dephosphorylation, by isolating them from soluble phosphatases (Levchenko et al., 2000). A notion that impinges on a hotly debated aspect of scaffold proteins: whether they promote or impede signal amplification. Conceptually, free kinases can activate multiple targets, so the signal is amplified exponentially along the pathway. Contrarily, when tethered onto a scaffold a kinase can only phosphorylate its accompanying substrate kinase, something that would prevent signal amplification. However, if the phosphatases levels are high, a situation in which a system based on freely diffusing kinases will be strongly down-regulated, the enhanced "local" concentration effect achieved by scaffolding will result in signal amplification, by increasing the chances for a successful encounter between kinases in the midst of surrounding high levels of deactivating phosphatases (Locasale et al., 2007; **Figure 2**).

In addition to these ideas, it is now well established that scaffold proteins serve a central role as spatial regulators of ERKs signals, acting in a sublocalization-specific fashion. In this respect, KSR1 acts preferentially upon ERKs signals originated in lipid rafts domains (Matheny et al., 2004). MP-1 acts at endosomes (Teis et al., 2002), Sef is ERKs main scaffold at the Golgi complex (Torii et al., 2004) and Paxillin at focal adhesions (Ishibe et al., 2003). Apparently, such spatial selectivity is important for the definition of ERKs substrate specificity. It has been demonstrated that the type of membrane from which Ras signals emanate dictates which substrates are amenable to be phosphorylated by ERKs, and this is achieved through the participation of defined scaffolds depending on the origin of Ras signals (Casar et al., 2009a). The molecular mechanism whereby scaffold proteins confer substrate specificity to ERKs, is based on the fact that scaffold proteins would facilitate the formation of ERK dimers, in such a way that one ERK molecule would bind to the scaffold and the other to the pertinent substrate (Casar et al., 2008). Thus, scaffold proteins serve as ERK dimerization platforms and in so doing agglutinate the assembly of the enzymatic complexes competent for the activation of ERKs cytoplasmic substrates. In support of this notion we detected that ERKs cytoplasmic substrates such as $cPLA_2$, RSK1, and PDE4, bind exclusively to ERK dimers, while a dimerization-deficient ERK2 mutant was incapable of interacting with cytoplasmic substrates (Casar et al., 2008, 2009b; Herrero et al., 2015). Accordingly, the overexpression of some scaffolds like KSR1, β-arrestin and Sef has been shown to promote ERKs functions at the cytoplasm (Sugimoto et al., 1998; DeFea et al., 2000; Tohgo et al., 2002; Torii et al., 2004) while attenuating those occurring at the nucleus. At this compartment, ERKs functions would be primarily undertaken in monomeric form (Casar et al., 2008; **Figure 3**).

Finally, a pivotal concept in the scaffolds theory is that for any given scaffold there exists an optimal concentration that yields maximum signal efficiency, resulting in a bell-shaped MAPK activation kinetics. In this process, sub-optimal MAPK

TABLE 1 | Locations and functions of ERK MAPK scaffolds in mammalian cells.

Scaffold	Subcellullar Localization	Functions
KSR1, 2	Cytoplasm, Plasma membrane	In resting cells, KSR, Kinase Suppressor of Ras, is bound to MEK in the cytoplasm. Upon Ras activation, KSR translocates with MEK1/2 to the plasma membrane and coordinates the assembly of a multiprotein complex containing Raf, MEK, and ERK which facilitates signal transmission (Roy and Therrien, 2002; Raman et al., 2007; Lavoie and Therrien, 2015). KSR1 acts to both potentiate and attenuate ERK cascade activation (McKay et al., 2009). Deficiency of KSR1 prevents oncogenic Ras signaling in mice (Lozano et al., 2003). KSR1 acts preferentially on ERK1/2 signals emanating from PM cholesterol-rich domains (Matheny et al., 2004). $cPLA_2$ activation is regulated by KSR1 when ERK1/2 are activated from lipid rafts (Casar et al., 2009a).
IQGAP 1	Cytoplasm, Focal adhesion, Cell-Cell junctions, Cytoskeleton	IQGAP1 binds B-Raf, MEK, and ERK and facilitates ERK activation by EGF (Roy et al., 2005). IQGAP1 regulates the phosphorylation of EGFr by ERK (Casar et al., 2009a). Other proteins that bind IQGAP1 include Cdc42 and Rac1, E-cadherin, β-catenin, calmodulin (White et al., 2009). IQGAP1 is over-expressed in some cancers, in some of these, high IQGAP1 levels is a sign of poor prognosis (Brown and Sacks, 2006; Jadeski et al., 2008). Blocking the interaction between IQGAP1 and ERK inhibits skin carcinogenesis driven by Ras-ERK pathway oncogenes (Jameson et al., 2013).
IQGAP 2	Cytoplasm, Cytoskeleton	IQGAP 2 associates with Cdc42, Rac1, F-Actin and calmodulin and regulates cell-cell adhesion. Deficiency of IQGAP2 predisposes to development of hepatocellular carcinoma and diabetes (Vaitheesvaran et al., 2014). Silencing of IQGAP2 contributes to gastric cancer metastasis (Jin et al., 2008).
IQGAP 3	Cytoplasm	IQGAP 3 interacts with ERK1 and enhances its phosphorylation following treatment with EGF (Nojima et al., 2008; Kunimoto et al., 2009). Overexpression of IQGAP3 promoted tumor cell growth, migration and invasion, whereas suppression of IQGAP3 in lung cancer reduces tumorigenicity (Yang et al., 2014). IQGAP 3 plays a role in FGFR1-Ras-ERK signaling, and loss of function of IQGAP3 affects both cell proliferation and cell motility (Fang et al., 2015).
Paxillin	Focal adhesion	Paxillin regulates ERK signaling at focal adhesions through other kinases such as Focal Adhesion Kinase (Ishibe et al., 2004). Paxillin—MEK-ERK complex serves as a regulator of HGF-stimulated FAK and Rac activation in the focal adhesions, thereby regulating tumor cell invasion, plasticity, and metastasis (Deakin et al., 2012). Paxillin is over-expressed in lung adenocarcinoma high-risk patients. Mutations in Paxillin have been associated with enhanced tumor growth and invasion in lung cancer (Mackinnon et al., 2011).
β arrestin 1 and 2	Cytoplasm	β-arrestins mediates ERK activation in clathrin-coated pits (DeFea et al., 2000). β-arrestins act as a scaffolds that bind C-Raf, MEK, and ERK and direct signaling to the cytosol preventing ERK translocation to the nucleus (DeWire et al., 2007; Shenoy and Lefkowitz, 2011) Dysregulation of β-arrestins expression, localization, or phosphorylation is associated with more aggressive cancer phenotypes and poorer prognosis in breast, prostate, lung, brain, and hematological tumors (Sobolesky and Moussa, 2013).
Sef 1	Golgi apparatus	Sef resides at the Golgi apparatus and binds active MEK/ERK complexes preventing ERK translocation to the nucleus but retaining it in the cytoplasm (Torii et al., 2004). Sef acts as a spatial regulator for MAPK signaling allowing phosphorylation to cytosolic substrates but not nuclear targets (Philips, 2004).
ß-Dystroglycan	Plasma membrane, Nucleus	ß-Dystroglycan interacts with MEK and active ERK, modulating ERK activity in response to integrin engagement on laminin (Spence et al., 2004). ß-Dystroglycan is involved in adhesion and adhesion-mediated signaling. Loss of the dystroglycan functions give rise to distinct disease phenotypes including muscular dystrophies and cancer (Mathew et al., 2013; Mitchell et al., 2013).
MP 1	Late Endosomes	MP1, MEK Partner-1, specifically binds to MEK1 and ERK1, but not MEK2 or EKR2 (Schaeffer et al., 1998). Over-expression of MP-1 increased ERK phosphorylation whereas down-regulation of MP-1 reduced MAPK signaling (Teis et al., 2002). MP-1 interacts with the adaptor protein p14 and enhances ERK signaling by targeting this complex to late endosomes (Teis et al., 2006)}.The MP1-p14 scaffold also enhances MEK activation by binding PAK1 to regulate cell adhesion and spreading on fibronectin (Pullikuth et al., 2005).

(Continued)

TABLE 1 | Continued

Scaffold	Subcellular Localization	Functions
RKIP	Cytoplasm	In unstimulated cells RKIP, Raf Kinase Inhibitor Protein, is bound to Raf and prevents MEK phosphorylation (Park et al., 2005). Following mitogenic stimulation, RKIP dissociates from Raf to allow MEK and ERK activation (Kolch, 2005; Shin et al., 2009) RKIP functions as a metastasis suppressor in multiple solid tumor types such as prostate and breast cancer (Keller, 2004). RKIP is down-regulated in some types of cancers and is associated with resistance of cancer cells to anti-neoplastic treatments (Granovsky and Rosner, 2008).
MORG 1	Cytoplasm	MORG 1, MAPK organizer, binds C-Raf, MEK, ERK, and MP1 and facilitates ERK activation when cells are stimulated with lysophosphatidic acid or serum, but not in response to EGF (Vomastek et al., 2004).
OSBP	Cytoplasm	Oxysterol-binding protein, OSBP is a sterol-binding protein that induces ERK activation regulating vesicle transport, lipid metabolism, and signal transduction (Chen and Wang, 2004).
RGS12	Cytoplasm, Plasma membrane	Regulator of G-protein signaling, RGS associates with NGF receptor tyrosine kinase TrkA, activates Ras, B-Raf, and MEK2 and facilitates their coordinated signaling to prolonge ERK activation (Willard et al., 2007). RGS12 modulates PDGF beta receptor signaling in smooth muscle cells (Sambi et al., 2006) and regulates osteoclastogenesis in bone remodeling and pathological bone loss (Yuan et al., 2015).
archvillin	Cytoplasm	Archvillin form a complex with B-Raf, MEK, ERK and 14-3-3 in smooth muscle cells to regulate differentiation and contractility (Gangopadhyay et al., 2004, 2009).
grb10	Cytoplasm, Plasma membrane	grb10 functions as a negative regulator in the insulin –stimulted ERK signaling interacting with Raf-1 and MEK in response to IGF-I or insulin (Charalambous et al., 2003; Langlais et al., 2004; Deng et al., 2008). *Grb10* loss promotes Ras pathway hyperactivation, which promotes hyperproliferation, (Mroue et al., 2015).
dyrk1a	Cytoplasm	dyrk1a prolongs the kinetics of ERK activation by interacting with Ras, B-Raf, and MEK1 to facilitate the formation of a Ras/B-Raf/MEK1 multiprotein complex. Dyrk 1a is required for promoting or maintaining neuronal differentiation and its overexpression contributes to the neurological abnormalities observed in Down syndrome patients (Kelly and Rahmani, 2005). Dyrk1a regulates cell cycle exit, oncogene-induced senescence, and cell differentiation and acts as an oncogene in myeloid leukemias and gliomas (Abbassi et al., 2015; Lee et al., 2016).
GIT1	Cytoplasm, Plasma membrane	GIT1 acts as a scaffold that exerts spatial control of ERK1/2 activation. GIT1 colocalizes with ERK1/2 at focal adhesions. GIT1 overexpression prolongs EGF stimulation of ERK1/2, and knocking down GIT1 expression inhibits EGF stimulated ERK1/2 activity (Yin et al., 2004, 2005).

activation occurs both when there are not sufficient scaffolds to unite all of the available MAPKs, MAPKKs, and MAPKKKs, and also when an excessive scaffold concentration scatters MAPKs, MAPKKs, and MAPKKKs in incomplete, thereby unproductive, complexes. This phenomenon has been termed "combinatorial inhibition" and "prozone effect" (Levchenko et al., 2000; Heinrich et al., 2002). Thus, it would be conceivable that by controlling fluctuations on scaffold concentrations, a biological system would find an efficient mode for regulating MAPKs signal output. Theoretically, maximum intensity for a MAPK signal would be attained only when scaffolds concentrations are at optimal levels. Thus, tilting scaffolds expression, either up or down, could be a valid means to attenuate MAPK signals. Noticeably, the expression of most scaffold proteins is rather stable and not subject to major, immediate changes in response to external stimuli and other factors that govern MAPKs activation. Something that does not preclude that alterations on scaffolds levels, when they do occur, can have profound, long-term effects on the biological outcomes mediated by MAPKs, even contributing to pathological processes. Indeed, it is well documented that some ERK scaffold proteins exhibit altered expression levels in certain types of tumors (www.oncomine.com; www.cBioportal.com).

COORDINATED SCAFFOLDS?

An intriguing aspect about ERKs scaffold proteins is that depletion, or overexpression, of any of them has dramatic effects on ERKs total signal intensity. This is somewhat counterintuitive, considering the sheer number of scaffold proteins and their, supposedly, localized mechanism of action. Conceptually, if we consider a cell in which ERK signals are tuned independently by 15 scaffold proteins, most of them acting in a sublocalization-specific fashion, any alteration on the expression of one of them

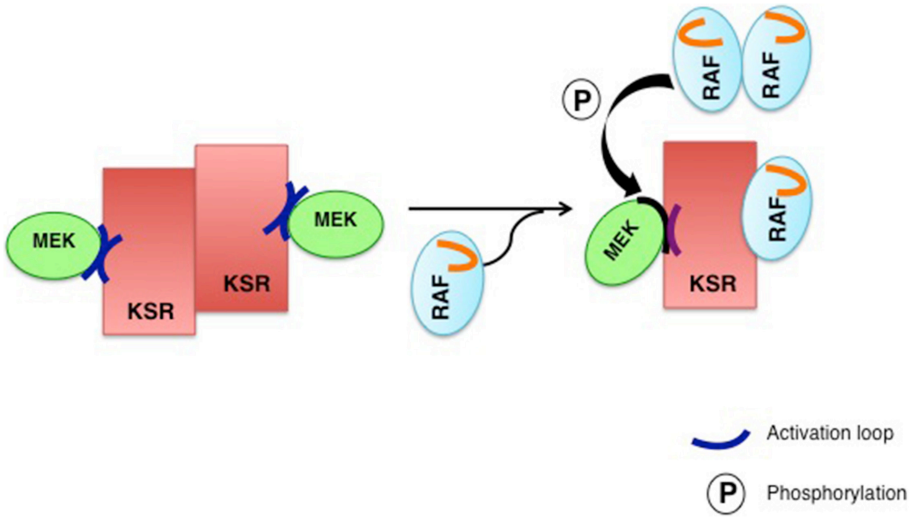

FIGURE 1 | Allosteric regulation of KSR2. A regulatory RAF interacts with KSR in *cis* to induce a conformational switch on MEK to expose its activation loop, subject to phosphorylation by RAF in *trans*. In the KSR2–MEK1 hetero-tetramer (left), the inaccessible activation segment of MEK1 is released through the interaction of KSR2 with RAF, induced by a conformational change, allowing a "catalytic" RAF to phosphorylate MEK (right).

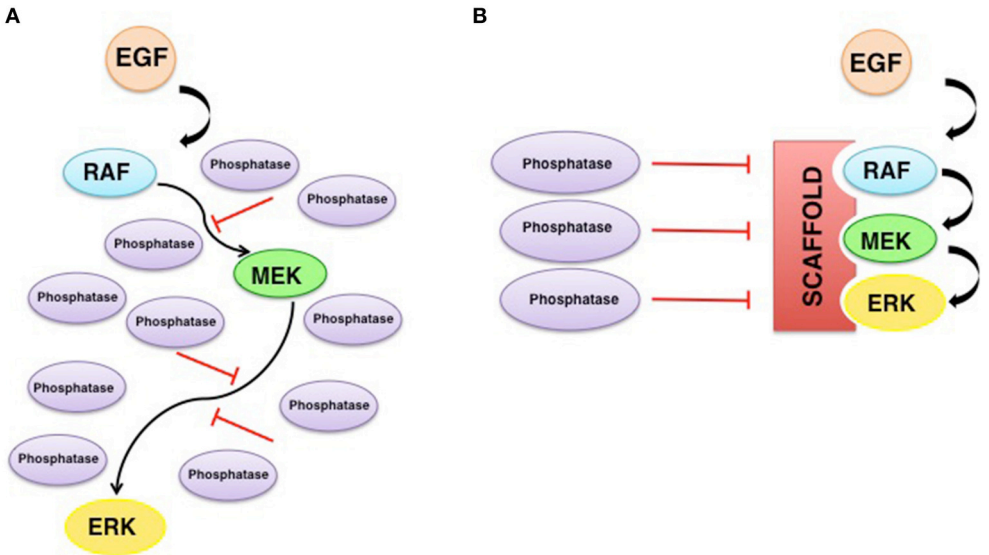

FIGURE 2 | Scaffolding promotes signal amplification in the presence of phosphatases. High phosphatase activity in the absence **(A)** or presence **(B)** of scaffold proteins. When there are no scaffolds, the signal will be strongly down-regulated by phosphatases. Scaffold proteins enhance the local concentration of kinases and shields them from dephosphorylation, facilitating signaling.

should only affect ERKs total activity by about one fifteenth. However, this seems not to be the case. For example, in several studies in which KSR1 levels are down-regulated, either by gene knock-out or using RNA interference, ERK activation levels consistently drop by over 80% (Nguyen et al., 2002; Lozano et al., 2003). A similar situation is observed for IQGAP1 (Roy et al., 2004; Jameson et al., 2013), and for MP1 (Sharma et al., 2005; Teis et al., 2006), just to mention a few cases. Apparently, tampering with scaffold proteins expression levels has far more profound effects than would be expected from proteins that, supposedly, influence ERK signals locally and partially.

One hypothetical explanation for this conundrum would be that scaffold proteins somehow influence the functions of other scaffold proteins. This can be easily envisioned, considering that overexpression of any scaffold should have an impact on other scaffold species that compete for the same pools of kinases, resulting in an increment on the number of incomplete scaffold complexes, for every scaffold, and therefore on less

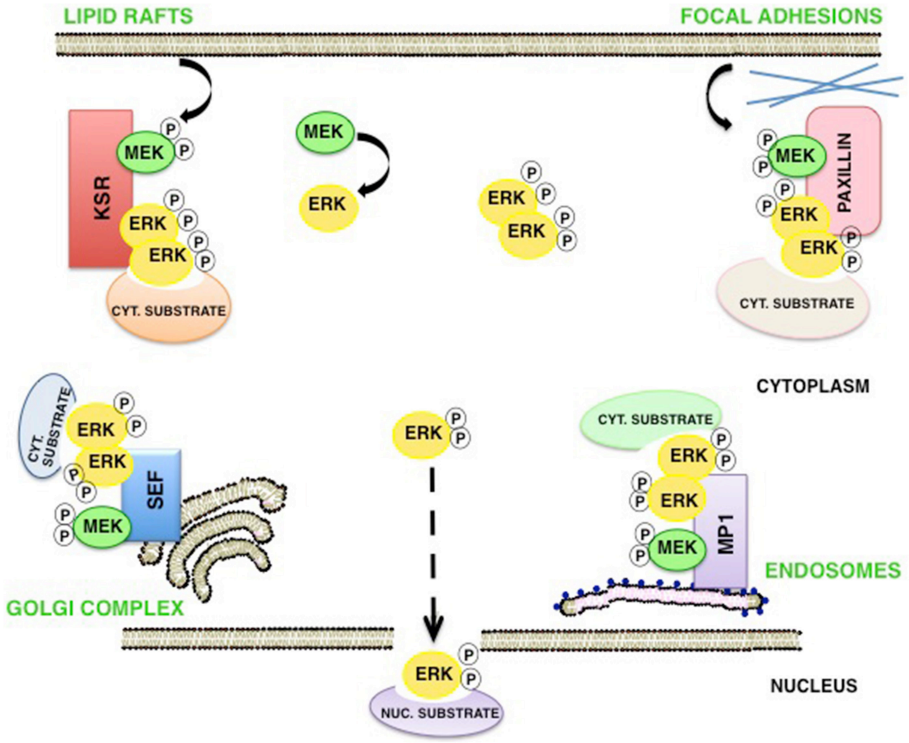

FIGURE 3 | Scaffold proteins as spatial regulators of ERK signaling. In response to stimulation, phosphorylated ERK monomers detach from MEK and may follow three destinies: (1) translocate as monomers to the nucleus; (2) dimerize freely in the cytoplasm, and (3) specific scaffolds act as dimerization platforms in a sublocalization–specific fashion, where ERK dimers are assembled and the new complexes can interact with different cytoplasmic pools of substrates.

efficient signaling overall. By the same token, depletion of scaffold A could even benefit signaling as mediated by scaffold B, by reducing the competition for the same kinases and thereby increasing the number of complete scaffold B complexes (**Figure 4**).

Other plausible explanations have a more mechanistic basis, for example in the case of multi-domain scaffolds, such as paxillin or those of the IQGAP family, involved in multiple signal transduction events (Deakin and Turner, 2008; Smith et al., 2015). It is not unlikely that under- or overexpression of these scaffolds can have an impact on other signaling pathways that, at their turn, may induce changes in the pattern of post-translational modifications of other ERK scaffold species. Many scaffolds, for example KSR1, MP1, and SEF, are subject to phosphorylation, acetylation, ubiquitylation, and other post-translational processes (www.phosphosite.com). In most cases, the functional consequences of these modifications are completely unknown, but it is quite conceivable that, one way or another, they could have some bearing on their behavior as ERK pathway scaffolds. Indeed, it is well documented that the role played by KSR in the RAS-ERK pathway is regulated by diverse phosphorylation events (Muller et al., 2001; Razidlo et al., 2004; Dougherty et al., 2009). Specifically, KSR performance as a RAS-ERK scaffold is regulated by calcium and cAMP signals via phosphorylation (Dougherty et al., 2009; Shen et al., 2013), while these type of signals are tightly regulated by IQGAP1 (Logue et al., 2011). Thus, perturbations on IQGAP1 expression levels might impact on ERK activation both as a direct consequence of IQGAP scaffolding and indirectly, via KSR scaffolding through its regulation by Ca^{2+}/cAMP signals.

Alterations on the expression of a given scaffold could also have broader consequences than expected if scaffold proteins don't act alone mechanistically. As previously mentioned, the prevailing notion is that scaffold proteins act as autonomous entities, regulating ERK signals generated by some specific stimulus, at defined subcellular localizations. However, it cannot be discarded that different scaffolds act in a coordinated fashion to regulate flux through the ERK cascade. Indeed, evidence in mounting showing that scaffold proteins can directly associate among themselves in macromolecular complexes. Several adaptor proteins, docking proteins and scaffold proteins of diverse types are well known to interact in order to form "macro" signaling platforms (Pan et al., 2012). In the case of scaffolds for the ERK pathway, associations between different entities have been demonstrated for: IQGAP1 and MP1 (Schiefermeier et al., 2014), MP1 and MORG1 (Vomastek et al., 2004), IQGAP1 and β-arrestin2 (Feigin et al., 2014), and paxillin and GAB1 (Ren et al., 2004). And this kind of interactions seems to be important for certain cellular processes. For example, the association between IQGAP1 and MP1 appears to be critical for the regulation of focal adhesion dynamics during

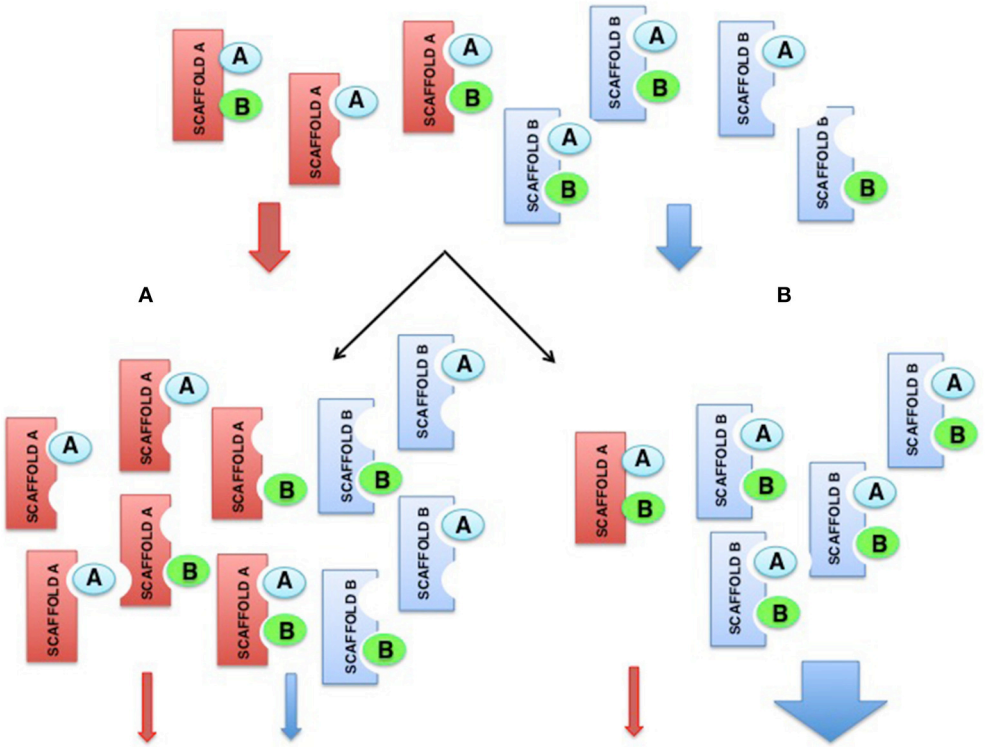

FIGURE 4 | Hypothetical model showing how alterations on scaffolds levels can impact on the functions of other scaffolds. (A) Overexpression of the red scaffold attenuates signals from itself and from the blue scaffold, that competes for the same pools of kinases. **(B)** Depletion of the red scaffold attenuates its own signals but promotes signaling by the blue scaffold as a consequence of the increment on available kinases that increase the number of complete blue scaffold complexes.

cellular migration (Schiefermeier et al., 2014). However, what orchestrates interactions among different scaffolds and how these interactions impact on the ability of each individual scaffold for regulating ERK signaling, are completely open questions at this moment.

Overall, the unveiling of this novel kind of associations is enough to start considering the existence of higher-order "macro-scaffolding" complexes, in which the participation, maybe in some coordinated fashion, of different scaffold species could add one further degree of complexity to the regulation of ERK signals. For example, considering that signals evoked by different stimuli, or emanating from distinct sub-localizations, could engage diverse scaffolds and target distinct pools of substrates (Casar et al., 2009b). It is conceivable that complexes formed by the association of two different scaffold proteins, and competent for "trans-phosphorylation" between the different kinase tiers, may serve as nodes for the integration of incoming, distinct spatially-defined signals, and for the subsequent diversification of outgoing signals with respect to substrate specificity (**Figure 5**). As an example, MORG facilitates ERK activation as evoked by serum but not by EGF (Vomastek et al., 2004). Contrarily, MP1 responds to EGF (Teis et al., 2002) but not to serum (Sharma et al., 2005). If MORG and MP1 directed ERK to different sets of substrates, EGF or serum stimulation would result in activation of just a narrow collection of substrates. However, a MORG-MP1 association would make available the whole spectrum of substrates both to serum- and to EGF-induced ERK signals, provided that trans-phosphorylation occurred between both scaffold complexes.

Furthermore, the possibility exists that an incomplete scaffold complex (missing one or more kinases) could interact in *trans* with another type of scaffold, also partially occupied, to allow trans-phosphorylation. This would enable the different scaffolds to complement, and compensate, each other's kinase deficiencies (**Figure 6**). In this fashion, incomplete scaffold complexes, apparently impaired for supporting efficient signaling, would be capable of contributing to the flux of signals. Thus, if scaffolds were to function cooperatively, signal optimization could be possible under situations in which different scaffold species, acting on their own, would be at a disadvantage. For example, when the levels of some kinase are limiting. This cooperation would be particularly advantageous in those cases in which the collaborating scaffolds exhibit markedly different affinities for the limiting kinase.

Such interdependence among different scaffold species, could offer a plausible explanation for the dramatic consequences on ERK signaling, frequently observed when tampering with the expression levels of most scaffold proteins. If proven to be

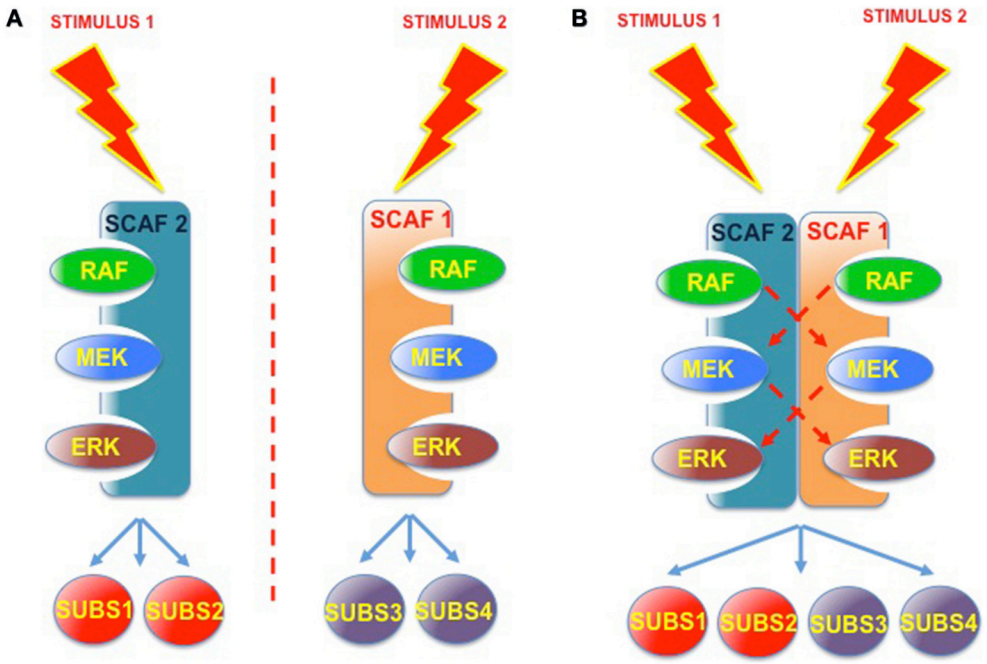

FIGURE 5 | Scaffold-Scaffold interactions as nodes for signal integration. (A) Working independently, defined scaffold proteins respond to specific stimuli and convey signals to a limited number of ERK substrates. **(B)** Scaffold complexes composed of two (or more) scaffold proteins, where trans-phosphorilation among the different kinase tiers would be feasible, would facilitate signal integration, serving as nodes for various incoming signals and for the diversification of outgoing signals with respect to the number of substrates.

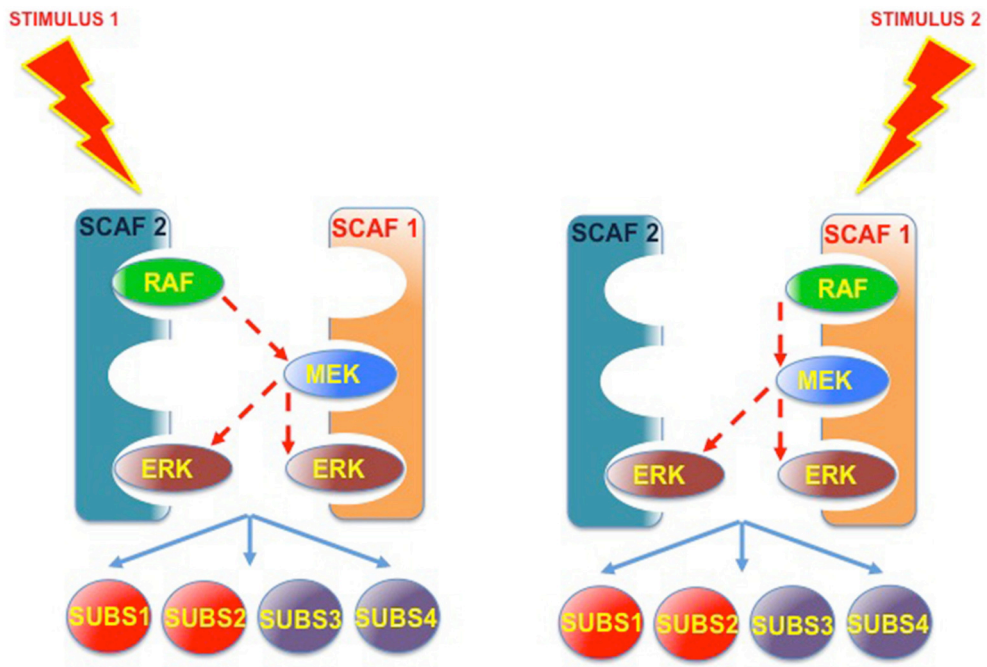

FIGURE 6 | Scaffold-Scaffold interactions may compensate deficiencies in kinases, facilitating signaling. Provided that trans-phosphorylation was possible, complexes formed by two (or more) partially occupied scaffolds would be able to complement each other's kinase deficiencies, so incomplete scaffold complexes, apparently impaired for supporting efficient signaling, would be capable of improving the flux of signals.

correct, associations among different scaffold proteins will add one further degree of regulation for an already tightly regulated cascade and could provide a novel means for manipulating ERK signals, even with therapeutic purposes.

AUTHOR CONTRIBUTIONS

Both PC and BC have made substantial, direct, and intellectual contribution to the work, and approved it for publication.

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REVIEW
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ERK1 and ERK2 Map Kinases: Specific Roles or Functional Redundancy?

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The MAP kinase signaling cascade Ras/Raf/MEK/ERK has been involved in a large variety of cellular and physiological processes that are crucial for life. Many pathological situations have been associated to this pathway. More than one isoform has been described at each level of the cascade. In this review we devoted our attention to ERK1 and ERK2, which are the effector kinases of the pathway. Whether ERK1 and ERK2 specify functional differences or are in contrast functionally redundant, constitutes an ongoing debate despite the huge amount of studies performed to date. In this review we compiled data on ERK1 vs. ERK2 gene structures, protein sequences, expression levels, structural and molecular mechanisms of activation and substrate recognition. We have also attempted to perform a rigorous analysis of studies regarding the individual roles of ERK1 and ERK2 by the means of morpholinos, siRNA, and shRNA silencing as well as gene disruption or gene replacement in mice. Finally, we comment on a recent study of gene and protein evolution of ERK isoforms as a distinct approach to address the same question. Our review permits the evaluation of the relevance of published studies in the field especially when measurements of global ERK activation are taken into account. Our analysis favors the hypothesis of ERK1 and ERK2 exhibiting functional redundancy and points to the concept of the global ERK quantity, and not isoform specificity, as being the essential determinant to achieve ERK function.

Keywords: intracellular signaling, MAP kinases, ERK1 and ERK2 isoforms, gene silencing, gene disruption, expression of isoforms in vertebrates, protein sequence evolution

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INTRODUCTION

The Ras/Raf/MEK/ERK cascade is a key signaling pathway which integrates extracellular clues from cell surface receptors to gene expression and regulation of multiple cellular proteins. ERK cascade plays a crucial role in multiple cellular processes such as cell proliferation, differentiation, adhesion, migration and survival. Therefore, it is essential for many physiological events including development, immunity, metabolism, and memory formation. The core of this pathway consists in activation of the cascade of three kinases Raf, MEK, and ERK. Raf and MEK are described to date as cytoplasmic kinases with a single well established substrate, however ERK is unique in this cascade as it phosphorylates multiple substrates in all cellular compartments (at least 270 substrates have been identified in proteomics screening von Kriegsheim et al., 2009). MEK and ERK can also be activated independently of Raf by the COT/TPL2 kinase (Johannessen et al., 2010) and by mos

during meiotic maturation (Nebreda et al., 1993). Integration of ERK signaling can also proceed from the regulation of scaffolding proteins which function mainly to bring several members of the cascade into close vicinity in order to increase the efficiency and strength of activation (reviewed in Roskoski, 2012; Cseh et al., 2014). Multiple isoforms have been described at each step of the Ras/Raf/MEK/ERK pathway (reviewed in Lefloch et al., 2009). At the levels of Raf and MEK, functional differences and tissue-specific expression among isoforms have been clearly established (see Section Search for Specific Functions of ERK Isoforms). On the other hand, ERK1 and ERK2 seem to be expressed ubiquitously and there are no obvious regulatory differences inferred from their protein sequences, their regulation or their sub-cellular localization. The aim of this review is to put into perspective for the first time the vast body of work that has attempted to find differential roles for ERK1 and ERK2 or tried to demonstrate their functional redundancy.

Prior to scrutinizing studies on ERK isoforms functions, we will recap the main traits of ERK1/2 regulation, action and role to aid in understanding the studies on isoform functions.

Overview on ERK Signaling

Before the molecular cloning of ERKs by Melanie Cobb's group (Boulton et al., 1990), ERK1 and ERK2 were known as two proteins respectively p44 and p42 MAPK rapidly phosphorylated in response to all mitogens (Kohno and Pouysségur, 1986; Sturgill et al., 1988). The essentiality of ERK signaling for cell proliferation of mammalian fibroblasts was first demonstrated by expression of antisense cDNAs or dominant negative mutants which inhibited global ERK activity (Pagès et al., 1993). Later, disruption of the *erk2* isoform was shown to induce early embryonic death (Hatano et al., 2003; Saba-El-Leil et al., 2003; Yao et al., 2003). In adult mice invalidation of both isoforms, led to animal death within 3 weeks by multiple organ failures (Blasco et al., 2011). Collectively, these experiments demonstrate the absolute requirement for a minimal ERK expression to permit proliferation and mammalian life.

Using quantitative proteomics, 284 ERK-interacting proteins have been identified, and 60 of these proteins changed their own binding to ERK upon induction of differentiation (von Kriegsheim et al., 2009). ERKs phosphorylate serine or threonine residues of substrates on the sequence PXS/TP. Many proteins possess this sequence and are not *bona fide* ERK substrates. Specificity is provided by docking motifs located at the back of the kinase (graphical representation on 3D structure in Busca et al. (2015). These docking interactions have been proposed to increase the local concentration of substrates to favor their phosphorylation when ERK is active. Two motifs on ERKs bind to substrates, 16 amino-acids that constitute the common docking site (CDS) also called D-recruitment site (DRS), and 7 amino-acids that constitute the F-recruitment site (FRS). The DEJL motif of substrates (*d*ocking site for *E*RK and *J*NK, *L*XL also called KIM, kinase interacting motif) binds to the DRS (Lee et al., 2004), whereas the DEF (FXFP) motif of substrates binds to the FRS (Liu et al., 2006). The duration of ERK activation can lead to phosphorylation of waves of substrates according to their docking sites content (Murphy et al., 2004). Of great importance for cell fate, ERKs phosphorylate multiple transcription factors, hence active ERKs translocate to the nucleus (Lenormand et al., 1993) mainly by passive diffusion. This ERK nuclear translocation is certainly a key process in ERK signaling. Indeed we demonstrated that retention of active ERK in the cytoplasm abolishes cell cycle progression and the onset of DNA replication (Brunet et al., 1999). ERK nuclear translocation is favored by ERKs binding to the FXFG motif of nucleoporins (Whitehurst et al., 2002), a motif that mimics the FXFP motif of ERKs substrates. Ideed, recently it was shown that ERK-mediated phosphorylation of nucleoporins regulates ERK translocation (Shindo et al., 2016). Others have indicated that ERKs entry into the nucleus may require active transport dependent on Ran, especially when ERKs are fused to beta-galactosidase (Adachi et al., 2000). ERK nuclear accumulation of ERKs requires synthesis of nuclear anchors (Lenormand et al., 1998), however nuclear translocation of ERKs can be regulated by the abundance of interactor proteins, for example over-expression of PEA-15 can sequester ERK in the cytoplasm (Formstecher et al., 2001). Similarly, it was demonstrated that Sef binds to activated forms of MEK, inhibits the dissociation of the MEK–ERK complex, and blocks nuclear translocation of activated ERK (Torii et al., 2004). PEA-15 and Sef expression do not prevent phosphorylation of cytoplasmic substrates by ERK, however they block activation of nuclear substrates.

MEK activates ERKs by dual phosphorylation on the threonine and tyrosine residues of the sequence $T^{185}EY^{187}$ (sequence of human ERK2). Inactivation of ERKs requires the removal of either one or both sites of the TEY motif. Tyrosine-specific phosphatases include PTP-SL, STEP, and HePTP whereas the threonine-specific phosphatases include protein phosphatase 2A and 2C. A large family of dual specificity phosphatases, the DUSPs, can inactivate ERKs. A coordinated action of all these phosphatases induced by ERK is required to shape the temporal ERK activity for proper mammalian development. Invalidation of a single ERK phosphatase such as DUSP6 can be sufficient to increase the basal ERKs phosphorylation level (Li et al., 2007; Maillet et al., 2008). However, DUSP6 invalidation may lead to minor cardiac abnormalities (Maillet et al., 2008) or a range of phenotypes in the same litter such as embryonic death, cranio-facial abnormalities or lack of obvious phenotype (Li et al., 2007). Hence it is difficult to sort out the precise role of each phosphatase to regulate the activation level of ERKs during development and adult homeostasis. Interestingly, in some cases such as the invalidation of DUSP5, phenotypes appear only upon challenging the pathway. Lack of DUSP5 in mouse embryo fibroblasts leads to increase nuclear phospho ERKs content, and lack of DUSP5 in mouse increases sensitivity to mutant Harvey-Ras (HRasQ61L)-driven papilloma (Rushworth et al., 2014). As described above, ERKs nuclear accumulation requires protein neo-synthesis; in fact some of these nuclear anchors are phosphatases since ERK accumulates in the nucleus in the dephosphorylated form (Volmat et al., 2001). DUSP5 is one of the phosphatases that drive ERK

nuclear accumulation and dephosphorylation (Mandl et al., 2005).

Pathological Consequences of Abnormal ERK Signaling

ERK pathway is misregulated *via* germline mutations in genes that encode components or regulators of the cascade, causing disease such as type1 neurofibromatosis and Noonan syndrome (pathologies clustered under the name rasopathies, Rauen, 2013). ERK pathway is also over-activated in many cancers. For example, at the receptor level, the HER2/Neu (EGF family) oncogene can be over-expressed or mutated leading to persistent activation of the pathway (Menard et al., 2004). Similarly EGFR receptor is often mutated in lung and colon cancers (Barber et al., 2004). At the level of Ras, point activating mutations of K-Ras are found in over 95% of pancreatic ductal adenocarcinomas for example (Bryant et al., 2014). Downstream of Ras, the B-Raf kinase is also mutated in many cancers such as at least 66% of melanomas (e.g., mutation B-Raf^{V600E}; Davies et al., 2002). At the level of MEK, somatic mutations have been found via next-generation sequencing of tumoral tissues. Interestingly, in the langerhans cell histiocytosis disease usually B-Raf is mutated; however when B-Raf is not mutated, MEK1 is activated by mutations in 50% of remaining cases (Brown et al., 2014), highlighting again the importance of ERK pathway in oncogenesis. It has also been shown that up to 8% of melanomas present activating mutations of MEK1 or MEK2 (Nikolaev et al., 2012). At the ERK level, amplification of the ERK2 gene has been found in tumors of patients treated with anti EGF-receptor kinase inhibitors. This amplification has been proposed to contribute to the treatment resistance (Ercan et al., 2012). On the contrary, loss of small chromosomal segment encompassing one allele of ERK2 has been observed in children that exhibit microcephaly, impaired cognition, and developmental delay (Samuels et al., 2008).

The very high prevalence of human cancers harboring constitutive activation of the ERK pathway has prompted a massive development of pharmacological inhibitors targeting members of the ERK cascade. After the great success of the tyrosine kinase inhibitor Imatinib to treat chronic myeloid leukemia (CML) by blocking the kinase activity of BCR-ABL, the hope was to obtain similar results when treating many cancers where the driver mutation was in the ERK kinase-cascade. With kinase inhibitors, CML patients are expected to have a normal life-expectancy (Jabbour, 2016). However, for cancers arising from mutations in ERK pathway, patients with mutations at the levels of receptors (HER/Neu, PDGF, and EGF-receptors) relapse after some months of treatment. At the level of Ras, targeted therapies have tried to block the anchoring of Ras at the plasma membrane unsuccessfully (Baines et al., 2011). At the level of Raf, inhibitors of the activated form of B-Raf have been approved recently to treat melanoma, for example PLX4032 (Bollag et al., 2010). Initially, most patients display dramatic improvements of the tumor burden, however rapidly resistances to the treatment arise, leading in most cases to reactivation of ERK pathway. Resistance can occur from amplification of tyrosine kinase receptors, acquisition of mutations in N-Ras, amplification of mutant B-Raf, alternative splicing of mutant B-Raf or even mutations in MEK protein. Moreover, it has been shown that inhibitors of B-Raf^{V600E} mutant can paradoxically activate the ERK pathway, especially in the presence of oncogenic Ras. This was unexpected since B-Raf acts downstream of Ras. However, B-Raf inhibitors drive the formation of B-Raf/C-Raf hetero dimers, where the drug-bound partner drives activation of the drug-free partner through scaffolding or conformational modifications leading to paradoxical activation of cRAF (Poulikakos et al., 2010; Hatzivassiliou et al., 2012). Medical hope in this field lays in pan-Raf inhibitors that target also Src-family of kinases and block all types of Raf dimers (Girotti et al., 2015) or combined therapies, for example with inhibitors at other levels of the ERK cascade.

MEK is mainly activated by Raf, however in some cells MEK has also been described to be activated by the TPL2/Cot pathway. Therefore, tumors have been shown to escape Raf inhibitors by re-activating TPL2/Cot (Johannessen et al., 2010). Furthermore, it has been shown that ERK can retro-phosphorylate MEK1 on threonine 292 to reduce its activation (Mansour et al., 1994; Saito et al., 1994), therefore any decrease of Raf activity by inhibitors, diminishes ERK activity, and as a loop, this decreased ERK activity reduces retro-inhibition of MEK therefore stabilizing a threshold of ERK activation. Considering these retro-controls, it was thought that MEK inhibitors would be better candidates to target cancers driven by activating mutations in ERK pathway. Interestingly, the activating lip of MEK kinase is uniquely structured allowing the design of very potent and specific kinase inhibitors (Ohren et al., 2004). Two families of MEK inhibitors have been designed: allosteric inhibitors acting via binding to the activating lip (e.g., PD184352) and more recently, classical competitors of ATP-binding that block MEK activity (e.g., E6201, Narita et al., 2014). Unfortunately, these inhibitors generate secondary effects in patients and therefore a reduction of dosage or duration of treatment is imposed, hence after an initial effectiveness, tumors relapse. Secondary effects observed during treatment with MEK inhibitors encompass hand and foot rash, diarrhea, nausea, retinopathy, visual disturbance, mental status change, alopecia, stomatitis, and verrucous keratoses (Welsh and Corrie, 2015). In 2013 the MEK inhibitor Trametinib was approved for the treatment of melanoma patients with unresectable or metastatic melanoma with BRAF mutations. As of 2016, many clinical trials are ongoing with several MEK inhibitors demonstrating the endeavor to cure cancers and rasopathies (for example: selumetinib/AZD6244; MEK162; Refametinib/BAY86-9766; Trametinib/ GSK1120212; GDC-0973; PD-0325901; RO5126766 and Cobimetinib/GDC-0973).

The resistance to treatments from Raf and MEK inhibitors has led to clinical trials for combined therapy such treatment with dabrafenib and trametinib for un-resectable or metastatic melanoma with a B-Raf^{V600E} or B-Raf^{V600K} mutations. Nevertheless, the majority of patients face relapse even with the combination of treatments by mechanisms which are not yet understood (reviewed by Queirolo et al., 2015).

Resistance to treatment by inhibitors of RTKs, Raf and MEK have finally led to target ERK itself. Interestingly even cells

resistant to MEK or Raf inhibition (for example by mutations in the kinase pocket of MEK or via amplification of K-Ras expression) were shown to be sensitive to ERK kinase inhibition (Hatzivassiliou et al., 2012; Morris et al., 2013). Specific ERK inhibitors are very difficult to design due to the high homology between ERK and CDK kinases pockets (these kinases belong to the same family of CMGC kinases Manning et al., 2002). For example, the CDK2 inhibitor purvalanol was shown to block activation of ERK at similar concentrations *in-cellulo* despite a higher affinity for purified CDK1/2 protein (Knockaert et al., 2002). As of 2016, five clinical assays are ongoing with four ERK inhibitors (MK-8353/SCH900353, BVD 523, RG7842/GDC0994, and CC-90003). No conclusive results have been reached up to date but these trials bring new and promising hope.

ORIGIN OF ERK ISOFORMS

In 1991 two isoforms of ERK were discovered in mammals: ERK1 (MAPK3) and ERK2 (MAPK1) (Boulton et al., 1991). At that time, two MAP kinases similar to mammalian ERK were discovered in budding yeast (S. cerevisiae), FUS3 and KSS1 (Courchesne et al., 1989; Elion et al., 1990) hence one could consider that two kinase isoforms were necessary from yeast to humans. However, only one kinase similar to ERK was discovered in fission yeast (S. pombe), spk1 (Toda et al., 1991). It is now established that mammalian ERK1/2 isoforms arose independently from budding yeast MAPK isoforms, therefore the comparative studies between FUS3 and KSS1 cannot be transposed to ERK1 and ERK2 studies. Indeed a yeast-specific whole genome duplication (WGD) led to emergence of FUS3 and KSS1 (Wolfe et al., 2015), whereas the vertebrate-specific WGD led to the emergence of ERK1 and ERK2. WGDs are essential events in the evolution that have been observed in all phylla of life, whereby an organism possesses initially two copies of its entire genome. After the duplication event, duplicate genes can have different fates that participate in speciation as described for paramecium (Aury et al., 2006). The origin of the duplication leading to emergence of ERK1 and ERK2 was determined to be early in the vertebrate phylum (Busca et al., 2015). First of all, in all tetrapod clades at least one animal expresses two ERKs that can be phylogenically classified into ERK1 and ERK2 by their protein and nucleic sequences. It is the case for all mammals analyzed so far, for turtles in the reptilian clade and for axolotl in amphibian clade. Among fishes, all teleost fishes express two ERKs and it has been shown that the most ancient branch of ray-finned fishes, the bichir, express ERK1 and ERK2. Demonstration of ERK1 and ERK2 presence at this evolutionary node is important since bichirs diverged from other vertebrates prior to teleost fishes that underwent an additional WGD. Hence ERK1 and ERK2 arose at least 400 million years ago and studies of ERK isoforms in fishes are relevant to mammalian ERK isoforms since they arose from the same duplication event (Busca et al., 2015). Further away in the vertebrate evolution tree, members of all classes of cartilaginous fishes express only one ERK which could be classified into the ERK2 group by three independent topological methods used to infer the phylogeny. At the base of vertebrates, hagfish and lampreys, belonging to the two most divergent vertebrate species compared to mammals, also express only one ERK isoform that is not possible to be classified as ERK1 or ERK2. In all invertebrates studied so far, only one ancestral ERK was identified; hence it is concluded that ERK1 and ERK2 arose from the early WGD at the base of the vertebrate phylogenic tree (representation in **Figure 1**). From ray-finned fishes to humans, ERK isoforms can be easily classified into ERK1 or ERK2 groups according to their coding sequences.

COMPARING STRUCTURES AND REGULATION OF ERK ISOFORMS

Gene Structure

In vertebrates, *erk2* gene is larger than *erk1* gene, this is especially obvious in mammals where *erk1* genes are on average 15 fold smaller than *erk2* genes in the same animal (sizes calculated from ATG initiating codon to stop codon; Busca et al., 2015). The first intron of mammalian *erk2* and the second intron of drosophila *erk* are very large (59 kb and 25 kb respectively). Clearly in drosphila, *erk* intron is among the largest ones, and requires specific factors for proper splicing (Ashton-Beaucage et al., 2010; Roignant and Treisman, 2010). These huge introns could provide a unique regulation during development. This point should be better understood in the future by genomic truncation of the mouse *erk2* gene's first intron for example.

Alternative Splicing in the Coding Sequence

R. Seger and his team reported that the intron 7 of several mammalian ERK1s is not always spliced properly. In rat genome the intron 7 sequence, if not spliced, is in phase with the coding sequence of exons 7 and 8, thereby increasing the size of ERK1 by 26 amino-acids to a predicted protein size of 45.8 kD. Indeed, a signal corresponding to a larger ERK protein has been detected in rat IEC-6 cells by western blot (Boucher et al., 2004). These ERK1 alternative splices were named ERK1b in rat and ERK1c in human and chimpanzee. Human ERK1c predicted size is 40.1 kD instead of 43.1 kD for the normally spliced ERK1 since non-splicing of intron 7 introducing a stop codon. Considering isoforms functionality, it has been reported that ERK1c mediates cell density-induced Golgi fragmentation (Aebersold et al., 2004).

It is tempting to wonder whether these alternative spliced forms provide a ground to explain why ERK1 and ERK2 isoforms have different functions and have been kept after the WGD. We consider that this is very unlikely for several reasons. First, ERK1b and ERK1c splice variants are expressed at very low levels (Aebersold et al., 2004; Boucher et al., 2004); secondly it is extremely puzzling that the sequence of intron 7 is not conserved at all across species: non-spliced intron 7 truncates human ERK1 while it increases the size of rat ERK1. On one hand, it is striking that the sequences of the correctly joined exons 7 and exon 8 are extremely conserved in all mammals, including the monotreme platypus which is phylogenically the most distant mammal to humans (**Figure 2A**). On the contrary, even when restricting the study to rodents, the protein sequences provided by intron 7 are

				ancestral ERK	ERK1	ERK2	
invertebrates	cnidarians			✓			
	worms			✓			
	insects			✓			
	echinoderms			✓			
vertebrates	lampreys / hagfish			✓			
	cartilaginous fishes					✓	
	bony fishes				✓	✓	
	tetrapods	amphibians	axololt		✓	✓	
			frogs			✓	
		reptiles and birds	turtles		✓	✓	
			lizards		✓		
			crocodiles			✓	
			birds			✓	
		mammals	marsupials		✓	✓	
			placentals		✓	✓	

FIGURE 1 | Expression of ERK proteins in animals. ERK1 and ERK2 proteins were classified as such upon phylogenic study of amino-acid coding sequences (same conclusions were reached with nucleotide sequences; Busca et al., 2015).In invertebrates only one *erk* gene was identified so far. Ancestral ERK corresponds to ERK protein sequence that cannot be classified into ERK1 or ERK2 group. Protein expression in vertebrate brains was described in the same study. Animal silhouettes are from phylopic (http://phylopic.org/).

absolutely not conserved. The total lack of conserved protein motifs renders the alternatively spliced ERK1s extremely unlikely to play a function in the cell (**Figure 2B**). In rodents, for example mouse ERK1b has a smaller molecular weight than mouse ERK1 (−0.67 kD) while rat ERK1b has a larger molecular weight than rat ERK1 (+2.66 kD). Finally, in transgenic mice that express only ERK1 cDNA (after disruption of all endogenous alleles of *erk1* and *erk2*), these splice variants do not exist, while mice live and reproduce normally (Frémin et al., 2015). Therefore, this alternative splices of ERK1 protein cannot provide a rationale for a distinct function between ERK1 and ERK2 proteins.

Protein Sequences

Protein sequences of ERK1 and ERK2 are 84% identical in a given mammal; human ERK1 is larger than human ERK2 due to an extension of 17 amino-acids at its N-terminal and 2 amino-acids at its C-terminal. The only described isoform-specific difference leading to functional difference resides in the N-terminal of mammalian ERK1. One report indicates that nuclear localization of ERK1 is slower than that of ERK2 due to the 20 amino-acids of ERK1 situated immediately after the poly-alanine stretch at N-terminal end (Marchi et al., 2008). However, no mechanism that could account for this difference has been reported so far, and again mice expressing only ERK1 are perfectly viable and fertile; hence this difference in rate of nuclear entry is not sufficient to block normal regulation of ERK signaling.

Expression Levels of ERKs

ERK2 is expressed at higher levels than ERK1 in most mammalian tissues (Busca et al., 2015; Frémin et al., 2015). One origin of this difference resides on the strength of the proximal promoters (1 kb upstream ATG codon), mouse *erk2* promoters being much stronger than mouse *erk1* promoter in transiently transfected NIH3T3 cells (Busca et al., 2015). However, the difference of strength between mouse *erk* promoters is larger than the steady state protein ratio measured (20% ERK1 and

A : The C-terminal end of rodents' ERK1 proteins is highly conserved (intron 7 is spliced)

```
                |              Exon 7                |              Exon 8                |stop
rat             LDLLDRMLTFNPNKRITVEEALAHPYLEQYYDPTDE PVAEEPFTFDMELDDLPKERLKELIFQETARFQPGAPEAP
guinea pig      LDLLDRMLTFNPNKRITVEEALAHPYLEQYYDPTDE PVAEEPFTFDMELDDLPKERLKELIFQETARFQPGASDAP
rabbit          LDLLDRMLTFNPNKRITVEEALAHLYLEQYYDPTDE PVAEEPFTFDMELDDLPKERLKELIFQETARFQPGAPEGP
kangaroo-rat    LDLLDRMLTFNPNKRITVEEALAHPYLEQYYDPTDE PVAEEPFTFDMELDDLPKERLKELIFQETARFQPGGPDAP
squirrel        LDLLDRMLTFNPNKRITVEEALAHPYLEQYYDPTDE PVAEEPFTFDMELDDLPKERLKELIFQETARFQPGAPEAP
mouse           LDLLDRMLTFNPNKRITVEEALAHPYLEQYYDPTDE PVAEEPFTFDMELDDLPKERLKELIFQETARFQPGAPEGP


platypus        LDLLDRMLTFNPNKRITVEEALAHPYLEQYYDPTDE PVAEEPFTFDMELDDLPKERLKELIFQETARFQPGHPGAP
```

B: The C-terminal end of rodents' ERK1b proteins diverge (intron 7 is not spliced)

```
                |              Exon 7                |          intron 7          |              exon 8                |stop
rat             LDLLDRMLTFNPNKRITVEEALAHPYLEQYYDPTDE VSRPPAAGRGISVPSVRPVPYCLCPQ PVAEEPFTFDMELDDLPKERLKELIFQETARFQPGAPEAP
guinea pig      LDLLDRMLTFNPNKRITVEEALAHPYLEQYYDPTDE VRQPPAAGQVGEAPPTPGQALLSLPP QPVAEEPFTFDMELDDLPKERLKELIFQETARFQPGASDAP
rabbit          LDLLDRMLTFNPNKRITVEEALAHLYLEQYYDPTDE VPWRRRQRWWGGGGGPSGGVGVSGAW GRGRFRGLAREPSCLCLCSRWPRSPSPSTWSWMIYPRSG
kangaroo-rat    LDLLDRMLTFNPNKRITVEEALAHPYLEQYYDPTDE VGQPLQHCGQTGIGVPHAGPGPYCLC SPSQWLRSPSLSTWSWMTYPRSG
squirrel        LDLLDRMLTFNPNKRITVEEALAHPYLEQYYDPTDE VRQPPAAGQAGRRGNTYPLVPVLSFT VFATPASSRGAVHF
mouse           LDLLDRMLTFNPNKRITVEEALAHPYLEQYYDPTDE VSRSPAAGRGASIPSAQPGPHRLCPL ASGRGAIHLRHGAG
```

FIGURE 2 | *erk1b* RNAs from related species drive synthesis of unrelated proteins domains. Genomic sequences corresponding to exon7-intron7-exon8 of MAPK3 (*erk1*) were retrieved from Ensembl release 83. First line: extension of the introns and exons, separated by vertical bars. "stop" indicates the final of ERK1 and ERK1b coding sequences. Sequences were aligned by multalin program with identity matrix (Corpet, 1988). Alignments were performed also with Tcoffee and Clustal Omega with the same results (not shown). Rat (*Rattus norvegicus*), guinea pig (*Cavia porcellus*), rabbit (*Oryctolagus cuniculus*), kangaroo-rat (*Dipodomys ordii*), squirrel (*Ictidomys tridecemlineatus*), mouse (*Mus musculus*), platypus (*Ornithorhynchus anatinus*). Letters in red, amino-acids highly conserved for a position among the protein sequences; letters in blue, amino-acids showing limited conservation; letters in black, amino-acids showing no conservation in the aligned sequences. **(A)** The C-terminal sequences of rodents' ERK1 proteins are highly conserved; platypus'ERK1 protein sequence is highly similar to rodents' ERK1s. **(B)** Rodents' ERK1b proteins display no significant conserved protein motifs after exon7.

80% ERK2, Lefloch et al., 2008); therefore further research is needed to understand the individual contribution of enhancers, RNA regulation and protein stability to establish the final ERK1/ERK2 protein ratio. One can note that mouse *erk1* RNA has only one short 3-prime UTR (632 bp) whereas mouse ERK2 RNA has a long 3-prime UTR (3777 bp) displaying also an alternative poly-adenylation site (transcripts mapk1-001 and -002 from Ensembl release 83). Does this long 3-prime UTR of ERK2 mouse mRNA provide additional regulation mechanisms? It is important to note that ERK expression is elevated, calculated to be in the μM range by several authors (reviewed in Fujioka et al., 2006). This concentration seems lower than that of MEK but markedly higher than that of Raf. More importantly, when proteomics measurements were performed systematically in mouse NIH3T3 cells, it was shown that ERK2 is among the 400 most expressed cell proteins (twice more expressed than PKA-catalytic subunit for example), and ERK1 is still among the 1500 most expressed cell proteins (10 fold more than p70-S6K for example; Schwanhausser et al., 2011). ERK protein expression is very stable and to our knowledge no stimulus-induced variations of protein quantities have been observed. The half-life of both ERK1 and ERK2 are very long, being of 68 and 53 h respectively as determined by proteomics analysis (Schwanhausser et al., 2011). ERK1 and ERK2 are expressed at different levels and apart from a clear difference in proximal promoter strength that was demonstrated recently, more work is needed to understand the regulation of ERKs protein expression.

Structural Changes upon Activation

The crystal structure of ERK2 protein was the second kinase structure to be resolved after PKA catalytic subunit (Zhang et al., 1994), at present many studies have described the crystal structure of ERK1 and ERK2 when bound to partners and small pharmacological inhibitors. Dual phosphorylation of ERK triggers dramatic conformational changes within the activation lip, reorganizing the substrate binding site to enable recognition of the proline-directed phosphorylation motif of substrates (Pro-X-pSer/pThr-Pro), and reorienting active site residues involved in catalysis (Xiao et al., 2014). Side by side comparison of ERK1 and ERK2 3D structures highlights their close homologies (Ring et al., 2011). However, it was shown, by measuring hydrogen/deuterium exchange, that constraints at the hinge between the lobes of ERK2 were released during activation, which does not seem to be the case for ERK1 (Ring et al., 2011). The functional consequences of this difference have not been currently deciphered since both kinases dramatically increase their catalytic activity upon phosphorylation.

Mechanism of ERKs Activation and Substrate Recognition

ERK1 and ERK2 are simultaneously activated by the same external growth factor agonists, indeed *in vitro* purified MEK1/2 can phosphorylate indiscriminately ERK1 and ERK2 (Robbins et al., 1993). In fact close analysis of isoforms upstream in the cascade failed to identify isoform-specific signaling cascades

(Lefloch et al., 2009). The ratio of active ERKs mimics exactly the ratio of ERK proteins expressed in the cell, furthermore when expression of one isoform is silenced, activation of the remaining isoform is increased. Collectively these observations indicate that MEK activates indistinctively ERK1 and ERK2 and that both compete each other for the upstream activating kinase (Lefloch et al., 2008). Both ERK1 and ERK2 were shown to translocate to the nucleus upon stimulation (Lenormand et al., 1993). Bacterially expressed ERK1 and ERK2 present similar specific activities *in vitro* (Robbins et al., 1993), and previous works of our group showed that this was also the case for immunoprecipitated epitope-tagged ERK1 and ERK2 from HeLa cells (Lefloch et al., 2008). These observations tend to indicate that ERK1 and ERK2 phosphorylate their substrates with the same efficiency, indeed it was shown that all the 284 interactors that bind to ERK2 also bind to ERK1 (von Kriegsheim et al., 2009). In addition, ERK1 and ERK2 share 22 out of 23 amino acids that have been demonstrated to directly interact with substrates (Lee et al., 2004; Liu et al., 2006), the sole difference being a conservative substitution: leucine^{155ERK2} into isoleucine^{175ERK1} (Busca et al., 2015). Therefore, if we consider these data on ERKs activation mechanisms and substrate recognition, no major differences seem to exist between ERK1 and ERK2.

"Dimerization" Domains

Two decades ago Khokhlatchev et al. identified surface residues that could interact to stabilize an ERK2-ERK2 dimer via studying ERK2 crystals (Khokhlatchev et al., 1998). To assist in the presentation of dimerization data in the literature we have drawn a 3D image of ERK1 (from Protein Data Bank (Berman et al., 2000) where the putative dimerization residues are highlighted in yellow and gold (**Figure 3**) on structure 4QTB (Chaikuad et al., 2014). These residues are located on the back of the kinase with a group of four leucines and the sequence PE/DHD that generates a protruding structure. ERK dimerization studies have already been extensively reviewed (Lee and Bae, 2012; Roskoski, 2012). Here we will summarize our current understanding of this process with an emphasis on differences among mammalian ERK1s and ERK2s.

Several studies have failed to observe dimerization of ERK molecules in cells. For example, studies from our group using real time fluorescence microscopy and fluorescence correlation spectroscopy failed to detect any dimer formation (Lidke et al., 2010). Similarly, Burack and Shaw failed to demonstrate ERK2 dimerization in live cells through FRET measurements between co-expressed yellow and cyan fluorescent ERK2s (Burack and Shaw, 2005). By using gel filtration chromatography coupled with multi-angle laser light scattering, Callaway et al. (2006) revealed that histidine-tagged ERK2 is overwhelmingly present as a monomer. Finally with an array of biochemical means, Kaoud et al. have shown that ERK2 without any tag was strictly monomeric, whereas only His-tagged ERK2 could partially form a dimer (Kaoud et al., 2011). More recently, Herrero et al. have reported that ERK2 from chicken embryo extracts does not form dimers unlike ERK2 from mouse cells. Since ERK2s from these two animals are over 99% identical (outside the alanine-rich N-term that is highly variable among all ERKs) and share the same "ERK-dimerization" domains it confirms that complex formation depends on the cellular context (partners) but it does not depend on the ERK2 protein itself (dimer formation).

The lack of ERK dimerization from these studies mentioned above does not invalidate the initial observation of complex formation and dimerization-mutants have proven to be very instructive. For example we showed that dimer^{mutant}-ERK1 activation by MEK and its nuclear entry were delayed (Lidke et al., 2010). Vomastek and co-workers have shown that dimer^{mutant}-ERK2 failed to associate with the Trp protein, a component of the nuclear pore (Vomastek et al., 2008). They further demonstrated that Trp-ERK2 association did not use classical ERK docking sites. More recently, Herrero et al. have shown that ERK's ability to form "dimers" was correlated to PEA15 expression level (Herrero et al., 2015); PEA15 is a well-known ERK partner (Formstecher et al., 2001). Even more interestingly, they have identified a small molecule inhibitor that blocks "dimerization" (it blocks the slower migration of stimulated ERK in native PAGE electrophoresis; Herrero et al., 2015). This inhibitor specifically blocked activation of cytoplasmic ERK substrates and impeded tumorigenesis driven by oncogenes of the RAS/ERK pathway and is the first of a new class of inhibitors that targets interactions of ERK with partners instead of blocking ERK activity. This inhibitor should also help to understand the exact nature of the interaction between ERKs and partner(s) via these "dimerization domains" (Herrero et al., 2015).

Regarding differences between ERK isoforms, the four leucines of the "dimerization" domain are conserved among ERKs of all tetrapods. Therefore, they cannot provide grounds for differences between ERK1 and ERK2. However, all mammalian ERK1s share the PEHD sequence, whereas all mammalian ERK2s have the sequence PDHD (the glutamic acid of ERK1 (E) is highlighted in gold in **Figure 3**). Changing glutamic-acid for aspartic-acid is considered a conserved substitution due to their overall negative charge in solution, therefore via their PEHD or PDHD sequences, ERK1s and ERK2s should bind similarly to interactors. Nonetheless, glutamic acid is bulkier since it has two carbon atoms on its side chain instead of one for aspartic-acid. Therefore, mammalian ERK1s may display a more restricted pattern of interactions with partners by requiring a deeper pocket since these sequences generate protruding structures (**Figure 3**). Interestingly, among all tetrapods, only ERK1s from squamates (snakes, lizards, and geckos) have the aspartic acid at this position. As we shall see later, these animals express only ERK1, which could indicate that the PDHD sequence is more universal than PEHD, being the sequence of tetrapods that express only ERK1 (squamates) and all tetrapods'ERK2s, including animals expressing only ERK2 (birds and frogs). However, invertebrates have only one ancestral ERK that present either glutamic or aspartic acid at this position, and even at the base of vertebrates, lamprey's only ERK has an aspartic acid whereas hagfish unique ERK has a glutamic acid. Therefore, the structural difference, PEHD in mammalian ERK1s vs. PDHD in mammalian ERK2s, is likely to have minor consequences on interaction with partners.

Although, many studies have failed to show ERK dimerization, the two "dimerization domains" play a role

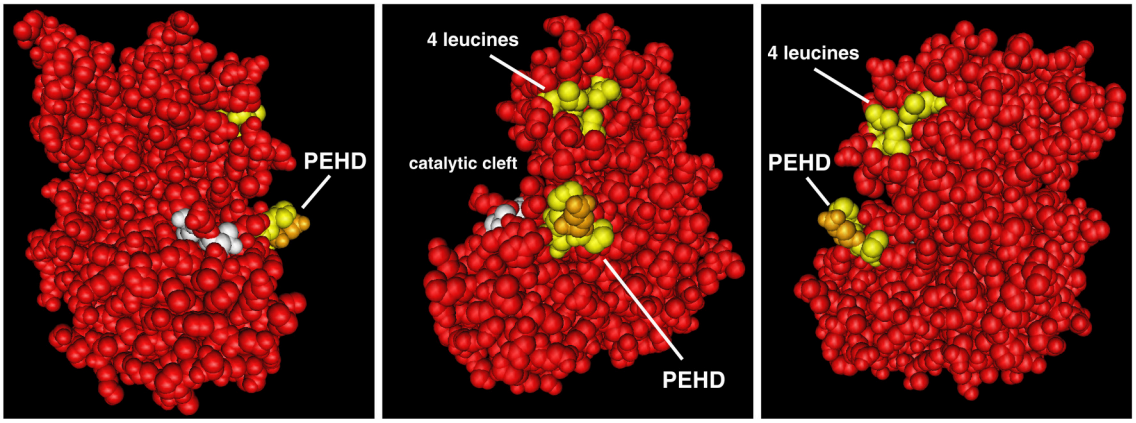

FIGURE 3 | Position of ERK "dimerization" domains on 3D representation of ERK1. The two domains of ERK implicated in "dimerization" are highlighted in yellow on the 3D structure of ERK1 (still-images of structure 4QTB from RCSB PDB, www.rcsb.org, Chaikuad et al., 2014 viewed with CN3D software). "4 leucines" of ERK1 are equivalent to leucines L333, L336, L341, and L344 of mouse ERK2 (Khokhlatchev et al., 1998). In the sequence PEHD (equivalent to $P^{174}DHD^{177}$ of mouse ERK2), the glutamic acid of mammalian ERK1s is highlighted in gold. On mammalian ERK2s this glutamic-acid is replaced by an aspartic acid that is less bulky.

in ERK response. The minor structural differences between ERK1 and ERK2 in the PE/DHD domain may trigger minor signaling differences that may be uncovered when partners that assemble with ERK via this domain will be fully identified.

SEARCH FOR SPECIFIC FUNCTIONS OF ERK ISOFORMS

Upstream of ERK in the signaling cascade, clear functional differences among isoforms have been demonstrated. For example, at the Raf level, B-Raf displays a greater specific activity than A-Raf and C-Raf, and partners of Raf kinases vary greatly (Desideri et al., 2015). Indeed B-Raf is already primed for MEK activation via constitutive phosphorylation of Ser^{445} and amino-acids negatively charged that mimic the phosphorylation status observed in activated A-Raf or C-Raf isoforms (Asp^{448} in B-Raf) (Tran et al., 2005). At the level of MEK1 and MEK2, only MEK1 can be regulated by multiple phosphorylation of its proline-rich domain. MEK1 can be retro-phosphorylated by ERK (Saito et al., 1994) in the context of phosphorylation by PAK kinase that transmits signaling from the cell matrix (Eblen et al., 2004).

Clear differences in the pattern of tissue-specific expression among isoforms as also been demonstrated upstream of ERK. For example, B-Raf has been described to be more expressed in cells of neuronal origin (Storm et al., 1990) and MEK2 was shown to be preferentially expressed in embryos and excluded from adult brains (Alessandrini et al., 1997; Di Benedetto et al., 2007). Taken together, all these observations indicate that the role of individual isoforms can be unique in the pathway; therefore the quest to understand putative functional differences between ERK1 and ERK2 is relevant to further understand all regulatory aspects of this signaling cascade.

In order to seek for specific ERK1 and ERK2 functions, their respective expression levels have been reduced by distinct means and phenotypical consequences have been analyzed. Expression of ERK1 and/or ERK2 has been silenced *via* expression of morpholinos in zebrafish, shRNA/siRNAs in cultured cells or disrupted by gene knock-out in mice. Since ERK1 and ERK2 proteins are usually not expressed at the same level, it is very important to take into account the relative level of both proteins to interpret the data. We want to emphasize that only the dual phosphorylated and activated forms of ERK1 and ERK2 define a common conserved epitope recognized by the same anti-phospho antibody (Busca et al., 2015). Indeed, the direct relationship between the ratio of phosphorylated ERKs and the quantitative expression ratio of ERKs has been previously shown (Lefloch et al., 2008), therefore the easiest way to evaluate the ratio between ERK1 and ERK2 in a biological sample consists in measuring the ratio between the dually-phosphorylated ERK isoforms. Ideally it would be of interest to add-back either isoform by recombinant vectors; unfortunately it is difficult to express successfully ERK cDNAs that can be activated as efficiently as the endogenous ERK. Usually a smaller proportion of transfected ERK is activated compared to endogenous protein (one example is found in Figure 6 of Radtke et al., 2013).

Morpholinos

Morpholinos (modified antisense oligo-nucleotides that are biologically stable) have been injected into zebrafish embryos to assess the contributions of ERK1 and ERK2 during zebrafish development. From the same laboratory, one study concluded that ERK1 and ERK2 target common and distinct gene sets during embryogenesis (Krens et al., 2008a) while another study concluded that cell migration defects during gastrulation were more pronounced upon ERK2 knock-down (Krens et al., 2008b). In this latter publication the authors indicate that morpholinos-mediated knock-down of ERKs could be rescued by co-injection of the corresponding mRNA. Strikingly, *erk2* mRNA cross-rescued ERK1 knockdown, but *erk1* mRNA was unable to rescue

ERK2 knockdown. These results tend to indicate that ERK1 and ERK2 play different roles in zebrafish development. A close look of the data reveals that ERK2 morpholinos reduce markedly more the global level of phospho-ERK than ERK1 morpholinos (measured by immuno-histochemistry with anti-phospho ERK antibody at 4.5 and 8 h post fertilization, Figure 6 in Krens et al., 2008b). Furthermore, when evaluating the expression level of six genes at 4.5 h post fertilization (goosecoid; antivin; vox; vent; notail; tbx6), injection of morpholinos targeting ERK1 reduced mildly the level of 5 genes, whereas morpholinos targeting ERK2 reduced strongly the level of all genes (Figure 10 in Krens et al., 2008a). Taken together, these two observations seem to indicate that ERK2 is more expressed than ERK1 in zebrafish embryos, consequently the different outcome observed when reducing ERK1 or ERK2 quantity could be linked to their different effectiveness to reduce global ERK activity, not due to isoform-specific functions. Injection of *erk1* RNA did not rescue anti-ERK2 morpholinos, whereas the opposite situation was effective. However, in this study no data indicate that *erk1* RNA re-established a normal level of global phospho-ERKs to conclude unambiguously an ERK1-specific effect.

Silencing by siRNA and shRNAs

Knock-down of ERK isoforms using specific siRNAs has been used in several studies to replace chemical inhibitors that inhibit ERK activity in cells. For example, in two lines of ovarian-cancers cells (HeyC2 and KGN), simultaneous diminution of ERK1 and ERK2 by a pool of siRNA was shown to reduce cell proliferation more effectively than MEK inhibition by either one of two chemical inhibitors (PD98059 and U0126; Steinmetz et al., 2004).

At least 27 publications have evaluated the role of individual ERK isoforms in biological processes via siRNA mediated knock-downs, and 4 more publications have combined the expression of shRNA directed to ERK2 with genomic *erk1* gene knock-out. It must be noted that removal of ERK1 alone induced phenotypical changes in 13 studies (**Table 1**); we can therefore conclude that ERK1 plays significant roles in many mammalian tissues that can be compensated in ERK1$^{-/-}$ animals. The fact that ERK1 has a functional role in mammals can also be deduced from the high stability of ERK1 protein sequence during evolution (Busca et al., 2015), if ERK1 had been dispensable its sequence would have derived rapidly.

ERK1-Specific Effects

Two studies have concluded that reduction of ERK1 expression was effective to induce phenotypes whereas reducing ERK2 expression was ineffective. In the study of Jung et al. the authors tested the role of ERK isoforms on the action of cordycepin on vascular smooth muscle cells (VSMC) (Jung et al., 2012). In VSMC, cordycepin increases the levels of p27KIP1 while it reduces both CDK4 levels and cell proliferation. These three actions of cordycepin were reversed by transfection of a single siRNA targeting ERK1 but not by a siRNA targeting ERK2. In these cells, the expression levels of ERK1 and ERK2 appeared very similar, or slightly higher for ERK2, if we look at the presented phospho-ERK immuno-blots. Unfortunately, the effectiveness of each siRNA to reduce the protein level of its target was not shown; hence it is not possible to correlate the decrease of global active ERK by the siRNAs with the phenotypical consequences. In the study of Bae et al. (2013), a single siRNA was also used against each ERK, and the effectiveness of each siRNAs to reduce ERKs levels was not presented, nor was shown the effectiveness of each siRNA to reduce the global active ERK levels. The authors claim that only transfection of siRNA targeting ERK1 reduced the IGF-1 mediated induction of two mucin genes, in NCIH292 airway epithelial cells (Bae et al., 2013). Altogether, the lack of controls in these two publications impairs concluding that only the decrease of ERK1 expression can trigger specific phenotypes (Jung et al., 2012; Bae et al., 2013).

Different Effects upon Silencing ERK1 or ERK2

Three publications propose different outcomes after knocking-down of ERK1 vs. ERK2 (one that will be discussed in the following paragraph on hepatocytes). In rhesus fibroblasts infected with rhadinovirus, Woodson and Kedes. conclude that knock-down of ERK1 seems to increase viral production whereas ERK2 accumulates preferentially into viral particles (respectively Figures 9, 10 in Woodson and Kedes, 2012). In these cells ERK2 is much more expressed than ERK1 as determined by phospho-ERK levels (Figure 6A of Woodson and Kedes, 2012) and in reality ERK2 does not accumulates preferentially in virions since the authors state in the caption of their Figure 8 that "intravirion ERK content reflects intracellular expression of ERK isoforms". Further, of concerns for interpreting the data in Figure 8 of Woodson and Kedes (2012), the total-ERK and phospho-ERK blots have been inverted.

In a study regarding human epithelioid malignant mesotheliomas (Shukla et al., 2011), the authors propose that ERK1 and ERK2 play different functions on the ground that gene expression is altered differently in stable clones expressing less ERK1 vs. stable clones expressing less ERK2. Unfortunately one cannot find measurement of the relative level of ERK1 and ERK2 since no phospho-specific antibodies were used, and this makes the interpretation of the data very difficult. Could their results be simply due to different contributions of ERK1 and ERK2 to global ERK activity? (Shukla et al., 2011).

ERK2-Specific Effects

In hepatocytes

Fourteen publications state that ERK2 silencing triggers biological consequences whereas ERK1 silencing does not. Among these studies, several were performed in hepatocytes with contrasting results that we have attempted to analyze. At first glance, in most experiments hepatocytes seem to express equivalent levels of ERK1 and ERK2, an ideal situation to compare the contribution of these isoforms to conduct ERK signaling. However, a closer look at the data reveals that it is clearly not the case in all hepatocytes cultures; for example in hepatocytes from a publication of Figure 3 in Frémin et al. (2009) there is clearly much more ERK2 than ERK1 (similarly in stellar HSC-T6 cells from the study of Figure 1C in Zhong et al., 2009). In a single publication, the ERK1/ERK2 ratio seems to vary between experiments and the reason for

TABLE 1 | Overview of studies using shRNA or siRNA transfections to study ERK1 vs. ERK2 signaling.

References	ERK1/2 ratio	Effect on phospho-ERKs	Cell line	Phenotypes studied
PHENOTYPES ONLY ERK1-DEPENDENT				
Zhong et al., 2009	E1 ≥ E2	Yes	Rat hepatic stellate HSC-T6	Cell proliferation, gene induction, hepatic fibrosis
Jung et al., 2012	E2 ≥ E1	Not done	Rat vascular smooth muscle cells	Cordycepin dependant block of cell proliferation
Bae et al., 2013	E2 > E1	Not done	Human pulmonary NCI-H292	IGF1-dependant MUC8 and MUC5B induction
PHENOTYPES ONLY ERK2-DEPENDENT				
Vantaggiato et al., 2006	E2 > E1	yes	Mouse embryo fibroblasts + NIH3T3	Cell proliferation, colony and tumor formation
Li and Johnson, 2006	E2 > E1	Yes	Mouse myoblasts C2C12	Myoblast proliferation and differentiation
Wille et al., 2007	E2 > E1	Yes	Hybridoma 1B6 T	IL-2 production from TCR stimulation
Bessard et al., 2008	E2 > E1	Yes	Rat hepatoma cell line, rat biliary epithel.	Hepatocytes proliferation
Carcamo-Orive et al., 2008	E2 > E1	Not done	Human mesenchymal stem cells	Proliferation and adipogenic differentiation
Li et al., 2009	E2 > E1	Yes	Mouse NIH3T3	TGF-beta1-induced collagen synthesis
Shin et al., 2010	E2 > E1	Not done	Human mammary gland MCF-10A	Epithelial-to-mesenchymal transformation
Botta et al., 2012	Not done	Not done	H. pancreatic ductal epithelial cells	Cell invasion, MMP RNA increase
Lee et al., 2013	E2 > E1	Yes	Human HSC-3 and MDA-MB-231	Expression of tumor-derived G-CSF
Radtke et al., 2013	E2 > E1	Yes	H. non-small cell lung carcinoma A549	HGF-induced cell motility, paxillin phosphorylation
Shin et al., 2013	E2 > E1	Yes	Mouse embryo fibroblasts	Increase $p19^{mArf}$ and $p16^{Ink4a}$, senescence
Bonito et al., 2014	E2 > E1	Yes	Human osteosarcoma cells U2OS	Expression of cytokine receptor sub-unit gp130
Gusenbauer et al., 2015	E2 > E1	Not done	H. squamous carcinoma cell SCC9	Amphiregulin upregulation by HGF
Chang et al., 2015	E2 > E1	Not done	H. monocytic leukemia cell line THP-1	LPS-induced G-CSF
PHENOTYPES ERK1 and ERK2-DEPENDENT				
Zeng et al., 2005	E2 > E1	Yes	Human ovarian epithelium pOSE	Cell viability
Lefloch et al., 2008	E2 > E1	Yes	Mouse NIH3T3	Cell proliferation
Wei et al., 2010	Not done	Not done	Human breast MCF7	Etoposide-induced G2/M arrest, ATM pathway
Wang et al., 2011	E2 > E1	Yes	Human chondrocytes	Osteoarthritis/cartilage breakdown
Wei et al., 2011	E1 = E2	Not done	Human breast MCF7	Hydroxy-urea induced DNA damage response
Shukla et al., 2011	Not done	Not done	Human mesotheliomas HMESO	Cell proliferation, migration and tumor growth
Woodson and Kedes, 2012	E2 > E1	Yes	Rhesus monkey fibroblasts	RRV virus production and localization inside virion
Qin et al., 2012	E2 > E1	Yes	Human A375 melanoma cells	Cell proliferation and cell death
Frémin et al., 2012	E2 ≥ E1	Yes	Rat hepatocytes	Survival, proliferation, differentiation state
Zhu et al., 2015	E2>E1	Yes	H. rhabdomyosarcoma RD	Enterovirus (EV71) replication
shRNA MEDIATED SILENCINGS AND *erk1* GENE DISRUPTION				
Fremin et al., 2007	E1 = E2	Yes	Primary murine hepatocytes	Cell proliferation
Frémin et al., 2009	E2 ≥ E1	Yes	Primary rat hepatocytes	Cell proliferation and survival
Voisin et al., 2010	E1 ≥ E2	Yes	Mouse embryo fibroblasts	Cell proliferation
Guegan et al., 2013	E2 > E1	Yes	H. hepatocellular carcinoma cells Huh-7	Cisplatin-induced cell death

"ERK1/2 ratio" (2nd column) was determined indirectly from western-blots probed with anti-phospho-ERK antibodies revealing active ERK levels, as demonstrated previously (Lefloch et al., 2008). "Effect on phospho-ERKs" (3rd column) indicates whether or not the impact of shRNAs or siRNAs on phospho-ERKs levels was evaluated by western-blot. Only the main phenotypes studied are presented (last column).

these changes is not explained. One explanation could be the very low level of active ERKs in most of the hepatocytes preparation, a low level that magnifies the background from antibodies that have some weak affinity for mono or even non-phosphorylated forms of ERKs. This situation has been encountered by Jung et al. in vascular smooth muscle cells, in cells where global ERK activity is elevated the immunoblot reveals much more phospho-ERK2 than phospho-ERK1 (Figure 4A in Jung et al., 2012); whereas there was not much difference between phospho-ERK1/ERK2 in the blot of their Figure 4B when ERK activity was much lower. The same conclusion can be drawn from Figure 1A in a study from Steinmetz et al. (2004), where extracts from the same cell line are loaded side by side, only the extracts with high level of phospho-ERK

demonstrate the clear prevalence of ERK2 (Steinmetz et al., 2004).

Furthermore, in hepatocytes it was shown for the first time that following ERK1 or ERK2 decrease, the remaining isoform was over-activated up to 11 fold (Fremin et al., 2007). Therefore, the global strength of ERK activation following removal of ERK1 or ERK2 needs to be measured, which is usually difficult to do when the activation levels are low, as it seems to be the case in most hepatocytes preparations. A clear example of this problem is shown in Figure 6D from a study by Frémin et al. (2012) where global phospho ERK seemed to be increased upon knock-down of ERK1 due to increased intensity of phospho-ERK2 band. Indeed, opposite consequences on phenotypes have been found; for example Zhong et al. (2009) have shown that decrease of ERK1 suppressed hepatic fibrosis and reduced cell proliferation. On the contrary Frémin et al. have concluded that only ERK2 silencing reduced hepatocytes proliferation (Fremin et al., 2007). In a later study, these authors have shown that ERK1 silencing could enhance hepatocyte survival (Frémin et al., 2009). More recently it has been shown that dual silencing of both ERK1 and ERK2 is required to maintain a highly differentiated state of hepatocytes, while survival and proliferation was suggested to be regulated via a complex interplay between ERK1 and ERK2 functions (Frémin et al., 2012). Similarly the fate of PC12 cells toward proliferation or differentiation was shown to be dependent on the strength and duration of ERK signaling, therefore a precise knowledge of the global level of active ERK is required all along these long lasting processes, to be able to effectively assign a function to ERK1 vs. ERK2 isoforms (Dikic et al., 1994; Traverse et al., 1994). Even small differences in ERK1/ERK2 expression level could be translated into differences of global ERK level, leading into different phenotypical consequences.

ERK2-specific effects in other cell lines

Twelve studies, independent of those using hepatocytes, have revealed phenotypes induced only by ERK2 decrease, it is essential to note that in 11 of them, cells express markedly more ERK2 which by itself could provide an explanation for the lack of effect of ERK1 knock down as demonstrated previously in NIH3T3 cells (Lefloch et al., 2008). In the last study out, the ratio cannot be determined by lack of phospho-ERK immunoblot (Botta et al., 2012).

In the studies of Chang et al. (2015), Gusenbauer et al. (2015), and Carcamo-Orive et al. (2008) unfortunately the effectiveness of siRNAs to reduce global ERK activation is not demonstrated, impeding to draw conclusions. Bonito et al. (2014) report that differences between both ERKs knock-down could not be attributed to quantitative differences because they extend their work to MCF7 cells which they claim to express the same level of ERK1 and ERK2. This remains to be demonstrated unambiguously, since unfortunately they did not measure phospho-ERK levels in those cells; furthermore the effect of ERK2 silencing triggered only a 30–40% diminution of GP130 expression, therefore a 10–20% decrease that could be caused by ERK1 silencing (if ERK1 was slightly less expressed than ERK2), would be difficult to demonstrate. Most of their conclusions were drawn from cell lines expressing markedly more ERK2 than ERK1 (Bonito et al., 2014). Finally, the studies of Li and Johnson (2006), Shin et al. (2013), and Li et al. (2009) have shown that only ERK2 silencing markedly reduces global ERK activity, providing a direct explanation for the lack of consequences after ERK1 knock-down.

Studies proposing ERK2-specific roles from add-back strategies

In MCF-10A breast cancer cells, Shin and co-workers elegantly demonstrate that the epithelium-mesenchymal transition is dependent on ERK activity via interaction with substrates containing DEF docking sites (Shin et al., 2010). However, these authors did not assess the effectiveness of the shRNAs to decrease global ERK activity, nor did they present the capacity of transfected ERK isoforms to re-establish the global ERK activity after knock-down. Direct comparison of ERK1 vs. ERK2 expression from transfected plasmids is rendered complicated by the use of different tags in ERK1 and ERK2 (measurement of expression with total ERK antibody could introduce an isoform-bias as discussed above). Furthermore, Figure S4 in Shin et al. (2010) reveals that ERK2 is much more expressed than ERK1 in those cells, which could explain why only ERK2 silencing impeded epithelio-mesenchymal transition. Vantaggiato and co-workers have also transfected ERK isoforms and showed that ERK1 expression was able to block Ras-induced increase of colony formation in contrast to transfected ERK2, however the size of transfected ERK1 is abnormally smaller than that of ERK2, rendering difficult the interpretation of the results (Vantaggiato et al., 2006). These authors have also shown that removal of ERK1 in a stable line of mouse embryo fibroblasts (MEFs) accelerates the rate of cell proliferation whereas removal of ERK2 decreases this parameter. This observation is surprising, first because the ERK1 removal in MEFs was demonstrated by others to slow cell proliferation (in five MEFs preparations of early cell culture passages; Voisin et al., 2010), and second because mice over-expressing ERK1 live and reproduce normally, even in the total absence of ERK2 protein (Frémin et al., 2015). Indeed Voisin et al. have demonstrated that individual loss of either ERK1 or ERK2 slows down the proliferation rate of fibroblasts to an extent reflecting the expression level of the kinases (Voisin et al., 2010). In A549 lung carcinoma cells, Radtke et al. (2013) concluded that only ERK2 mediates HGF-induced motility. In these cells ERK2 is markedly more expressed than ERK1, and in Figure 5A of the paper only shERK2 is shown to reduce global ERK activity which could explain why only an ERK2-mediated effect was observed. In an add-back experiment (Figure 6 in Radtke et al., 2013) HA-tagged ERK proteins are hugely expressed and furthermore only transfected HA-ERK2 was markedly stimulated upon HGF treatment. This can explain again why only ERK2 mediates the action of HGF (Radtke et al., 2013).

One well controlled study reporting ERK2-specific effect

In HSC-3 cells, Lee et al. report that a decrease in ERK1 expression reduces more effectively global ERK activity than ERK2 decrease, but only ERK2 knockdown reduces G-CSF mRNA levels (Figure 5 in Lee et al., 2013). For the first time,

this study clearly favors a distinct role for ERK isoforms, however in the same study with another cell line (MDA-MB-231) the same authors report a direct correlation between the global ERK activation (reduced significantly only by ERK2 knockdown) and G-CSF mRNA levels.

Redundant Effects

If we consider the remaining 10 studies that report a biological consequence after reducing ERK1 quantity, all of them have also revealed a specific phenotype upon ERK2 protein diminution. Most of these studies (7 out of 10) reveal a redundant role of both ERK isoforms. Unfortunately, for the study of Zeng et al. (2005) (siRNAs in cancerous ovarian cells) and for two publications from the group of D. Tang (siRNA in MCF7 cells, Wei et al., 2010, 2011) the decrease in phospho-ERKs was not measured, rendering impossible to correlate the higher effectiveness of ERK1 knock-down to a slower ovarian cell proliferation for example. Surprisingly the same blot was used in both papers from the group of D. Tang to illustrate the effectiveness of each shRNA to reduce expression of their targets.

Fortunately several publications have presented the effectiveness of knock-downs to regulate global ERK activity, allowing to correctly interpreting the data. For example, the production of EV71 viral particles in Rhabdomyoscarcoma cells was shown to be equally diminished by reduction of ERK1 or ERK2 expression (Zhu et al., 2015). Here, both siRNAs effectively reduced the global level of active ERKs, despite the fact that these cells seem to express more ERK2. Pharmacological inhibition of MEK activity by U0126 inhibitor was more effective to reduce global ERK phosphorylation level, and was shown to further reduce the production of viral particles (Zhu et al., 2015). In human melanoma cell line A375, Qin et al. have shown that silencing of ERK1 or ERK2 reduces the levels of active ERK and killed the cells by similar apoptosis induction. Silencing of ERK isoform was performed with lentiviral particles that express shRNAs. Interestingly enough, ERK1 silencing was sufficient to reduce the global levels of active ERK, although the expression level of ERK1 is somewhat lower than that of ERK2 in these cells (Qin et al., 2012). In human chondrocytes infected with lentivirus expressing shRNA, Wang et al. (2011) showed that knock-down of either ERK1 orERK2 reduces the mRNA levels of MMP3 and MMP13 and type II collagen, while double knock-down of ERK1 and ERK2 acted synergistically. Furthermore, there is a correlation between the effectiveness of the shRNAs to reduce the global levels of active ERK and the consequences on MMPs and collagen II expression.

Conclusions from Studies with siRNAs and shRNAs

Overall nearly all publications claiming a specific role of ERK2 can be re-interpreted by observing that higher reduction of global ERK activity upon ERK2 silencing induces more effects than upon ERK1 silencing. Doubts persist only in studies that cannot be carefully interpreted by lack of assessment of the contribution of each isoform to the global ERK activity. Only in one out of two cell lines of the study of Lee et al. (2013) was demonstrated an ERK2-specific effect. In the few cell lines studied that express more ERK1 than ERK2, ERK1 was demonstrated to be the pre-eminent isoform driving defined phenotypes. Therefore, in all but one study, the observed phenotype develops proportionally to the reduction of the global ERK activity.

Of special interest is the work of Wille et al. who used stable expression of shRNA targeting ERK1 or ERK2. The stable clones generated expressed various levels of shRNAs, producing a quantitative range of knock-downs in mouse 1B6 hybridoma cells (Wille et al., 2007). These authors demonstrate a strict correlation between global ERK activity and a signal output (IL-2 production) irrespective of the targeted isoform.

Similarly, in NIH3T3 it has been demonstrated that ERK1 knock-down decreases cell proliferation only when ERK2 is lowered at the same time to a threshold level. In these NIH3T3 fibroblasts, ERK activity is mainly provided by the ERK2 isoform that represents 80% of the total ERK pool (Lefloch et al., 2008). Globally, experiments knocking-down ERK1 and ERK2 by siRNAs and shRNAs converge to reveal that ERK1 and ERK2 contribute to ERK signaling according to their contribution to global ERK activation; therefore suggesting their functional redundancy at least for the read-out reported.

erk1, erk2 Gene Disruption

Several studies have tried to uncover functional differences between ERK1 and ERK2 by gene knock out in mice. Mouse ERK1 was disrupted by removal of exon 3 (Pages et al., 1999). ERK1$^{-/-}$ animals lived and reproduced normally in striking contrast to *erk2* disruption that led to early embryonic death (Hatano et al., 2003; Saba-El-Leil et al., 2003; Yao et al., 2003). The opposite fates of ERK1 vs. ERK2 invalidations initiated the quest to discover whether these two conserved kinases play distinct roles. Lack of ERK2 led to placental failure that could account for the early lethality (Hatano et al., 2003; Saba-El-Leil et al., 2003). When the placental defect was rescued by tetraploid-aggregation, ERK2-deficient fetus grew as well as littermate controls for 5 more days, up to E13.5 (Hatano et al., 2003), however this rescue did not allow animals to be born alive. To remove the possibility that lethality was due to a delayed placental failure, the group of Meloche has recently shown that *erk2* disruption in the epiblast still led to lethality (they induced a CRE-dependent ERK2 knock-out in the whole embryo except placenta; Frémin et al., 2015). Indeed in a different mouse genetic background, it was shown that *erk2* disruption did not form mesoderm (Yao et al., 2003). Overall, ERK2 is absolutely required for mammal life at several stages of development. In adult mice already lacking ERK1, inducible invalidation of ERK2 led to death by multiple organ failures within weeks (Blasco et al., 2011).

In order to correctly interpret the data from genomic disruptions of ERK isoforms, one should at least analyze the contribution of ERK1 and ERK2 to the global ERK activity in wild-type tissues. Ideally total ERK activity of tissues from wild-type animals should be compared with tissues from mice lacking one ERK isoform, since lack of one isoform is partially compensated by over-activation of the remaining one.

erk1 Disruption

At least 17 studies describe consequences of ERK1 sole removal in mice. ERK1 being less expressed than ERK2 in most mouse tissues (Frémin et al., 2015), phenotypes are usually not dramatic and great care needs to be taken to interpret the data. For example it was initially reported that lack of ERK1 impeded terminal differentiation of CD4CD8 thymocytes (Pages et al., 1999), however studies with congenic mice (different genotypes in the same litter) led to conclude that thymocytes proliferate and differentiate normally in mice lacking ERK1 (Fischer et al., 2005; Nekrasova et al., 2005). Goplen and co-workers have shown by western-blot analysis that the lack of ERK1 decreased by half the global ERK activity in airway tissues thereby impairing mice lacking ERK1 to develop airway inflammation and hyper-reactivity to experimental asthma (Goplen et al., 2012). **Table 2** illustrates the multiple phenotypes observed in mice lacking ERK1, such as resistance to obesity (Bost et al., 2005), increase in long-term memory (Mazzucchelli et al., 2002) and hyper-activity in open field (Selcher et al., 2001). Considering these multiple studies, although mice lacking ERK1 live and reproduce normally, ERK1 is an essential kinase which role is can be revealed upon challenging the ERK pathway. For example, mice lacking ERK1 develop less skin papilloma generated by DMBA and TPA treatment than wild-type mice (Bourcier et al., 2006).

erk2 Disruption

Since the absence of ERK2 without artificial compensation is lethal for mice, hemizygote disruption ($ERK2^{+/-}$) and hypomorphic mice were generated. The hypomorphic animals expressed about 20–40% less ERK2 than wild-type ones due to insertion of the neomycin resistance cassette in the 5-prime region of the *erk2* gene (Satoh et al., 2007). The mere presence of the neo cassette reduced ERK2 expression, but unfortunately the active ERK levels were not presented in this study. Nonetheless, gene dosage was indirectly demonstrated since it was impossible to generate an animal expressing only a single hypomorphic allele of ERK2 whereas a smaller increment in ERK2 expression via a single wild-type ERK2 allele was sufficient to sustain life (Satoh et al., 2007). Spatial working memory was normal in animals expressing less ERK2, however mutant mice showed a deficit in long-term memory in classical fear conditioning (Satoh et al., 2007). Lips et al. compared hemizygote disruption of ERK2 with total disruption of ERK1 in ischemic injury studies. Global ERK was immuno-precipitated from $ERK2^{+/-}$ and $ERK1^{-/-}$ hearts to measure kinase activity on MBP substrate (Lips et al., 2004). Mice lacking both alleles of ERK1 or only one allele of ERK2 presented very similar levels of ERK activity in hearts as observed in the western blots presented, however these authors claim that only $ERK2^{+/-}$ mice presented an increased infart area in heart after ischemic injury. The infart surface represented the 30% of the heart in WT mice and it increased up to the 40% $ERK2^{+/-}$ mice. Considering that mouse hearts express more ERK2 than ERK1 in their study, they may have missed a small difference in ERK immuno-precipitated kinase activity that could explain the lack of statistically significant effect in $ERK1^{-/-}$ mice compared to the mild effect in $ERK2^{+/-}$ (Lips et al., 2004). In an independent study from the same laboratory, neither mice lacking both alleles of ERK1 nor mice lacking one allele of ERK2 showed a reduction in pathologic or physiologic stimulus-induced cardiac growth (Purcell et al., 2007). In that study, the impact of gene disruption on phospho-ERK level was negligible and could explain this lack of effect (Figure 1B of Purcell et al., 2007). However, expression of the phosphatase DUSP6 reduced markedly ERK activity in the heart and predisposed mice to heart decompensation and failure (Purcell et al., 2007).

Chen and co-workers have invalided *erk1* and *erk2* genes in mouse embryonic stem cells (ESCs) by Talen or Crisp/Cas9 technologies (Chen H. et al., 2015) (respectively Transcription Activator-Like Effector Nucleases and Clustered Regularly Interspaced Short Palindromic Repeats). These authors established readily $erk1^{-/-}$ and $erk2^{-/-}$ cells. However, double disruption of *erk1* and *erk2* genes was impossible unless ERK1 expression was induced from a cDNA stably integrated. MEK inhibition by small chemical inhibitors is known to promote self-renewal of embryonic stem cells, here ERK expression irrespective of the isoform, and ERK kinase activity were demonstrated to be necessary for ES-cells self-renewal and genomic stability (Chen H. et al., 2015).

Tissue Specific *erk2* Disruption

Tissue specific disruption of *erk2* is necessary to bypass early embryonic lethality. For this purpose exon 3 of ERK2 was surrounded by two loxP sites, and upon expression of the Cre recombinase, exon 3 was deleted to generate a non-functional ERK2 protein (Fischer et al., 2005). Lines of mice expressing Cre recombinase in a tissue at a specific stage of development were crossed with mice harboring ERK2 gene with loxP sites on each side of exon 3 (in **Table 2** mice are listed by the promotor driving Cre expression, which defines the cells were recombination occurs). Alternatively, the recombinase can be ubiquitously expressed, but its expression can be pharmacologically switched on. For example tamoxifen has been shown to activate CRE in a timely fashion in Cre-ERT2 mice. Indeed, in adult Cre induction by tamoxifphen led to death by multiple organ failures upon deletion of ERK2 in mice already lacking ERK1 constitutively (Blasco et al., 2011). Finally, Cre recombinase can be expressed by viral infection of airway cells with adenoviruses encoding Cre (Blasco et al., 2011).

Out of 19 publications presenting tissue-specific disruption of *erk2*, we shall limit our detailed analysis to the 4 studies where ERK1 was shown to be more expressed than ERK2 in targeted cells, and the 5 studies where ERK2 was shown to be more expressed than ERK1 (ratio evaluated by western-blot displaying phospho-ERK levels, **Table 2**). In other publications, the expression ratio between isoforms cannot be evaluated, impeding proper isoform-assignment to the observed phenotypes.

Among cells expressing somewhat more ERK1, Blasco et al. indicate that lung tumor development driven by $K\text{-}Ras^{G12V}$ is not impaired upon invalidation of a single isoform; however disruption of both ERK1 and ERK2 appears necessary to block tumor progression (Blasco et al., 2011). Richardson et al. describe that macrophages can proliferate and differentiate by expressing either ERK1 or ERK2. In mice expressing ERK1, removal of *erk2* gene by Cre-recombinase does not change the profile

TABLE 2 | Overview of studies using genomic disruption to study ERK1 vs. ERK2 signaling.

Refernces	ERK1/2 ratio	Effect on phospho-ERKs	Promoter of Cre recombinase	Main phenotypes
***erk1* GENE DISRUPTION IN WHOLE MICE**				
Pages et al., 1999	E2 > E1	Yes		Thymocytes differentiation
Selcher et al., 2001	E1 = E2	Yes		Behavior, activity in the open field, fear, learning, fear acquisition
Mazzucchelli et al., 2002	E2 > E1	Yes		Long-term memory, rewarding properties of morphine
Nekrasova et al., 2005	Variable	Yes		Thymocytes differenciation, priming encephalomyelitis
Bost et al., 2005	E1 = E2	Not done		Adipose tissue development, obesity and insulin resistance
Agrawal et al., 2006	not done	Not done		Thymocytes Th1 polarization, immune response, encephalomyelitis
Ferguson et al., 2006	Not done	Not done		Psychomotor sensitization to cocaine, behavioral plasticity
Cestari et al., 2006	Not done	Not done		Memory reconsolidation, fear conditioning
Bourcier et al., 2006	E2 > E1	Yes/No		Cutaneous lesions, TPA+DMBA induction of skin papillomas
Nakazawa et al., 2008	E2 > E1	Yes		NMDA-induced retinal injury
Alter et al., 2010	E2 > E1	Yes		Formalin-induced long-term heat hypersensitivity, pain models
Lee et al., 2010	E2 > E1	Yes		Adiposity and adipogenesis, insulin resistance
Cisse et al., 2011	Not done	Not done		Secretion of N1 fragment of cellular prion protein PrP(c)
Jager et al., 2011	Not done	Not done		Obesity, insulin resistance, liver steatosis, glucose uptake
Saulnier et al., 2012	Not done	Not done		Osteopetrosis, differentiation of hematopoietic stem cells
Goplen et al., 2012	E1 = E2	Yes		Thymocytes Th2 differentiation, asthma
***erk2* GENE DISRUPTION IN WHOLE MICE**				
Saba-El-Leil et al., 2003				Embryonic lethality, placenta development
Yao et al., 2003				Embryonic lethality, mesoderm differentiation
Hatano et al., 2003				Embryonic lethality, placenta development
Lips et al., 2004	Not done	Yes (IP)		(Loss of only one erk2 allele) ischemia-reperfusion injury, infarction
Purcell et al., 2007	E2 > E1	Yes		(Loss of only one erk2 allele) cardiac hypertrophic growth response
***erk1* AND *erk2* DISRUPTION IN CELLS**				
Chen H. et al., 2015	E2 > E1	Not done		Self-renewal, genome stability and pluripotency of mouse ESCs
***erk2* GENE DISRUPTION IN TISSUES**				
Satoh et al., 2007	Not done	Not done	EIIA–Cre + partial ERK2	Long term memory, fear conditioning
Newbern et al., 2008	Not done	Not done	Wnt1:Cre (neural crest)	Developmental defects
Samuels et al., 2008	Not done	Not done	hGFAP–(neural progenitor)	Proliferation, differentiation, cognition, memory formation
Satoh et al., 2009	E2 = E1	Yes	EIIA-Cre	Re-epithelization, burn healing, keratinocytes proliferation
Hamilton et al., 2013	E2 > E1	Yes	Get embryonic stem cells	Pluripotency-associated transcripts
Ulm et al., 2014	On 2 gels	Not clear	MLC2v-CRE (cardiomyocyte)	Hypertrophic remodeling of cardiomyocytes, apoptosis
Frémin et al., 2015	E2 > E1	Yes	Sox2:Cre (whole embryo)	Embryo development with normal placenta
***erk2* GENE DISRUPTION IN TISSUES IN MICE LACKING *erk1* GENE**				
Fischer et al., 2005	Not done	Not done	Cd4-Cre and Lck-Cre	CD4 and CD8 T-cell lineage commitment
Ishii et al., 2012	Not done	Not done	CNP-Cre	Myelin growth, oligodendrocyte differentiation

(Continued)

TABLE 2 | Continued

Refernces	ERK1/2 ratio	Effect on phospho-ERKs	Promoter of Cre recombinase	Main phenotypes
Fan et al., 2009	E2 > E1	Yes	Cyp19-Cre	Oocyte activation, ovulation, luteinization
D'Souza et al., 2008	Not done	Not done	dLck-iCre	CD8 T-cell activation, proliferation and survival
Matsushita et al., 2009	Not done	Not done	Col2a1-Cre and Prx1-Cre	Lineage specification of osteo-chondroprogenitor, osteoblast
Srinivasan et al., 2009	Not done	Not done	Tie2-Cre	Endothelial cell proliferation, migration during angiogenesis
Imamura et al., 2010	Not done	Not done	Nestin-Cre	Cortical brain development
Blasco et al., 2011	E1 ≥ E2	Yes	Cre-adenoV + inducible Cre-ERT2	K-Ras induced tumors in lungs, mice death upon KO in adulthood
Sebastian et al., 2011	Not done	Not done	Col2a1-Cre	Growth of cartilaginous skeletal elements, synchondrosis closure
Fyffe-Maricich et al., 2011	Not done	Not done	hGFAP–Cre+ NG2–Cre (=Cspg4Cre)	Differentiation/proliferation of oligodendrocytes, myelination
He et al., 2011	E1 > E2	Yes	Mx1-Cre	Osteoclast differentiation, adhesion, migration, bone resorption
Satoh et al., 2011b	Not done	Not done	Nestin-Cre	Brain development, behavior
Satoh et al., 2011a	E2 > E1	Yes	Nestin-Cre and EIIA-Cre	Social behaviors and learning disabilities
Kehat et al., 2011	E1 ≥ E2	Yes	Nkx2.5-Cre	Cardiac hypertrophy, lengthening vs thickening of myocytes
Otsubo et al., 2012	E2 > E1	Yes	Nestin-Cre	Responses to pain models
Chan et al., 2013	Not done	Not done	Mx1-Cre	Hematopoietic stem cells proliferation, differentiation, aplasia
Staser et al., 2013	Not done	Not done	Mx1-Cre	Hematopoietic stem cells proliferation and differentiation (HSPCs)
Richardson et al., 2015	E1 ≥ E2	Yes	Lyz2-Cre	Proliferation of bone marrow progenitors, macrophages induction
O'Brien et al., 2015	Not in all	Not in all	Nav1.8-Cre	Inflammatory pain, sensory neurons proliferation-differentiation
Chen Z. et al., 2015	Not done	Not done	Osx-Cre	Chondrocyte terminal differentiation, enchondromas

"ERK1/2 ratio" and "Effect on phospho-ERKs" (2nd and 3rd column) are presented as in ***Table 1****.The promoters driving Cre-recombinase in mice are presented in 4*th *column. Upon crossing these mice with erk2$^{floxed/floxed}$ mice, erk2 gene will be invalidated in the tissues expressing the recombinase. Only the main phenotypes studied are presented (last column).*

of macrophages, however in mice lacking ERK1, the only macrophages that were encountered were those that did not lose ERK2 (failure of Cre-recombinase to invalidate *erk2* gene; Richardson et al., 2015). In osteoclasts, He et al. (2011) have demonstrated that ERK1 is markedly more expressed than ERK2 and only *erk1*$^{-/-}$ osteoblast display a clear reduction of the global ERK activity. Indeed only in *erk1*$^{-/-}$ cells they observed that the ERK substrate p90RSK is less phosphorylated and osteoclast differentiation and bone resorptive activity is reduced. Kehat et al. (2011) have shown that global invalidation of ERK1 and ERK2 was necessary to regulate the balance between eccentric and concentric growth of the heart. They did not assess the contribution isoforms to regulate this balance.

Focusing on ERK2-targeted cells/tissues that express more ERK2, for example, in granulosa cells of mouse ovaries, Fan et al. showed that invalidation of ERK2 or ERK1 partially reduced global ERK activation whereas invalidation of both isoforms abrogated ERK activity (Figure S1A of Fan et al., 2009). Then they demonstrated that maturation of ovocytes and birth of mice pups was abrogated only when both isoforms were invalidated. Interestingly, oocyte maturation occurred nearly normally when global ERK signaling in granulosa cells was provided either by a single ERK1 allele or two alleles of *erk2* (no experiment with only one allele of *erk2* was presented). Considering the care of these authors to show global activation levels in different mice lines, these results clearly argue for a redundant role of ERK isoforms in mouse granulosa cells (Fan et al., 2009).

Satoh et al. invalidated *erk2* in mice brain and observed abnormal social behavior of these mice and impairment of long-term memory (Satoh et al., 2011a). *erk2* disruption led to increased ERK1 activation measured by anti phospho-ERK antibody. To study ERK1 contribution, these authors injected a MEK inhibitor intraperitoneally and observed that 1 h later the levels of phospho-ERKs in the hippocampus, cortex and cerebellum were reduced. In animals lacking ERK2, the inhibitor did not modify the phenotype observed, this result was interpreted by the authors as a failure of ERK1 to drive these behavioral and memory features (especially because ERK1 was over-activated in these brain regions of *erk2*$^{-/-(brain)}$ mice; Satoh et al., 2011a). An alternative explanation could be that

a minimal threshold of global ERK activity for phenotype was already reached in animals lacking ERK2, impeding further behavioral damage. Indeed these authors did not show that the limit of behavioral damage could be increased further than what was observed after *erk2* disruption. With the same model, Ostubo et al. studied the nociceptive response in mice and also implicated ERK2 but not ERK1, again because pharmacological inhibition did not increase the $erk2^{-/-(\text{brain})}$ phenotype (Otsubo et al., 2012). However, in the same laboratory, Satoh et al. showed that the sole deficiency of ERK2 in mice disrupted mildly brain development, however further invalidation of ERK1 aggravated the phenotype leading to death within 1 day after birth (the pups failed to breast-feed in double knock-out mice). In these brain areas, ERK2 was more expressed than ERK1. Hence with this model of single vs. dual invalidation, these authors conclude that total ERK activity, and not a specific ERK isoform, governs cellular behaviors to ensure proper brain development (Satoh et al., 2011b). Hamilton and co-workers invalidated ERK2 expression in mouse embryonic stem cells that express more ERK2 and showed that cells lacking ERK2 displayed enhanced self-renewal capacity and remained even more undifferentiated (Hamilton et al., 2013). Interestingly, these phenotypes were reversed by re-expression of either ERK1 or ERK2 arguing again in favor of isoform redundancy.

In the remaining 13 publications that study consequences of *erk2* gene disruption by recombinase in a specific tissue, the ratio between isoforms was not presented. However, 6 of these studies aimed at determining the role of global ERK activity, not isoform contributions in biological processes (Fischer et al., 2005; Matsushita et al., 2009; Srinivasan et al., 2009; Sebastian et al., 2011; Chan et al., 2013; Chen Z. et al., 2015), and 4 studies demonstrated that there is virtually no phenotype in single mutants whereas in the double *erk1*+*erk2* knockout phenotypes are exacerbated, re-enforcing the notion of isoform functional redundancy (Imamura et al., 2010; Satoh et al., 2011b; Ishii et al., 2012; Staser et al., 2013). Three remaining publications conclude for a specific role of ERK2 (out of 13 that did not quantify isoform ratios), however from our point of view, the lack of measurement of ERK1/ERK2 ratio does not allow to draw clear cut conclusions (D'Souza et al., 2008; Fyffe-Maricich et al., 2011; O'Brien et al., 2015). Furthermore, in a given tissue Cre recombinase expression may not invalidate alleles in all cells. Therefore, it is mandatory to measure to which extent global ERK activity is effectively decreased following mice crossings. This is particularly obvious in light of the minimal decrease of ERK activity observed by O'Brien et al. in dorsal root ganglia of mouse invalidated for ERK2 (Figure 5A of O'Brien et al., 2015). In that study, only phenotypes of animals lacking both isoforms allow to conclude that ERK is essential for sensory neuron biology.

Conclusions from *erk1, erk2* Gene Disruptions in Mice

When taking into account the isoform ratio and effectiveness to reduce global ERK activity, the studies presenting disruption of *erk1/2* alleles in mice, give overwhelmingly credit toward functional redundancy between ERK1 and ERK2.

Isoform Loss in Vertebrates

As described above, silencing experiments in cells and gene disruption in mice provide strong arguments for a functionally redundant role of ERK isoforms, furthermore, deletion of *erk1* gene in laboratory mice is compensated by increase of endogenous ERK2 activity to allow normal development and reproduction. Therefore, one can wonder why all mammals analyzed so far have kept expression of both ERK1 and ERK2? We have used another approach to investigate this question by obtaining insights from vertebrate evolution (Busca et al., 2015).

First it is striking that cartilaginous fishes, birds and frogs do not possess *erk1* genes, confirming that vertebrate life is compatible with total loss of one isoform. All other vertebrates analyzed so far possess *erk1* and *erk2* genes. The high expression level of ERK proteins, and the availability of anti-phospho ERK antibodies allowed to determine expression of isoforms in all vertebrates (the phospho ERK epitope is 100% conserved in vertebrates; Busca et al., 2015). In reptiles, two species of turtles express both ERK1 and ERK2, but crocodiles do not express ERK1 at detectable levels, and more surprisingly no ERK2 protein was detected in squamates (snakes lizards and geckos). In lizards, ERK2 was not present in all brain areas studied and all adult tissues tested (it is important to note that ERK is highly expressed in the brain). To confirm unambiguously this observation, only siRNA targeting ERK1 reduced ERK protein expression in lizard embryo fibroblasts. As controls, the pools of siRNA targeting ERK1 or ERK2 were able to reduce the level of their respective RNAs. The strength of *erk* proximal promoters were compared (about 1 kb upstream initiating ATG). In lizards, *erk2* promoter is markedly weaker than *erk1* promoter, whereas it is the opposite for mouse promoters (Busca et al., 2015). Therefore, in vertebrates, ERK signal can be provided either by both isoforms or only by ERK1 or ERK2. One reason explaining the predominance of isoforms lies at least on the strength of their respective promoters.

The question can be turned up-side down: why most vertebrates express functionally redundant ERK isoforms? As presented above, ERK1 and ERK2 isoforms appeared about 400 million years ago in the course of whole genome duplication (WGD) in early vertebrates. Most duplicate genes are lost progressively during evolution of species; however after a WGD the loss of duplicated genes is slower than gene loss after local duplication (Blomme et al., 2006). Indeed, from studying WGD in paramecium, it was shown that duplicates with no divergent functions can be kept for of millions of years; interestingly genes were shown to be kept longer as duplicates if they are highly expressed and/or if they belong to signaling cascades (Aury et al., 2006; Brunet et al., 2006). Therefore, considering that ERK1 and ERK2 are both highly expressed and signaling molecules, they are good candidates to be kept as duplicates for many millions of years, even without being endowed with novel functions nor sub-functionalization. Nonetheless, duplicates can still be lost when dosage is provided by one isoform, already cartilaginous fishes, birds and frogs have lost the gene one *erk* isoform. Will a wild mammal lacking one *erk* gene ever be found?

Phylogenic studies revealed that ERK1 and ERK2 nucleotide sequences evolved at similar rates, but the sequences of ERK1 proteins evolved faster than those of ERK2 proteins. This could indicate a less important/dispensable role of ERK1. However, it should be noted that among all vertebrate MAP kinases, ERK2 sequences are the most stable ones, and the rate of ERK1 sequences evolution is still very low, in the range measured for all p38-MAP kinases (Li et al., 2011). By aligning sequences on ERK1's 3D structure, it was shown that the few positions displaying variable amino-acids among mammalian ERK1s are all located on the back of the kinase, away from domains that bind to activators and substrates and away from kinase effector domains. Therefore, positions essential for all known functions are invariant in vertebrate ERK1 and ERK2 sequences, arguing against isoform-specific functional differences. In most vertebrates, and especially in mammals, *erk2* genes are much larger than *erk1* (15 fold) providing a structural explanation (and not a functional one), for the slower evolution rate of ERK2 protein sequences, since larger genes can recombine more easily to purify mutations during evolution (Marais et al., 2005; Liao et al., 2006).

Overall these phylogenic studies accredit the hypothesis that ERK1 and ERK2 are functionally redundant kinases, whose protein domains essential for function remain extremely conserved across evolution.

Replacement of *erk2* in Mice

In the three studies that have described the lethality of *erk2* gene disruption in mice (Hatano et al., 2003; Saba-El-Leil et al., 2003; Yao et al., 2003), it was suggested that ERK1 is insufficiently expressed at some developmental stages to complement ERK2 loss to convey properly ERK signaling. To validate this hypothesis, Frémin and co-workers intended to express *erk1* cDNA in the first exon of endogenous mouse *erk2* gene, which should have allowed expression of ERK1 at the levels of endogenous ERK2 since the half-life of mouse ERK mRNAs and mouse ERK proteins were shown to be very similar (Schwanhausser et al., 2011). Unfortunately, neither *erk1* cDNA, nor a control *erk2* cDNA, drove expression to levels near those of endogenous *erk2*, precluding the use of these constructs to generate mice.

However, these authors generated transgenic mice expressing ERK1 under the control of the ubiquitously expressed chicken beta-actin promoter (Frémin et al., 2015). In these mice, transgenic-ERK1 was expressed in all early embryonic stages to all adult tissues at elevated levels. Upon crossing these mice with $erk2^{+/-}$ mice, it was possible to obtain mice lacking both alleles of *erk2* at Mendelian rates. They found that mice lacking ERK2 lived and reproduced normally, establishing that ERK1 protein can replace ERK2 throughout all mammalian life. It is very interesting to note that ERK1 seemed to be more expressed in transgenic-ERK1 animals than ERK2 in wild-type animals, however the global level of ERK activity was very similar in both animal lines (Frémin et al., 2015). In few tissues such as the heart, transgenic-ERK1 expression was much higher than ERK2 expression in WT-animals, but global active ERK seemed nearly identical in transgenic-ERK1 animals and WT ones (Figure S3H in Frémin et al., 2015). This observation confirms the high resiliency of ERK signaling to perturbations such as over-expression of one component, at least in the absence of challenging stimuli. In mouse embryo expressing transgenic-*erk1* in $erk2^{f/f}$ background, the highly expressed ERK1 protein did not change the expression of endogenous ERK2 but it captured most of the activation from upstream MEK, illustrating clearly again the resiliency of the pathway (ERK2 activation is markedly diminished, Figure S3C in Frémin et al., 2015). This observation demonstrates once more the equivalence of ERK1 and ERK2 isoforms to receive MEK activation. Furthermore, upon crossing animals missing different alleles of ERK isoforms, these authors have demonstrated that the extent of placental and embryonic development is strictly correlated to global ERK activity (Frémin et al., 2015).

The lethality of *erk2* loss in mice can be compensated when ERK1 expression is increased in all tissues. Then, there is a tight correlation between global ERK activity and the phenotypes observed, irrespectively of the isoform expressed. Taken together these observations demonstrate functionally redundant roles for ERK1 and ERK2 by this gene-replacement strategy.

CONCLUSIONS/PERSPECTIVES

Deregulation of the ERK signaling cascade is devastating for many patients. Many clinical trials with inhibitors of Raf, MEK and ERK are ongoing to overcome cancer, stressing the importance in understanding all aspects of this signaling cascade.

At the level of Ras, several cancers are triggered almost exclusively via mutation of a single isoform such as only K-Ras mutations found in pancreatic cancers for example (Bryant et al., 2014). At the level of Raf, pharmacological inhibitors targeting a single isoform have been approved to treat patients. At the level of MEK, distinct retro-phosphorylations provide a rationale for having two isoforms. Overall, isoform specificities play a significant role in this pathway, therefore the quest in understanding why two isoforms convey ERK signaling appears of great importance.

With regards to protein sequences, ERK1 and ERK2 are very stable across evolution, with variations in amino-acids only at positions that are neutral for function. Presently, neither ERK isoform-specific agonists, nor isoform-specific substrates have been found. These observations point again toward functional redundancy of ERK1 and ERK2. However, these kinases are not redundant *per se* since disruption of *erk2* without compensation by $ERK1^{transgenic}$ is lethal in mice. Indeed, ERK1 and ERK2 are expressed ubiquitously but not at the same level in most tissues. Clear differences between ERK1 and ERK2 are obvious at the gene level. For example, in mammals *erk2* genes are on average 15 times larger than *erk1* genes, and in mice the 3-prime UTR of *erk2* is both 6 times larger than *erk1* and possess an alternative poly-adenylation site. These differences indicate that ERK2 has the capacity to be more exquisitely regulated than ERK1. Apart from these differences in expression levels, we cannot exclude that very fine specializations exist between ERK1 and ERK2, such as a slightly stronger affinity for a substrate or an interactor

protein. Only replacement of all exons of one isoform with those of the other isoform may uncover these fine differences; however it might be very complicated to draw conclusions if the replaced exons modify protein stability and change even slightly the global ERK quantity/activity ratio!

The studies presented here using knock-down approaches or gene disruptions overwhelmingly demonstrate a direct correlation between a biological consequence and the global level of ERK activation, irrespectively of the isoform. In cells with complex fate such as a balance between proliferation and differentiation, not all combinations have been studied so far. For example one would have liked to compare knock-out of a single *erk2* allele with knock-out of both *erk1* alleles; pending these two conditions would decrease global ERK activation to the same extent. Among three add-back studies presented here, one concluded that transfection of either ERK1 or ERK2 could reverse the phenotype of ERK2 loss, whereas the two other studies were inconclusive since it was not demonstrated that individual ERK transfections re-established the active ERK levels. In our hands it has proven difficult to re-express ERK that can be activated as efficiently as endogenous ERK (efficiency measured as the percentage of the transfected kinase that is effectively activated). Similarly, Meloche and co-workers did not succeed to express ERK1 or ERK2 at normal levels from cDNA inserted in mouse *erk2* locus (Frémin et al., 2015). Further work is needed to understand the means by which ERK proteins are highly expressed and regulated.

In more than 75 studies, the decrease of ERK expression was shown to trigger biological phenotypes, therefore it is surprising that over-expression of ERK1 in mice does not cause apparent phenotypical changes (Frémin et al., 2015)! We predict that under challenging conditions, phenotypes will be discovered in this ERK1-transgenic model. In fact, despite the elevated quantity of ERK1 in these mice, global ERK activation level seemed normal due to the robustness of the pathway via multiple retro-inhibitions. However, ERK quantity needs to be regulated, at least under challenging conditions. For example *erk2* gene amplification in humans was demonstrated to be the cause of tumor resistance to cancer treatment (Ercan et al., 2012). Therefore, more research is needed to understand the relevance of global ERK quantity for proper signaling under challenging conditions.

Lack of ERK is lethal in vertebrates but mice have been generated without ERK1 protein or without ERK2 protein, and tetrapods (vertebrates with four limbs) express either both ERKs or only ERK1 or only ERK2. In many studies, a direct correlation between the global quantity of ERK activation and phenotypical consequences has been established. Taken together, these data strongly suggest that ERK1 and ERK2 are functionally redundant.

AUTHOR CONTRIBUTIONS

PL, RB work in the laboratory of JP, in the IRCAN institute of Nice. PL wrote the main body of the text that was amended by RB and corrected in detail by JP.

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REVIEW
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Redundancy in the World of MAP Kinases: All for One

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The protein kinases ERK1 and ERK2 are the effector components of the prototypical ERK1/2 mitogen-activated protein (MAP) kinase pathway. This signaling pathway regulates cell proliferation, differentiation and survival, and is essential for embryonic development and cellular homeostasis. ERK1 and ERK2 homologs share similar biochemical properties but whether they exert specific physiological functions or act redundantly has been a matter of controversy. However, recent studies now provide compelling evidence in support of functionally redundant roles of ERK1 and ERK2 in embryonic development and physiology. In this review, we present a critical assessment of the evidence for the functional specificity or redundancy of MAP kinase isoforms. We focus on the ERK1/ERK2 pathway but also discuss the case of JNK and p38 isoforms.

Keywords: signal transduction, MAP kinases, ERK1/2, JNK, p38, mouse genetics, functional redundancy

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INTRODUCTION

Mitogen-activated protein (MAP) kinase pathways are evolutionarily conserved signaling modules that play a key role in transducing extracellular signals into intracellular responses (Meloche, 2012). These signaling modules are found in plants, fungi and animals (Kultz, 1998). In mammals, 14 MAP kinase genes have been identified that define 7 distinct MAP kinase pathways. The best-characterized MAP kinase pathways are the extracellular signal-regulated kinase 1(ERK1)/2, cJun NH_2-terminal kinase 1 (JNK1)/2/3, and p38α/β/γ/δ pathways. Phylogenetic analysis of the evolutionary history of MAP kinase genes suggests that vertebrate MAP kinases originated from 3 precursors and have expanded through gene duplication during early vertebrate evolution (Li et al., 2011). Thus, invertebrate species have less MAP kinases than vertebrate species. For example, humans express two ERK isoforms, ERK1 and ERK2, whereas Drosophila expresses the single ortholog Rolled. The expansion of vertebrate MAP kinase genes raises the important question of whether mammalian MAP kinase isoforms have evolved unique physiological functions or are used interchangeably to reach a threshold of global kinase activity. The current review addresses this question.

ERK1 AND ERK2, TWO HOMOLOGOUS KINASES WITH SIMILAR BIOCHEMICAL PROPERTIES

ERK1 and ERK2 are the effector kinases of the prototypical Ras-ERK1/2 MAP kinase pathway. This signaling pathway processes information from a wide range of extracellular stimuli to regulate cell proliferation, differentiation and survival (Pearson et al., 2001). ERK1 and ERK2 isoforms are encoded by distinct genes, which are located on chromosomes 16q11 and 22q11 in human, respectively (Li et al., 1994). They are co-expressed

in almost all cell types and tissues, although their relative abundance varies considerably from one tissue to another (Boulton and Cobb, 1991; Boulton et al., 1991; Fremin et al., 2015). Of note, some discrete regions of the adult mouse brain express exclusively *Erk1* or *Erk2* mRNA, suggesting that a single ERK isoform mediates cellular responses in these areas (Di Benedetto et al., 2007). The two ERK proteins display 83% amino acid identity overall and 100% similarity in residues involved in catalysis and docking interactions with substrates (Boulton et al., 1990, 1991; Busca et al., 2015). They share similar biochemical properties and are activated by the upstream kinases MEK1/2 with comparable efficiency *in vitro* (Robbins et al., 1993). Hydrogen/deuterium exchange mass spectrometry has revealed distinct patterns of activation-induced changes in conformational mobility between ERK1 and ERK2 (Ring et al., 2011). However, these differences in internal protein motions do not appear to significantly impact protein kinase activity and selectivity. ERK1 and ERK2 both recognize the same minimal Ser/Thr-Pro primary sequence determinant on substrates, with a preference for a proline at P-2 position (Gonzalez et al., 1991), and have almost identical *in vitro* intrinsic kinase activity (Lefloch et al., 2008). They phosphorylate hundreds of substrates (Yoon and Seger, 2006; Courcelles et al., 2013) and, with the exception of a few anecdotal reports (Chuang and Ng, 1994; Hanlon et al., 2001; Hwang et al., 2009), no evidence has been provided for a difference in substrate specificity between the two isoforms. Quantitative proteomics analysis of the ERK1 interactome in agonist-stimulated PC12 cells led to the identification of 284 ERK1-interacting proteins (von Kriegsheim et al., 2009). Notably, all proteins tested also interacted with ERK2 in co-immunoprecipitation assays. Thus, the two ERK isoforms display similar biochemical properties.

EVIDENCE FOR SPECIFIC REGULATORY MECHANISMS AND FUNCTIONS OF ERK1 AND ERK2

The question of whether ERK1 and ERK2 exerts specific functions or act redundantly has been a subject of intense research and controversy over the years. The unavailability of activated alleles or selective pharmacological inhibitors of ERK1 and ERK2 has complicated the analysis of their functions. Expression of phosphorylation-defective or catalytically inactive mutants of ERK1 or ERK2 has been used to successfully probe the functions of the kinases, but these mutants exert dominant interfering effects on both isoforms (Pages et al., 1993). The development of small molecule pharmacological inhibitors of MEK1/2, such as PD98059 and U0126, has provided invaluable tools for dissecting out the role of the ERK1/2 MAP kinase pathway in numerous cellular responses (Dudley et al., 1995; Favata et al., 1998; Fremin and Meloche, 2010). However, these reagents could not be used to discriminate the roles of each isoform.

A number of studies have reported that ERK1 and ERK2 are regulated differentially in response to specific extracellular stimuli or cellular contexts (Papkoff et al., 1994; English and Sweatt, 1996; Kashiwada et al., 1996; Sarbassov et al., 1997; Matos et al., 2005; Wollmann et al., 2005; Aceves-Luquero et al., 2009; Chernova et al., 2009). However, these results should be interpreted with caution as they rest on the use of non-quantitative immunoblotting assays to monitor the activating phosphorylation of ERK isoforms. In the vast majority of studies, ERK1 and ERK2 were found to be co-activated in response to various extracellular agonists (Lewis et al., 1998). Detailed kinetic analyses in mouse fibroblasts have revealed that the two ERK isoforms are coordinately phosphorylated and enzymatically activated in response to mitogenic factors (Meloche, 1995). Intriguingly, the scaffold protein MP1 (MEK Partner 1) was proposed to interact preferentially with MEK1 and ERK1, and to specifically enhance ERK1 activation (Schaeffer et al., 1998). MP1 was later shown to form a heterodimeric complex with the adaptor protein p14, which is required to localize MP1 to late endosomes and promote the endosomal activation of both ERK1 and ERK2 isoforms (Wunderlich et al., 2001; Teis et al., 2002). Conditional deletion of the *p14* gene in the mouse epidermis further demonstrated that p14 is required for the global activation of ERK1 and ERK2 in the epidermis (Teis et al., 2006), refuting the isoform-specific regulatory function of the MP1-p14 scaffolding complex. It has also been reported that the nucleocytoplasmic trafficking of ERK1 is slower than that of ERK2 because of a unique sequence located in the N-terminal extremity of ERK1 (Marchi et al., 2008, 2010). As a consequence, ERK1 would have a reduced capability of transducing proliferative signals to the nucleus. However, this model requires rigorous validation by other groups and is inconsistent with genetic studies of the individual roles of ERK1 and ERK2 in cell proliferation (see below).

The generation of genetically-engineered mouse models bearing inactivating mutations in the *Erk1* and *Erk2* genes and the advent of RNA interference (RNAi) technology has allowed analysis of the phenotypical consequences of the specific depletion of ERK1 or ERK2 in animals and cells. *Erk1*$^{-/-}$ mice develop normally, are viable and fertile, and display no observable phenotype (Pages et al., 1999). In contrast, invalidation of the *Erk2* gene in mouse severely compromises the formation of ectoplacental cone and extra-embryonic ectoderm, which give rise to mature trophoblast derivatives in the fetus (Saba-El-Leil et al., 2003). Therefore, *Erk2* disruption leads to embryonic lethality early in mouse development after the implantation stage at embryonic day (E) 6.5 (Hatano et al., 2003; Saba-El-Leil et al., 2003; Yao et al., 2003). These observations suggested for the first time that ERK1 and ERK2 could exert specific biological functions *in vivo*. Further analysis of ERK1- or ERK2-deficient mice has fueled the idea of isoform-specific functions. Thus, it has been proposed that ERK1 specifically regulates adipocyte differentiation (Bost et al., 2005), skin homeostasis and carcinogenesis (Bourcier et al., 2006), cocaine-sensitive long-term depression of excitatory synaptic transmission (Grueter et al., 2006), splenic erythropoiesis (Guihard et al., 2010), and osteoclast differentiation (He et al., 2011; Saulnier et al., 2012). A defect in thymocyte maturation was originally described in ERK1-deficient mice (Pages et al., 1999) but subsequent studies failed to confirm this phenotype (Fischer et al., 2005; Nekrasova et al., 2005). Contradictory findings have also been reported about the specific role of ERK1 in emotional

learning and memory (Selcher et al., 2001; Mazzucchelli et al., 2002). Heterozygous inactivation and conditional deletion of *Erk2* have been used to study the role of the kinase in specific tissues. These studies have suggested that ERK2 preferentially regulates cardiac myocytes survival (Lips et al., 2004), CD8 T cell proliferation and survival (D'Souza et al., 2008), neural development and associated cognitive functions and memory (Satoh et al., 2007; Samuels et al., 2008), production of brain collagen (Heffron et al., 2009), nociceptive sensitization (Alter et al., 2010), oligodendrocyte differentiation (Fyffe-Maricich et al., 2011), and social behaviors (Satoh et al., 2011a).

In vitro studies of cells depleted of ERK1 or ERK2 expression by genetic disruption or RNAi have also contributed to the idea of isoform-specific functions. For example, ERK1-deficient keratinocytes show an impaired proliferative response to mitogenic factors (Bourcier et al., 2006). On the other hand, depletion of ERK2 was reported to specifically impair terminal differentiation of skeletal myoblasts (Li and Johnson, 2006), replication of hepatocytes (Fremin et al., 2007; Bessard et al., 2008), transforming growth factor-beta-induced collagen synthesis (Li et al., 2009), Ras-dependent epithelial-to-mesenchymal transition (Shin et al., 2010), hepatocyte growth factor-induced lung cancer cell migration (Radtke et al., 2013), oncogenic Ras-induced senescence (Shin et al., 2013), and regulation of gp130 expression (Bonito et al., 2014). However, it should be emphasized that several other studies have documented that both ERK isoforms similarly contribute to the cellular response being studied (Liu et al., 2004; Wille et al., 2007; Lefloch et al., 2008; Dumesic et al., 2009; Voisin et al., 2010; Wei et al., 2010). Intriguingly, one group even proposed that ERK1 and ERK2 exert antagonistic effects on cell proliferation (Vantaggiato et al., 2006). This model was disproved in subsequent studies (Lefloch et al., 2008; Voisin et al., 2010).

The ERK1 gene has been reported to undergo alternative splicing to encode the Erk1b transcript in the rat (Yung et al., 2000) and ERK1c transcript in human (Aebersold et al., 2004). A subsequent study proposed that ERK1c specifically regulates Golgi fragmentation during mitosis in a non-redundant manner with ERK1 and ERK2 (Shaul and Seger, 2006). Analysis of ERK1 nucleotide sequences indicates that the Erk1b and ERK1c transcripts derive from the retention of an intronic sequence between exon 7 and exon 8 of the gene. Notably, the mouse *Erk1* gene contains an intron of 79 nucleotides at this position, but no evidence has been reported that this intron sequence can be translated in mouse tissues. The lack of an ERK1b isoform in the mouse raises doubts about the physiological importance of this isoform.

FUNCTIONAL REDUNDANCY OF ERK1 AND ERK2: LESSONS FROM GENETIC STUDIES

The observation that specific ablation of ERK1 or ERK2 causes distinct phenotypes in cells or mice has been interpreted by many authors as evidence for isoform-specific functions of the two kinases. However, these studies did not take into account the global level of ERK1/2 activity in the analysis of the phenotypes. This is a crucial point since ERK1 and ERK2 are expressed at different levels in cell lines and tissues, with ERK2 being the predominant isoform in most tissues. Accordingly, this may explain why depletion of ERK2 usually results in stronger phenotypes than the loss of ERK1. The impact of the total activity of ERK1 and ERK2 on phenotypic outcomes was analyzed quantitatively in three *in vitro* studies. In a first study, Wille et al. generated an epi-allelic series of stable ERK1 and ERK2 knockdown mouse T cell lines obtained by shRNA lentiviral infections (Wille et al., 2007). They showed that T-cell receptor-stimulated interleukin-2 production was dependent on both total and phosphorylated ERK levels, with a similar contribution of ERK1 and ERK2. In another study, Lefloch et al. have used RNAi to silence the expression of ERK1 and ERK2 in NIH 3T3 fibroblasts and examine their relative roles in cell proliferation and immediate-early gene expression (Lefloch et al., 2008). Depletion of ERK2 slowed down the proliferation of NIH 3T3 cells, whereas reduction of ERK1 expression had no effect. Interestingly, by clamping the expression of ERK2 to a limiting level, they showed that depletion of ERK1 further restrains cell proliferation, demonstrating that both isoforms positively contributes to cell proliferation. Silencing of either ERK1 or ERK2 expression was sufficient to inhibit the serum-dependent transcriptional induction of immediate-early genes in this model. Importantly, these authors established that ERK1 and ERK2 have similar intrinsic kinase activity and demonstrated that the relative expression level of the two ERK proteins correlates with their ratio of activation state. Our group used a robust genetic approach to analyze the individual roles of ERK1 and ERK2 in cell proliferation using primary mouse embryonic fibroblasts (MEFs) as model (Voisin et al., 2010). We showed that individual loss of either ERK1 or ERK2 decreases the proliferation rate of MEFs. The impact of ERK2 deficiency was more severe, consistent with its higher level of expression in these cells. Genetic disruption of both *Erk1* and *Erk2* genes resulted in complete G1 arrest and premature replicative senescence. By combining genetic disruption of *Erk1* or *Erk2* with RNAi depletion of the alternate isoform, we were able to demonstrate that the rate of MEF proliferation is strongly correlated with the global level of phosphorylated ERK1/ERK2, which is dictated by the relative expression of the two isoforms. Altogether, these findings provided strong evidence for a redundant role of ERK1 and ERK2 in promoting cell proliferation.

In vivo analyses of genetically-engineered mutant mice also suggested that ERK1 and ERK2 have redundant functions in specific tissues. Conditional inactivation of *Erk2* in the developing neural crest leads to craniofacial abnormalities and conotruncal cardiac defects, which are exacerbated by the additional deletion of *Erk1* (Newbern et al., 2008). Similarly, *Erk1* deficiency enhances the abnormal neurogenesis phenotype in central nervous system-specific *Erk2* conditional knockout mice (Satoh et al., 2011b). Genetic deletion of *Erk1* and *Erk2* genes in hematopoietic cells coupled to reconstitution studies with catalytically active or inactive ERK1 and ERK2 also revealed that ERK1 and ERK2 play redundant kinase-dependent functions

in the maintenance of hematopoietic stem cells and adult hematopoiesis (Chan et al., 2013; Staser et al., 2013).

We have used complementary genetic approaches to rigorously address the question of ERK1 and ERK2 specificity or redundancy in embryonic development (Fremin et al., 2015). In a first approach, we examined the impact of the progressive deletion of *Erk1* and *Erk2* alleles on the development of the placenta and embryo *per se*. We found that the weight of the placenta and surface of the labyrinth is strictly correlated with the total activity of ERK1/2 as monitored by anti-phospho-ERK1/2 immunoblotting analysis. Quantitative analysis of various embryonic phenotypes (embryo size, weight, digit length) also revealed a tight relationship between the extent of development of embryos with different combinations of *Erk1* and *Erk2* alleles and total ERK1/2 activity in embryonic tissues. As a second approach, we asked whether ERK1 can substitute for ERK2 in mouse embryonic development. We found that ubiquitous expression of an *Erk1* transgene fully rescues the placental and embryonic defects observed in ERK2-deficient embryos. ERK1-only mice grow normally, are fertile and do not display any overt phenotype. Expression of transgenic ERK1 also rescued the proliferation defect of ERK2-deficient MEFs and restored normal phosphorylation of a panel of ERK1/2 substrates. Our study provides compelling and definitive evidence for a functionally redundant role of ERK1 and ERK2 kinases during development (Fremin et al., 2015). Interestingly, Aiodi et al. recently reported a similar functional redundancy of the upstream kinases MEK1 and MEK2. Knock-in of *Mek2* at the *Mek1* locus rescued the placental phenotype of MEK1-deficient mice (Aoidi et al., 2016). These observations reinforce the notion that MEK1 and MEK2 isoforms activate ERK1 and ERK2 indiscriminately.

Differences in the phenotypes of *Erk1* and *Erk2* null mice are attributable to differences in expression levels, with ERK2 being the predominant isoform. The higher expression level of *Erk2* in most mammalian tissues can be related to a stronger promoter, although further regulation by post-transcriptional mechanisms cannot be ruled out (Busca et al., 2015). Importantly, ERK1 and ERK2 proteins have a long half-life of over 50 h (Schwanhausser et al., 2011) and no evidence of stimulus-induced change in protein levels has been reported, indicating that ERK1/2 protein expression levels are not subject to regulation by feedback mechanisms. Furthermore, genetic disruption of a single ERK isoform does not result in increased expression of the other isoform as documented by immunoblotting analyses (Saba-El-Leil et al., 2003; Voisin et al., 2010; Fremin et al., 2015).

The above findings underscore the concept that a threshold of global ERK1/2 activity determines developmental progression and phenotypic outcome (**Figure 1**). ERK1 and ERK2 provide a pool of functionally interchangeable kinases available for activation and different thresholds of ERK1/2 activity are required for executing different developmental decisions in specific cellular contexts. In most cells or tissues where ERK2 is the predominant isoform, loss of ERK2 results in a greater decrease of total ERK1/2 activity associated with a broader spectrum of phenotypic manifestations as observed in many studies. Overexpression of the non-predominant ERK1 isoform is sufficient to replenish the pool of ERK kinases, restore global ERK1/2 activity and rescue the ERK2-associated defects. Thus, phenotypes resulting from the depletion or genetic deficiency of a single MAP kinase isoform should be cautiously interpreted in the context of global MAP kinase activity.

The group of Philippe Lenormand recently reported the most detailed analysis of the expression and evolution of ERK1 and ERK2 protein sequences in vertebrates (Busca et al., 2015). Interestingly, they found that the *Erk1* gene has been lost in all bird lineages and some amphibians, whereas squamates only express ERK1 isoform, despite the presence of both *Erk1* and *Erk2* genes. The finding that tetrapods can live by expressing only ERK1 or ERK2 provides further demonstration of the functional redundancy of ERK isoforms in animal physiology.

REDUNDANCY OF MAP KINASES: THE CASE OF JNK1 AND JNK2

The JNK pathway provides another example where different groups have reported contradictory conclusions about the specificity or redundancy of closely related MAP kinase isoforms. JNK1 and JNK2 are ubiquitously expressed in the mouse although their expression levels vary across tissue types. Mice deficient in either *Jnk1* or *Jnk2* gene exhibit distinct phenotypes, suggesting that individual JNK isoforms may serve different signaling functions (Davis, 2000). In addition, $Jnk1^{-/-}$ and $Jnk2^{-/-}$ MEFs proliferate at different rates, a phenotype that has been related to the expression levels of *cJun* (Tournier et al., 2000; Sabapathy et al., 2004). Specifically, JNK1-deficient MEFs have lower cJun levels and proliferate more slowly than wild type MEFs as opposed to JNK2-deficient MEFs that express higher levels of cJun and proliferate faster. These findings have led to the hypothesis that JNK1 and JNK2 have distinct and opposite roles in regulating cJun expression and cell proliferation (Ronai, 2004; Sabapathy and Wagner, 2004).

The group of Roger Davis has revisited the proposed negative regulatory role of JNK2 on cJun expression and cell proliferation using a chemical genetic approach (Jaeschke et al., 2006). They introduced the M108G mutation in JNK2 to enlarge the ATP binding pocket and render the kinase sensitive to the small molecule inhibitor 1-NM-PP1 (Bishop and Shokat, 1999). The mutant $Jnk2^{M108G}$ allele was integrated at the endogenous *Jnk2* locus by homologous recombination to generate mice expressing analog-sensitive JNK2 kinase. Analysis of MEFs bearing different combination of *Jnk1* and *Jnk2* alleles revealed that pharmacological inhibition of JNK2 in JNK1 proficient cells caused no change in cJun expression or cell proliferation, contrary to the results obtained in $Jnk2^{-/-}$ cells. However, both genetic ablation and pharmacological inhibition of JNK2 in $Jnk1^{-/-}$ cells reduced cJun levels and inhibited cell proliferation. These results demonstrated that JNK1 and JNK2 act redundantly to increase cJun expression and promote cell proliferation. The most likely explanation for the misleading phenotype of $Jnk2^{-/-}$ cells is that loss of JNK2 leads to increased JNK1 function by a compensatory adaptation mechanism. This adaptation is not observed upon acute inhibition of the kinase and/or in conditions where protein expression is maintained. This study

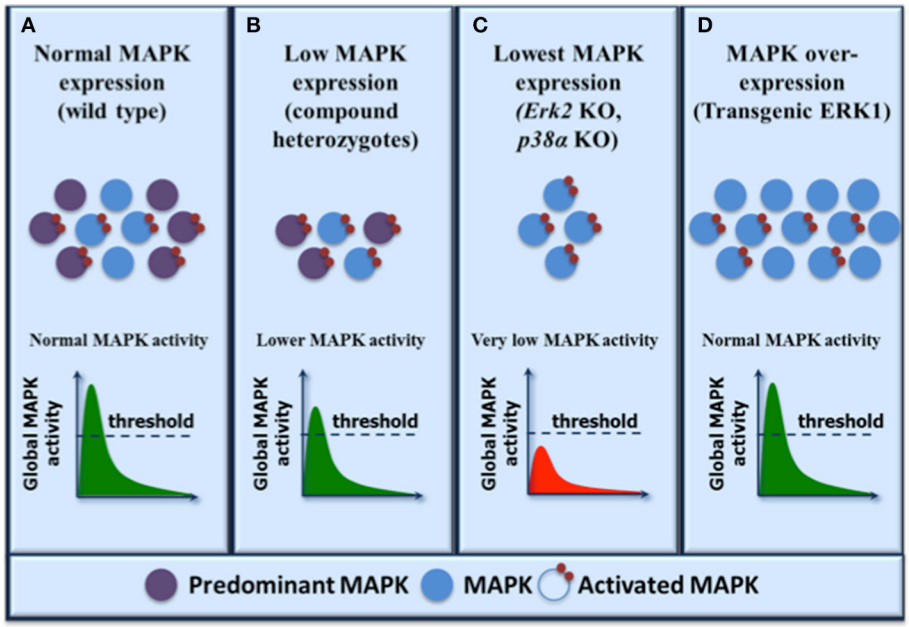

FIGURE 1 | Functional redundancy within the MAP kinase family: a model to reconcile biochemical and genetic evidence. The expression levels of MAP kinase isoforms are shown on the top and the resulting activation levels are illustrated graphically as global MAP kinase activity. A threshold of activity is required for normal biological output. MAP kinase isoforms are activated by upstream MAP kinase kinases indiscriminately and the resulting global MAP kinase activity depends on the level of expression of the individual isoforms. **(A)** In normal physiological conditions, both MAP kinase isoforms are activated with the predominantly expressed kinase contributing to most activity. **(B)** Reduced MAP kinase expression results in decreased global MAP kinase activity but the activity remains above threshold resulting in normal phenotypic outcome as exemplified by compound heterozygotes (ERK1/2, JNK1/2, or p38α/β). **(C)** Further reduction of MAP kinase expression (by depletion of the predominant kinase isoform) lowers global MAP kinase kinase activity below the threshold and results in developmental defects (ERK2 or p38α) or deficient cell proliferation (ERK2, JNK1). **(D)** Overexpression of the less predominant kinase restores global MAP kinase activity above threshold and rescues the phenotypes associated with the loss of the predominant kinase (transgenic ERK1). Note that in this model, the maximum level of MAP kinase activity is dictated by upstream activators such that the same global activity is observed under normal or MAP kinase overexpression conditions.

also highlighted the importance of using multiple experimental approaches to interpret the phenotypes of mouse mutants, as discussed above for ERK1 and ERK2.

P38α/β AND P38γ/δ KINASES HAVE OVERLAPPING ROLES

The p38 MAP kinase pathway regulates numerous cellular processes including adaptation to environmental stress, innate immunity; cell cycle progression and cellular differentiation (Cuenda and Rousseau, 2007; Cuadrado and Nebreda, 2010; Trempolec et al., 2013). The mammalian p38 kinase family is composed of four members, p38α, p38β, p38γ, and p38δ, of which the p38α and p38β isoforms are the closest related isoforms with 75% amino acid identity (Cuenda and Rousseau, 2007). The two isoforms are ubiquitously expressed, although p38α is the predominant isoform in most tissues. p38α and p38β are commonly activated by a wide variety of environmental stresses or inflammatory cytokines and share similar substrate specificity, suggesting overlapping functions. Gene disruption studies have revealed that *p38α* and *p38β* mouse mutants exhibit distinct phenotypes. Specifically, loss of p38α is embryonic lethal owing to defects in placenta morphogenesis (Adams et al., 2000; Allen et al., 2000; Mudgett et al., 2000; Tamura et al., 2000). Conditional deletion of p38α in the mouse embryo bypasses the embryonic lethality but mice die shortly after birth as a result of lung dysfunction (Hui et al., 2007). In contrast, the p38β knockout is viable with no obvious phenotype (Beardmore et al., 2005). This suggests, but does not prove, that the two kinases may have specific roles in certain tissue types.

The group of Angel Nebreda used a combination of genetic approaches to address the question of the specificity and redundancy of p38α and p38β isoforms (del Barco Barrantes et al., 2011). Their work suggested a specific role for p38α in placental development since the placental defects resulting from p38α deficiency could not be rescued by expression of a p38β knock-in allele under transcriptional control of the endogenous p38α promoter. On the other hand, several embryonic phenotypes including defects in heart development, spina bifida, and exencephaly were observed in compound *p38α* and *p38β* deficient embryos but were absent in single gene knockouts, indicating that that the two isoforms can compensate for each other with respect to these defects (del Barco Barrantes et al., 2011). These results demonstrate that p38α and p38β have overlapping functions suggesting functional redundancy of the two MAP kinase isoforms. Consistent with this idea, the phenotypes observed were found to be dependent on the

dosage of p38α and p38β. Specifically, embryos with a single *p38β* knock-in allele in the p38 knockout background developed to E18.5 and showed rescue of spina bifida and exencephaly defects, but not heart defects. Upon increased dosage from the two additional endogenous *p38β* alleles, the heart anomalies were rescued and, more importantly, some of the animals survived to adulthood, thereby overcoming the lung defects observed in p38α-deficient animals (del Barco Barrantes et al., 2011). These results are reminiscent of our observations in which specific tissues requiring high levels of global ERK1/2 activity showed defective development in absence of the predominantly expressed ERK2 isoform, which could be completely rescued by overexpression of ERK1 thus confirming that the two kinases are interchangeable and that gene dosage is crucial (Fremin et al., 2015). In the case of p38 isoforms, the possibility also exists that the placental defects that persist in p38β knock-in mice are simply the consequence of insufficient p38β expression. The use of a transgenic approach in which higher ubiquitous expression levels of p38β can be achieved may rescue this phenotype in which case this would demonstrate that the two kinases are redundant.

Further evidence that p38α and p38β act redundantly comes from work demonstrating that embryos lacking both *p38α* and *p38β* genes are deficient in sex determination due to reduced expression of the testis-determining gene *Sry* (Warr et al., 2012). In another study, compound loss of p38α and p38β was shown to compromise Met signaling to p53 in the developing liver. The loss of p53 Ser 389 phosphorylation by p38 MAP kinases in mutant livers resulted in increased hepatocyte death (Furlan et al., 2012). In these two studies, the phenotypes associated with the loss of p38α and p38β were absent in mice deficient in one of the two isoforms.

The two other members of the p38 MAP kinase subfamily also exhibit overlapping functions. p38γ and p38δ share 70% amino acid identity (Cuenda and Rousseau, 2007) and mice deficient for a single p38γ or p38δ isoform show no obvious phenotype under normal physiological conditions (Sabio et al., 2005, 2010; Remy et al., 2010; Risco and Cuenda, 2012). However, disruption of both *p38γ* and *p38δ* genes has unveiled key roles of p38γ and p38δ isoforms in tissue regeneration, innate immune responses, inflammation, and tumorigenesis (Escós et al., 2016). Analysis of compound p38γ and p38δ deficient mice revealed that both kinases are required for physiological and pathological cardiac hypertrophy (Gonzalez-Teran et al., 2016). The role of p38γ and p38δ in the inflammatory response was documented in various experimental mouse models such as lipopolysaccharide-induced septic shock and acute liver failure (Risco et al., 2012; Gonzalez-Teran et al., 2013) and collagen-induced arthritis models (Criado et al., 2014). Similarly, in models of cancer associated with chronic inflammation, such as colitis-associated cancer (Del Reino et al., 2014) and the two-step chemical skin carcinogenesis model (Zur et al., 2015), deficiency in p38γ and p38δ was shown to decrease cytokine production and immune cell infiltration, resulting in decreased tumor burden. Together, these studies suggest that p38γ and p38δ isoforms may exert functionally redundant roles. More rigorous genetic approaches similar to those used to demonstrate functional redundancy of the ERK1/2, JNK1/2, or p38α/β should be used to formally address this question for p38γ and p38δ.

CONCLUDING REMARKS

We have used a combination of genetic approaches together with quantitative analysis of embryonic phenotypes and ERK1/ERK2 activity to demonstrate that ERK1 and ERK2 isoforms are functionally redundant in mouse development and physiology. This conclusion is consistent with the discovery of animal species that express only ERK1 or ERK2 and with studies showing that the two isoforms share similar biochemical properties and substrate specificity. Similarly, by combining multiple experimental approaches, other studies have revealed that JNK and p38 MAP kinase isoforms exert functionally redundant roles. These findings clearly illustrate the importance of using multiple genetic, pharmacological and phylogenetic analyses to define the physiological functions of related signaling proteins.

The question of MAP kinases redundancy has far-reaching implications. Dysregulation of the ERK1/2, JNK1/2/3, and p38α/β/γ/δ pathways has been causally linked to human congenital syndromes and to a variety of diseases including cancer, arthritis, fibrosis, cardiomyopathies, and neurodegenerative diseases. Small molecule inhibitors of the ERK1/2 pathway have been approved for the treatment of $BRAF^{V600E}$ metastatic melanoma and other inhibitors of MAP kinase pathways are undergoing clinical evaluation. It is therefore crucial to determine whether the direct or downstream targets of these inhibitors have specific or redundant functions.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct, and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Regulation of Muscle Stem Cell Functions: A Focus on the p38 MAPK Signaling Pathway

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Formation of skeletal muscle fibers (myogenesis) during development and after tissue injury in the adult constitutes an excellent paradigm to investigate the mechanisms whereby environmental cues control gene expression programs in muscle stem cells (satellite cells) by acting on transcriptional and epigenetic effectors. Here we will review the molecular mechanisms implicated in the transition of satellite cells throughout the distinct myogenic stages (i.e., activation from quiescence, proliferation, differentiation, and self-renewal). We will also discuss recent findings on the causes underlying satellite cell functional decline with aging. In particular, our review will focus on the epigenetic changes underlying fate decisions and on how the p38 MAPK signaling pathway integrates the environmental signals at the chromatin to build up satellite cell adaptive responses during the process of muscle regeneration, and how these responses are altered in aging. A better comprehension of the signaling pathways connecting external and intrinsic factors will illuminate the path for improving muscle regeneration in the aged.

Keywords: muscle stem cells, satellite cells, p38 MAPKs, aging, epigenetics, tissue regeneration

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INTRODUCTION

The regenerative capacity of adult skeletal muscle depends on a resident population of muscle stem cells called satellite cells, which are located between the sarcolemma and the basal lamina. In healthy adult muscles, satellite cells reside in a dormant quiescent state and are characterized by the expression of the paired-box transcription factor Pax7 (Chang and Rudnicki, 2014; Comai and Tajbakhsh, 2014). Different stimuli, such as injury or disease, are known to induce satellite cell activation from quiescence and expansion as myoblasts; these cells will subsequently either exit the cell cycle, differentiate and fuse to generate new muscle fibers (i.e., myogenesis) or undergo self-renewal and return to quiescence to replenish the stem cell pool (Brack and Rando, 2012; Wang et al., 2014).

The transcriptional regulation of muscle cell specification has been well characterized. Pax3 and Pax7 are thought to be the principal regulators of muscle cell specification and tissue formation during embryonic development. In adult satellite cells, whereas Pax7 is highly expressed, Pax3 is only highly expressed in satellite cells of a subset of muscles, such as the diaphragm, but lowly expressed in other muscles (Soleimani et al., 2012a). Recent studies analyzing the role of Pax7 in the maintenance and expansion of adult muscle stem cells have demonstrated that long-term Pax7 ablation leads to the loss of adult satellite cells, which results in impaired muscle regeneration after injury confirming that $Pax7^{+}$ satellite cells are sufficient and required for adult muscle repair

(Lepper et al., 2011; Sambasivan et al., 2011; von Maltzahn et al., 2013; Brack, 2014). Furthermore, inactivation of Pax7 in satellite cells of adult mice also leads to diminished heterochromatin condensation (Gunther et al., 2013), suggesting a role for Pax7 in chromatin organization; of note, this has also been recently proposed for Pax3 (Bulut-Karslioglu et al., 2012).

Satellite cells can be activated by many signals from the regenerative microenvironment, including those mediated by adhesion molecules, necrotic cues released from the damaged fibers, or by growth factors and cytokines produced by neighboring cells, including interstitial cells, resident macrophages, fibroblasts, and microvasculature-related cells (Giordani and Puri, 2013; Judson et al., 2013; Pannerec et al., 2013; Brancaccio and Palacios, 2015). Several signaling cascades, including the p38 mitogen-activated protein kinase (MAPK) and the AKT pathways, can transmit these extracellular cues to the myogenic cell nucleus (Cuenda and Cohen, 1999; Wu et al., 2000; Keren et al., 2006; Serra et al., 2007), and regulate expression and activity of the muscle-specific regulatory factors (MRFs, that belong to the bHLH family of transcription factors: Myf5, MyoD, myogenin and MRF4). In cooperation with ubiquitously-expressed E proteins and myocyte enhancer factor 2 (MEF2) transcriptional regulators, MRFs bind to E-boxes and MEF2-boxes of muscle promoters and induce the expression of muscle-specific genes (Lluis et al., 2006; Singh and Dilworth, 2013; Segales et al., 2015). Satellite cell-dependent myogenesis is also controlled by several epigenetic mechanisms, such as chromatin remodeling, DNA methylation and covalent modification of histones and transcription factors. The cross-talk between the basic muscle-specific transcriptional and epigenetic machineries allows the coordinated induction or repression of distinct subsets of genes in order to advance through the myogenic program (Dilworth and Blais, 2011; Segales et al., 2015).

In this review, we will sum up the existing knowledge about the transcriptional and epigenetic regulation of postnatal myogenesis, describing the epigenetic status of adult muscle stem cells and the changes in chromatin structure needed to coordinately regulate the myogenic gene expression program. We will also discuss the main signal transduction pathways that transmit extracellular signals to the nucleus of myogenic cells, focusing especially on the p38 MAPK pathway. Finally, we will address how alterations in these signaling cascades contribute to the reduced regenerative capacity of aged satellite cells and whether this defect can be counteracted by pharmacological manipulation of these pathways.

TRANSCRIPTIONAL AND TRANSLATIONAL REGULATION OF MYOGENESIS

Myogenesis dependent on satellite cells is a well-defined multi-step process characterized by the sequential activation of MRFs: Myf5 and MyoD are expressed in undifferentiated proliferating myoblasts, while myogenin and MRF4 are induced at the early and late phases of differentiation, respectively (Sartorelli and Caretti, 2005; Singh and Dilworth, 2013). Pax7 is expressed in quiescent satellite cells and is also critical for their cell cycle progression by regulating genes involved in cell proliferation, while preventing differentiation (Olguin et al., 2007; Soleimani et al., 2012a; von Maltzahn et al., 2013). Pax7-expressing quiescent satellite cells induce the expression of Myf5 and MyoD upon their activation (Mckinnell et al., 2008), thereby allowing successive rounds of cell proliferation (Olguin and Olwin, 2004); instead, downregulation of Pax7 prior to myogenin activation facilitates exit the cell cycle and differentiation entry (Olguin et al., 2007; Olguin, 2011; Bustos et al., 2015). Translational control of MRFs' expression also accounts for the transition through the sequential myogenic stages: (1) In quiescent satellite cells the expression of the Myf5 protein is avoided by sequestration of the Myf5 mRNA in messenger ribonucleoprotein granules and by the action of the microRNA-31, which blocks Myf5 translation (Crist et al., 2012); (2) MyoD protein expression is also prevented in quiescent satellite cells by the action of tristetraprolin (TTP), a protein that promotes the degradation of MyoD RNA (Hausburg et al., 2015); (3) Moreover, a global mechanism of repression of translation, mediated by the phosphorylation of the eukaryotic initiation factor eIF2α at serine 51, preserves the quiescent state of satellite cells, as cells that cannot phosphorylate eIF2α exit quiescence and activate the myogenic program (Zismanov et al., 2016).

Once MyoD mRNA has been correctly translated, the initiation of the differentiation program requires the association of MyoD with E proteins and their binding to E boxes of muscle gene promoters, to activate their expression. This is actively inhibited in proliferating myoblasts by different mechanisms. Initially, in proliferating myoblasts, the formation of functional MyoD/E protein heterodimers is prevented by the inhibitor of differentiation (Id), an HLH protein that lacks the basic DNA-binding domain and interacts with either MyoD or E proteins, thus inhibiting myogenic differentiation. When myoblasts exit the cell cycle Id expression is downregulated, allowing functional heterodimers to be formed and promoting muscle differentiation-specific gene expression (Puri and Sartorelli, 2000). Other proteins, such as the bHLH factors Twist, masculine, Mist1, ZEB, Hey1, and I-mfa proteins, act as repressors of MRFs either by direct association with them or by sequestering their functional partners (Buas et al., 2010; Siles et al., 2013). Moreover, in proliferating myoblasts, the transcriptional repressor Snai1 excludes MyoD from muscle-specific promoters. During differentiation, Snai1/2 expression is downregulated by the action of two specific microRNAs, miR-30 and miR-206, allowing access for MyoD to its target genes (Soleimani et al., 2012b).

Interestingly, recent genome-wide analyses of MyoD binding in C2C12 myogenic cells and primary myoblasts have revealed that MyoD also binds to a large number of promoters of genes that are not regulated during muscle differentiation, in both myoblasts and myotubes (Cao et al., 2010; Soleimani et al., 2012b). This genome-wide MyoD binding correlates with local histone hyperacetylation, suggesting that MyoD could play a role in reprogramming the epigenetic landscape by recruiting histone acetyltransferases (HATs) to regions throughout the genome.

EPIGENETIC REGULATION OF MYOGENESIS

As mentioned above, muscle gene expression is also regulated by different epigenetic mechanisms, including DNA methylation, chromatin remodeling, post-translational modifications of histones and regulation by a network of noncoding RNA. These epigenetic modifications are usually dynamic and reversible and can be associated with gene activation or repression (reviewed in Bergman and Cedar, 2013).

EPIGENETIC STATUS OF QUIESCENT SATELLITE CELLS

The capacity to maintain quiescence is essential for the long-term preservation of a functional stem cell pool in the majority of tissues and organs. In stem cells, genes implicated in lineage determination and progression are found in a poised state: although transcriptionally silent, they contain active histone marks that keep them prepared to be expressed after receiving differentiation signals (Puri et al., 2015; Rumman et al., 2015). In quiescent satellite cells, chromatin is kept in a transcriptionally permissive state, with many genes harboring the activation H3K4me3 mark at the transcription start sites (TSS), including MyoD and Myf5, and a few number of genes labeled with the inhibitory H3K27me3 mark (Liu et al., 2013). Thus, instead of being only found on actively transcribed genes, the H3K4me3 mark is also present in inactive genes that will likely be transcribed at later time points, for example, following satellite cell activation (Guenther et al., 2007). Furthermore, Liu and colleagues have also described that quiescent satellite cells contain more than 1800 genes with their transcription start site (TSS) marked by bivalent domains (e.g., both H3K27me3 and H3K4me3 marks), many of them corresponding to lineage-specific genes (Mikkelsen et al., 2007). Of interest, in quiescent satellite cells, the only myogenic transcription factor having bivalent domains is *Pax3* whereas, *Pax7* is only marked by H3K4me3 (**Figure 1**). In contrast, myogenin which is not marked by either H3K4me3 or H3K27me3 in quiescent satellite cells, shows a significant enrichment of the H3K4me3 mark at its TSS upon cell activation (Liu et al., 2013). Together, these data suggest an interplay between the Trithorax complex (TrxG; responsible of H3K4me3) and the polycomb repressive complexes (PRCs; responsible of H3K27me3). Additionally, H3K9 methyltransferase PRDM2/RIZ, which is highly expressed in quiescent satellite cells, binds to thousands of promoters in G0 synchronized C2C12 myoblasts, including myogenic and cell cycle regulators (Cheedipudi et al., 2015a,b). PRDM2 interacts with Ezh2, the catalytic subunit of PRC2, and regulates its association with a novel G0-specific bivalent domain identified in the Ccna2 locus (Cheedipudi et al., 2015a). Ezh2, in turn, is needed for homeostasis of the adult muscle stem cell pool (Juan et al., 2011). Mice lacking Ezh2 specifically in satellite cell have reduced muscle mass, fewer satellite cells post-birth, and impaired regeneration following muscle injury. These differences can be explained by defects in the proliferative capacity of satellite cells (Woodhouse et al., 2013), and by impaired maintenance and/or return to quiescence after injury (Juan et al., 2011). Moreover, recent studies showed that preservation of muscle stem cell quiescence is also dependent on the repression of senescence pathways by Polycomb proteins (Sousa-Victor et al., 2014a). Indeed, derepression of the senescence regulator $p16^{INK4a}$ (*Cdkn2a*) in satellite cells of geriatric mice causes loss of quiescence and entry into irreversible senescence, demonstrating that the repression of *Cdkn2a* mediated by polycomb proteins is needed to maintain the quiescent state of satellite cells in muscle homeostatic conditions (revised in Sousa-Victor et al., 2015).

Additional methylation events regulate the activity of satellite cells throughout myogenesis. One layer of epigenetic regulation is performed by direct interaction of the arginine methyltransferase Carm1 with Pax7. In quiescent satellite cells Carm1 binding to Pax7 is inhibited; in contrast, when satellite cells are activated, Carm1 interacts and methylates Pax7. Methylated Pax7 directly binds to the Thritorax complex resulting in its recruitment to the Myf5 promoter, leading to H3K4 methylation, Myf5 expression and myogenic commitment (Kawabe et al., 2012). Finally, a very recent study has shown that the histone methyltransferase Suv4-20H1 is necessary to maintain satellite cell quiescence by causing a condensed state of the heterochromatin through the transcriptional repression of MyoD (Boonsanay et al., 2016). Indeed, Suv4-20H1 binds directly to the MyoD Distal Regulatory Region enhancer and catalyzes the transcriptionally repressive H4K20me2 mark to enforce quiescence. Moreover, ablation of Suv4-20H1 specifically in satellite cells resulted in changes in chromatin structure accompanied by increased MyoD expression.

In addition to muscle injury, low stress exercise can also activate satellite cells, via accelerated Wnt signaling (Fujimaki et al., 2014). Indeed, the upregulation of canonical Wnt/β-catenin signaling pathway modifies the structure of chromatin at the *Myod1* and M*yf5* promoters, which results in an increased expression of both genes and a higher number of proliferating satellite cells. Of interest, in a recently published genome-wide analysis of p38α binding at promoters, the Wnt signaling pathway appeared as one of the principal signaling cascades modulated by p38α (Segales et al., 2016). This finding highlights the importance of p38α-dependent regulation of myogenesis, since modulation of Wnt signaling has been shown to critically regulate different aspects of satellite cell functions *in vivo* (expansion, switching from proliferation to differentiation or cell motility; Le Grand et al., 2009; Bentzinger et al., 2014; Murphy et al., 2014).

Interestingly, several recent whole transcriptome analysis of quiescent and proliferating muscle stem cells have shown that the expression of DNA methyltransferases is deregulated, since DNMT1 was markedly increased in activated satellite cells compared to quiescent satellite cells, whereas DNMT3a and all Tet isoforms were downregulated (Pallafacchina et al., 2010; Liu et al., 2013; Ryall et al., 2015a). These observations suggest that a regulation of the DNA methylation status, on top of regulating differentiation (see below) may be critical for initiating the MRF transcriptional program and/or regulating cell cycle in the transition between quiescence and proliferation.

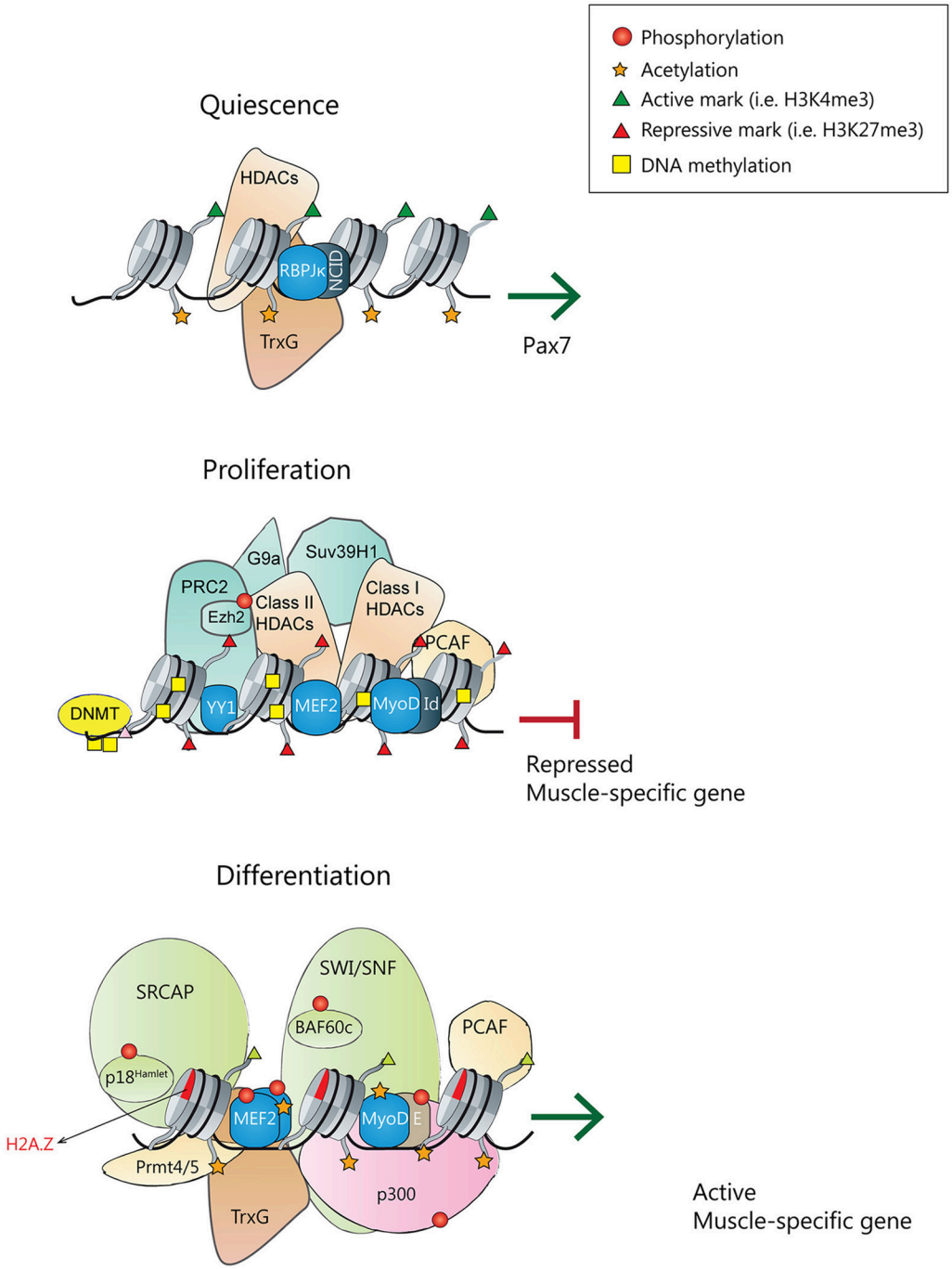

FIGURE 1 | Transcriptional and epigenetic regulators of satellite cell quiescence, proliferation and differentiation. (Top) During homeostasis, quiescent satellite cells express Pax7. Pax7 promoter is active, holding active chromatin marks, and being transcriptionally regulated by the Notch signaling pathway with the Notch intracellular domain (NICD) interacting with the effector protein recombining binding protein-Jκ (RBPJκ) (Wen et al., 2012), and although not demonstrated, probably populated by active chromatin remodelers and HATs. **(Middle)** In quiescent and proliferating satellite cells, muscle-specific gene promoters are repressed. MyoD is associated with several repressors (like Id) and Sir2 in a complex that also contains pCAF. MyoD, YY1, and MEF2 factors recruit the PRC2 complex, Suv39H1, and class I/II HDACs. DNMTs associate and methylate the DNA, and chromatin is populated with repressive histone marks. **(Bottom)** Upon differentiation cues, transcriptionally active muscle-specific promoters contain active phosphorylated MyoD/E heterodimers, phosphorylated MEF2 dimers and SRF transcription factors. In collaboration with arginine methyltransferases Prmt4/5, the SWI/SNF remodeling complex, HATs and Thritorax complexes will be recruited. DNA will be demethylated, and chromatin acetylated and populated with active histone marks.

EPIGENETIC REPRESSION OF THE MYOGENIC-DIFFERENTIATION GENE PROGRAM IN PROLIFERATING SATELLITE CELLS

Epigenetic events are crucial to maintain satellite cells in a quiescent or proliferating state and prevent their premature differentiation (**Figure 1**). DNA methylation is thought to be one of the main repressive systems acting on muscle gene loci (reviewed in Carrio and Suelves, 2015; Laker and Ryall, 2016). Indeed, DNA methyltransferase inhibitors (such as 5-azacytidine) can induce the transdifferentiation of fibroblasts into myoblasts (Taylor and Jones, 1979; Lassar et al., 1989); moreover, treatment of C2C12 myoblasts with 5-azacytidine promotes myogenesis, resulting in myotubes with enhanced maturity as compared to myotubes from untreated cells (Hupkes et al., 2011). Whereas the DNA methylation status of quiescent satellite cells has not been investigated in high detail, it has been shown that, when myoblasts differentiate into myotubes, a subset of genes display changes in their DNA methylation status, most of them becoming hypomethylated (Carrio et al., 2015). Other studies have proved that DNA methylation helps to restrict myogenin activation until both MEF2A and SIX1 transcription factors are co-expressed, in embryonic myogenesis and in adult myoblasts, and that binding of SIX1 and MEF2A is required for demethylation at the myogenin locus (Palacios et al., 2010b). Additionally, Ezh2 targets the DNA methyltransferases DNMT3a and DNMT3b to specific genes, thus linking the two repressive mechanisms: DNA and histone methylation (Vire et al., 2006). However, it is still unclear how DNA methylation is regulated during myogenesis or if this regulation affects only a subset of genes. A recent study showed the presence of 5-methylcytosine and 5-hydroxymethylcytosine within specific gene regions of the Notch signaling pathway in myoblasts, myotubes and mature skeletal muscle (Terragni et al., 2014). Notch signaling is crucial for the regulation of several developmental stages, including the quiescence and proliferative states of satellite cells (Bjornson et al., 2012; Mourikis et al., 2012; Qin et al., 2013). Terragni et al. identified that several Notch signaling genes, such as *Notch1* and its ligands *Dll1* and *Jag2* were hypomethylated and hydroxymethylated in skeletal muscle lineages, and propose that hypomethylation and/or hydroxymethylation could directly regulate the expression of these genes, thus placing key myogenic processes regulated by Notch signaling (e.g., satellite-cell maintenance and self-renewal; see Mourikis and Tajbakhsh, 2014) under epigenetic control (Terragni et al., 2014). Therefore, is crucial to determine how these modifications are regulated by specific DNA methyltransferases and demethylases that act during myogenesis.

Another important epigenetic mechanism to repress muscle-differentiation gene expression is the post-translational modification of histones. In proliferating satellite cells, the promoters of genes important for muscle differentiation contain histones that are hypoacetylated and marked with H3K9me2, H3K9me3, and H3K27me3. These repression marks are catalyzed by histone deacetylases (HDACs) and histone lysine methyltransferases from the PcG and Suv39H1 families (Palacios and Puri, 2006; Segales et al., 2015). In proliferating myoblasts, the transcriptional regulator Ying Yang 1 (YY1) recruits the histone H3K27 methyltransferase Ezh2 to muscle genes promoters, thus preventing their expression (Caretti et al., 2004). Another lysine methyltransferase, G9a, catalyzes the repressive H3K9me2 mark on MyoD target promoters and also directly methylates MyoD at lysine 104 (K104) to repress its transcriptional activity (Ling et al., 2012).

Muscle-specific gene expression can also be repressed by the action of HDACs, which remove acetyl groups from histone tails and also maintain transcription factors in a deacetylated state. In the absence of promyogenic signals, class I HDACs interact with MyoD and several members of class II HDACs interact with MEF2 repressing its activity, thus avoiding premature expression of skeletal muscle differentiation genes (Lu et al., 2000). Moreover, there is another class of HDACs called Sirtuins (or class III HDACs; Sir2), whose activity depends on NAD^+ levels and forms a repressor complex with pCAF and MyoD, suggesting that Sir2 works as a redox-sensor regulating muscle-specific genes expression in response to metabolic changes (Fulco et al., 2003). Gene transcription can also be regulated by other chromatin modifications, such as by switching canonical histones with histone variants or expressing specific histone isoforms in a cell type-specific manner (Happel and Doenecke, 2009). For instance, in undifferentiated myoblasts, MyoD expression is repressed by the homeobox protein Msx1, that interacts with the repressive histone methyltransferase G9a and also binds specifically to the histone variant H1b, which is enriched at the core enhancer region of *Myod1* (Lee et al., 2004).

EPIGENETIC REGULATION OF SKELETAL MUSCLE DIFFERENTIATION

For myogenic differentiation to take place, the expression of the differentiation-inhibitory gene program operating during myoblast proliferation has to be repressed. In fact, several cell cycle inactivating mechanisms are known act together with early muscle differentiation events. For instance, cell cycle exit is regulated by the retinoblastoma protein pRb, which represses the E2F transcription factors and its implicated in the recruitment of H3K9me3 and H3K27me3 methyltransferases at cell cycle-associated genes (Blais et al., 2007). Furthermore, the small chromatin-binding protein p8, which interacts with MyoD, the histone acetyltransferase p300 and the RNA helicase p68, negatively regulates the cell cycle and promotes myogenic differentiation (Sambasivan et al., 2009). Additionally, in myoblasts that are induced to differentiate, *Pax7* promoter presents increased levels of H3K27me3 repression mark, mediated by Ezh2 (Palacios et al., 2010a).

Following reception of differentiation-promoting signals, the epigenetic pattern of muscle-specific genes is quickly modified, and the Polycomb-mediated repressive H3K27me3 marks are substituted by the transcriptionally permissive H3K4me3 mark (**Figure 1**). H3K27me3 removal is mediated by the histone demethylase UTX, which can be recruited to muscle specific

loci by the homeodomain transcription factor Six4 and/or the histone chaperone Spt6 (Seenundun et al., 2010; Wang et al., 2013). Importantly, a very recent study using UTX specific deletion in satellite cells demonstrated that demethylation of H3K27 is required for myofiber regeneration (Faralli et al., 2016). Indeed, the demethylase activity of UTX has been proved to be necessary for myogenin expression, which leads to differentiation of myoblasts. Thereby, the authors uncovered a crucial role for the enzymatic activity of UTX in muscle-specific gene activation during tissue regeneration, and a physiological role for active H3K27 demethylation *in vivo* (Faralli et al., 2016). In parallel, the combined action of histone demethylases KDM1A/LSD1A and JMJD2A/KDM4A permits the removal of the H3K9me2/3 repressive marks deposited by KMT1A/Suv39h1 in proliferating myoblasts (Choi et al., 2010; Verrier et al., 2011). Moreover, the expression of several key repressors of transcription, such as the H3K27 methyltransferase Ezh2 and the H3K9 methyltransferase G9a, is downregulated at the onset of muscle differentiation (Asp et al., 2011; Ling et al., 2012). On the other hand, through its association with p38-phosphorylated MEF2D, the TrxG complex is recruited to muscle-specific promoters, such as *myogenin* and *Ckm*, and deposits the transcriptionally permissive mark H3K4me3 (Rampalli et al., 2007; Mckinnell et al., 2008). Other histone methyltransferases are also involved in myogenesis regulation; for instance, expression of the H3K4 histone methyltransferase Set7/9 increases during differentiation, and this is required to express muscle contractile proteins and for myofibril assembly (Tao et al., 2011). In addition, Set7/9 interacts with MyoD to enhance the expression of muscle-specific genes and prevents H3K9 methylation at muscle promoters mediated by Suv39h1. Thus, many mechanisms seem to work coordinately to efficiently remove the transcriptionally-repressive marks.

In undifferentiated myoblasts, muscle gene promoters are partially occupied by inactive transcription factors forming a complex with HDACs and HATs. Active HDACs and Sirtuins deacetylate HATs and inhibit their acetyltransferase activity, as demonstrated for Sirt2 and pCAF/Kat2b (Fulco et al., 2003). Importantly, the redox balance of the myoblast can regulate Sirt2 activity: upon receiving differentiation signals, its $[NAD^+]/[NADH]$ ratio decreases, leading to Sirt2 inhibition and enabling pCAF to acetylate several target proteins including histones, MyoD and MEF2 (Sartorelli and Caretti, 2005). In a recent study, a role in the regulation of myoblast differentiation has been ascribed to Sirt3, a mitochondrial NAD+ dependent deacetylase (Abdel Khalek et al., 2014). Sirt3 expression increases when C2C12 cells reach confluence and is maintained elevated during differentiation. Indeed, depletion of Sirt3 blocks differentiation, causing high levels of ROS and a decreased manganese superoxide dismutase activity (Abdel Khalek et al., 2014). Notably, sirtuins have also been found to play an important role during muscle stem cell activation, when satellite cells undergo a metabolic switch from fatty acid oxidation to glycolysis. This metabolic reprogramming is associated with reduced intracellular NAD^+ levels and lower activity of the histone deacetylase Sirt1, which leads to increased H4K16 acetylation and induction of muscle gene transcription (Ryall et al., 2015b). Furthermore, mice with muscle-specific ablation of the Sirt1 deacetylase domain have smaller myofibers and impaired muscle regeneration. Of note, the IGF1/AKT signaling pathway leads to p300 phosphorylation, which promotes its interaction with MyoD and the acetylation of muscle gene promoters (Serra et al., 2007). Thereby, the inhibition of HDACs and Sirtuins is coupled to HAT activation, which results in the activation of muscle transcription factors in response to different extracellular cues. Recent studies have also demonstrated that, upon differentiation, the HAT p300 is recruited at distinct Myod1 regulatory regions, resulting in an increased histone acetylation (Hamed et al., 2013). The authors also show that p300 directly participates in the early regulation of Myod1 enhancer, and shed light on how p300 histone acetyltransferase activity is associated to enhancer activation and, consequently, gene transcription (Hamed et al., 2013).

Chromatin remodeling is also essential for myogenesis, as illustrated by the recruitment of the SWI/SNF chromatin remodeling complex to muscle-specific loci, which depends on p38 MAPK activity (Simone et al., 2004). MyoD and the SWI/SNF subunit BAF60c are found to the promoters of MyoD target genes in myoblasts, previous to activation of transcription, while the ATPase subunit Brg1 is recruited only when cells are induced to differentiate, thus allowing chromatin remodeling and muscle genes transcription (Forcales et al., 2012). Furthermore, the histone arginine methyltransferases Prmt5 and Carm1 are also involved in the recruitment of the SWI/SNF complex to different muscle gene promoters, such as *myogenin* and *Ckm* (Dacwag et al., 2009). Interestingly, it has been recently shown that the regulatory sequences of genes expressed at late time points of myogenesis lie in close physical proximity, despite these genes being located on different chromosomes (Harada et al., 2015). Of note, formation of these inter-chromosomal interactions requires MyoD as well as functional Brg1. The late myogenic gene interactions are associated with the repression of these genes at early times of differentiation, suggesting that this higher-order chromatin organization constitutes a mechanism that contributes to temporal regulation of gene expression during muscle differentiation (Harada et al., 2015).

There is increasing evidence indicating that the substitution of canonical histones with histone variants can also regulate gene expression and muscle differentiation. For instance, the chromatin-remodeling complex SNF2-related CBP activator protein (SRCAP) modulates the replacement of histone H2A for the H2A.Z variant, which is associated with active transcription. At the onset of skeletal muscle differentiation, the SRCAP subunit $p18^{Hamlet}$, which deposites H2A.Z on chromatin, is recruited to the myogenin promoter in a p38 MAPK-dependent manner (Cuadrado et al., 2010). Additionally, upon induction of muscle differentiation, the canonical histone H3 is cleaved and the histone chaperones Asf1 and HIRA recruit the variant histone H3.3 to the *Myod1* promoter, allowing MyoD transcriptional activation. Moreover, histone H3.3 is deposited at myogenin and other muscle-specific promoters thanks to other histone chaperone, Chd2, and MyoD itself (Yang et al., 2011; Harada et al., 2012). Another histone variant that has been proved to be essential for the activation of the myogenic program is the macroH2A1.2 (mH2A1.2), which is characterized by

the presence of a ∼25 kDa evolutionarily conserved carboxyl-terminal domain called the macro domain. Indeed, activation of muscle enhancers is dependent on mH2A1.2, as it regulates both H3K27 acetylation and recruitment of the transcription factor Pbx1 at prospective enhancers (Dell'orso et al., 2016).

THE p38 MAPK SIGNALING PATHWAY

Cell response to external stimuli requires the integration and activation of intracellular mediators and effectors and signal transduction mechanisms, which strongly depend on post-translational modifications of proteins, among which phosphorylation is particularly relevant. A paradigm of intracellular signaling is the activation of mitogen-activated protein kinases (MAPKs), as they seem to participate in most signal transduction pathways. The MAPK superfamily of intracellular serine/threonine protein kinases is evolutionary conserved, and in mammals it principally includes the extracellular signal regulated kinases (ERKs), the c-Jun N-terminal kinases/stress activated protein kinases (JNKs/SAPKs), the p38 MAPKs, the ERK5 or big MAPKs and atypical MAPKs like ERK3/4, ERK7, and Nemo-like kinase (NLK) (Reviewed in Cargnello and Roux, 2011).

p38, a subgroup of the MAPKs, was firstly described as a transducer of the response to environmental stress conditions and as a critical mediator of inflammatory cytokines, but many different non-stress stimuli can also activate p38 MAPK signaling, leading to the regulation of multiple cellular processes, including senescence, apoptosis, cell-cycle arrest, regulation of RNA splicing, tumor development or differentiation of various cell types such as neurons, adipocytes, cardiomyocytes and myoblasts (reviewed in Cuenda and Rousseau, 2007; Igea and Nebreda, 2015). Mammalian cells have four p38 MAPK family members: MAPK14 (p38α), MAPK11 (p38β), MAPK12 (p38γ), and MAPK13 (p38δ). p38α is ubiquitously expressed usually at high levels, whereas p38β is expressed at lower levels and p38γ and p38δ have more restricted expression patterns (Cuenda and Rousseau, 2007; Cuadrado and Nebreda, 2010). All p38 MAPKs are phosphorylated and activated by the MAPK kinase MKK6. Others p38 MAPK kinases are MKK3, which activates p38α, p38γ, and p38δ, and MKK4 that in some cases can activate p38α. Once activated, p38 MAPKs phosphorylate serine/threonine residues of their substrates, which include several transcription factors as well as protein kinases (see Zarubin and Han, 2005). The identification of physiological substrates for p38α and p38β has been facilitated by the availability of specific pyridinyl imidazole inhibitors, such as SB203580/SB202190 and the reported inhibitor of the four p38 isoforms (BIRB0796) (Kuma et al., 2005; Bain et al., 2007). More than 100 proteins have been found to be directly phosphorylated by p38α and many of them are implicated in the regulation of gene expression (Cuenda and Rousseau, 2007; Cuadrado and Nebreda, 2010). In addition, the p38α pathway can regulate the production of extracellular signaling molecules, such as cytokines, chemokines, and growth factors. Recent reports have provided evidence for a dual role of p38α in regulating cancer progression, as, depending on the cell type and the tumoral stage, it can act either as tumor suppressor or as tumor promoter by facilitating tumor cell survival in response to chemotherapeutic treatments (Reviewed in Igea and Nebreda, 2015).

MAPKs activation has also been related to the differentiation capacity of various stem cell types. Particularly, the p38 pathway acts as one of the main controllers of muscle stem cells' fate decisions (Keren et al., 2006; Lluis et al., 2006; Segales et al., 2015). Indeed, several studies recapitulating myogenesis *in vitro* using different cellular models (myoblast cell lines or satellite cell-derived primary myoblasts) have demonstrated an active role of the p38 MAPK pathway in each myogenic stage, where its function as a regulator of the myoblast proliferation-to-differentiation transition is particularly relevant, as p38 induces cell cycle withdrawal and the expression of muscle-specific genes (Keren et al., 2006; Lluis et al., 2006; Liu et al., 2012; Doles and Olwin, 2015; Segales et al., 2015).

p38 MAPK SIGNALING IN SATELLITE CELL-DEPENDENT MYOGENESIS

Different stimuli can activate p38 MAPK in satellite cells, including inflammatory cytokines, such as TNF or amphoterin/HMGB1, growth factors such as TGFβ or cell-to-cell contact (reviewed in Guasconi and Puri, 2009; Krauss, 2010). The role of p38 MAPKs pathway during myogenesis has been extensively studied, especially *in vitro*. Pioneer studies from the groups of Cuenda, Bengal and Puri showed the requirement of this pathway for myogenic differentiation *in vitro* through distinct mechanisms (Cuenda and Cohen, 1999; Zetser et al., 1999; Wu et al., 2000). It has been proven that p38α turns off the proliferation-promoting JNK pathway by upregulating the JNK phosphatase MKP-1 leading to downregulation of cyclin D1 expression, cell cycle exit and allowing the onset of muscle differentiation (Perdiguero et al., 2007b). Besides this, independent studies have shown that the p38 MAPK signaling pathway is a critical regulator of skeletal muscle differentiation and fusion. In fact, the fusion of myoblasts into myotubes and the induction of muscle-specific genes are prevented by treatment with the p38α/β inhibitors, whereas forced activation of p38 MAPK by ectopic expression of a constitutively active mutant of MKK6 is sufficient to override the inhibitory factors present in proliferating cells and to induce both the expression of differentiation markers and the appearance of multinucleated myotubes (Lluis et al., 2006). Notably, *in vitro* studies using satellite cells lacking individual p38 family members showed that the four p38 isoforms are not completely redundant during myogenesis, and uncovered a predominant role of the p38α isoform in myogenic differentiation and fusion (Ruiz-Bonilla et al., 2008; Wang et al., 2008; Liu et al., 2012), with p38α regulating the whole myogenic gene expression program at multiple steps (see below) and promoting myoblast fusion by upregulation of tetraspanin CD53 (Liu et al., 2012), with p38γ signaling contributing to proliferation by preventing premature differentiation through induction of a repressive MyoD transcriptional complex (Gillespie et al., 2009), whereas

p38β and p38δ seemed to be dispensable for these processes (Perdiguero et al., 2007a; Ruiz-Bonilla et al., 2008).

Importantly, p38α/p38β are also required for activation of quiescent satellite cells and MyoD induction (Jones et al., 2005). Moreover, when satellite cells are activated, signaling by p38α leads to inactivation of tristetrapolin (TTP) and stabilization of MyoD RNA (Hausburg et al., 2015). Activated satellite cells enter the cell cycle and a subset undergoes asymmetric division to replenish the muscle stem cells pool. Interestingly, it has been recently published that p38α/β MAPK are asymmetrically activated in only one daughter cell, in which MyoD is induced, allowing cell cycle entry and generating a proliferating myoblast. In contrast, MyoD induction is prevented in the other daughter cell by the absence of p38α/β MAPK signaling, renewing the quiescent satellite cell pool (Troy et al., 2012).

A role of p38α in skeletal muscle growth and regeneration *in vivo* has also been reported. Using conditional satellite cell p38α-null mice the authors confirmed previous *in vitro* studies and demonstrated that p38α restrains postnatal proliferation and promotes timely myoblast differentiation (Brien et al., 2013). p38α ablation caused a postnatal growth defect together with an augmented number of satellite cells, due to increased progenitor proliferation postnatally. Moreover, muscle regeneration after a cardiotoxin-induced injury was delayed in the absence of p38α, with further enhancement of the satellite cell population (Brien et al., 2013). The absence of p38α was accompanied by increased p38γ phosphorylation, and p38γ inhibition *ex vivo* significantly diminished the myogenic defect. As muscle regeneration *in vivo* can occur quite effectively in the absence of p38γ (Ruiz-Bonilla et al., 2008), but is defective in the absence of p38α (Brien et al., 2013), p38α arises as the master kinase for reprogramming gene expression during proliferation-to-differentiation transition of satellite cells, both *in vitro* and *in vivo*.

Similar to mice, an essential role for p38 MAPK has been demonstrated in the regulation of human satellite cells (huSCs) regenerative capacity (Charville et al., 2015). The authors found that p38 was upregulated in activated muscle stem cells compared with quiescent satellite cells. Moreover, reversible inhibition of p38 in cultured human satellite cells prevented differentiation and promoted expansion of huSCs. These expanded satellite cells showed an enhanced self-renewal and engraftment potential in comparison to freshly isolated satellite cells or cells cultured without p38 inhibitors.

p38 MAPK SIGNALING IN THE TRANSCRIPTIONAL AND EPIGENETIC REGULATION OF MYOGENESIS

p38 MAPK pathway is crucial for the onset of muscle differentiation, as it modulates the expression and/or activity of many of the players involved in the transcriptional and epigenetic regulation of myogenesis (**Figure 2**). p38α/β MAPKs promote MEF2 transcriptional activity and MyoD/E47 heterodimer formation by direct phosphorylation of MEF2 and E47 (Zetser et al., 1999; Lluis et al., 2005), which in turn enhances RNA Pol II recruitment to myogenic loci, thus initiating the differentiation program. Furthermore, by phosphorylating the chromatin-associated protein BAF60c, p38α/β kinases contribute to the assembly of the myogenic transcriptosome on the chromatin of muscle loci by promoting the recruitment of SWI/SNF chromatin remodeling complex (Simone et al., 2004; Serra et al., 2007; Forcales et al., 2012), and ASH2L-containing mixed-lineage leukemia (MLL) methyltransferase complex (Rampalli et al., 2007). Through phosphorylation, p38α also recruits SNF2-related CBP activator protein (SRCAP) subunit $p18^{Hamlet}$ to muscle loci, which is in turn required for H2A.Z accumulation and transcriptional activation (Cuadrado et al., 2010).

p38α can also repress the expression of Pax7 in differentiating muscle stem cells by phosphorylation of Ezh2, which promotes the interaction between YY1 and PRC2 creating repressive chromatin on the *Pax7* promoter, therefore regulating the decision of satellite cells to proliferate or differentiate (Palacios et al., 2010a; Mozzetta et al., 2011). By contrast, activation of p38γ in satellite cells suppresses MyoD transcriptional activity by direct phosphorylation, via association with the H3K9 methyltransferase KMT1A, thus also affecting this myogenic decision (Gillespie et al., 2009). Moreover, two recent studies have shown that that activation of p38α/β downstream kinase Msk1, via phosphorylation of serine 28 on histone H3, regulates a chromatin exchange between Ezh2- and Ezh1-containing PRC2 complexes at the onset of differentiation (Stojic et al., 2011; Mousavi et al., 2012). Interestingly, downregulation of Ezh1blocks MyoD recruitment to the *myogenin* promoter, thereby impairing muscle differentiation. Furthermore, in differentiating myoblasts, Ezh1-containing complexes have also been associated to Pol II recruitment and transcriptional activation, questioning the prevalent view of PRC2 complexes as chromatin repressors (Mousavi et al., 2012). Thus, depending on the engagement of specific p38 isoforms, the p38 MAPK pathway can either induce or repress gene expression in satellite cells, Interestingly, in addition to all these nuclear functions, p38α has been recently shown to have an unexpected set of cytoplasmic substrates during myoblast differentiation, probably implicated in its own activation (Knight et al., 2012).

In response to osmotic stress, Hog1, the p38 MAPK homolog in yeast, has been shown to activate transcription by acting directly at chromatin (De Nadal et al., 2004; Pokholok et al., 2006). Equally, upon exposure to different kind of stresses, mammalian p38 MAPK can bind to some stress-responsive loci, highlighting that the interaction of the MAPK with target promoters can induce gene expression (Ferreiro et al., 2010). During myogenic differentiation, p38α has also been found to bind certain muscle-specific genes such as myogenin, muscle creatine kinase, and myosin heavy chain (Simone et al., 2004; Palacios et al., 2010a). Importantly, it has been recently demonstrated that p38α exerts its promyogenic function at least in part by binding and acting at chromatin (Segales et al., 2016). Genome-wide localization analysis linked to gene expression profiling have shown that p38α binds to a large number of active promoters during the myoblast proliferation-to-differentiation transition, confirming the importance of kinase signaling pathways in directly regulating transcription (Segales

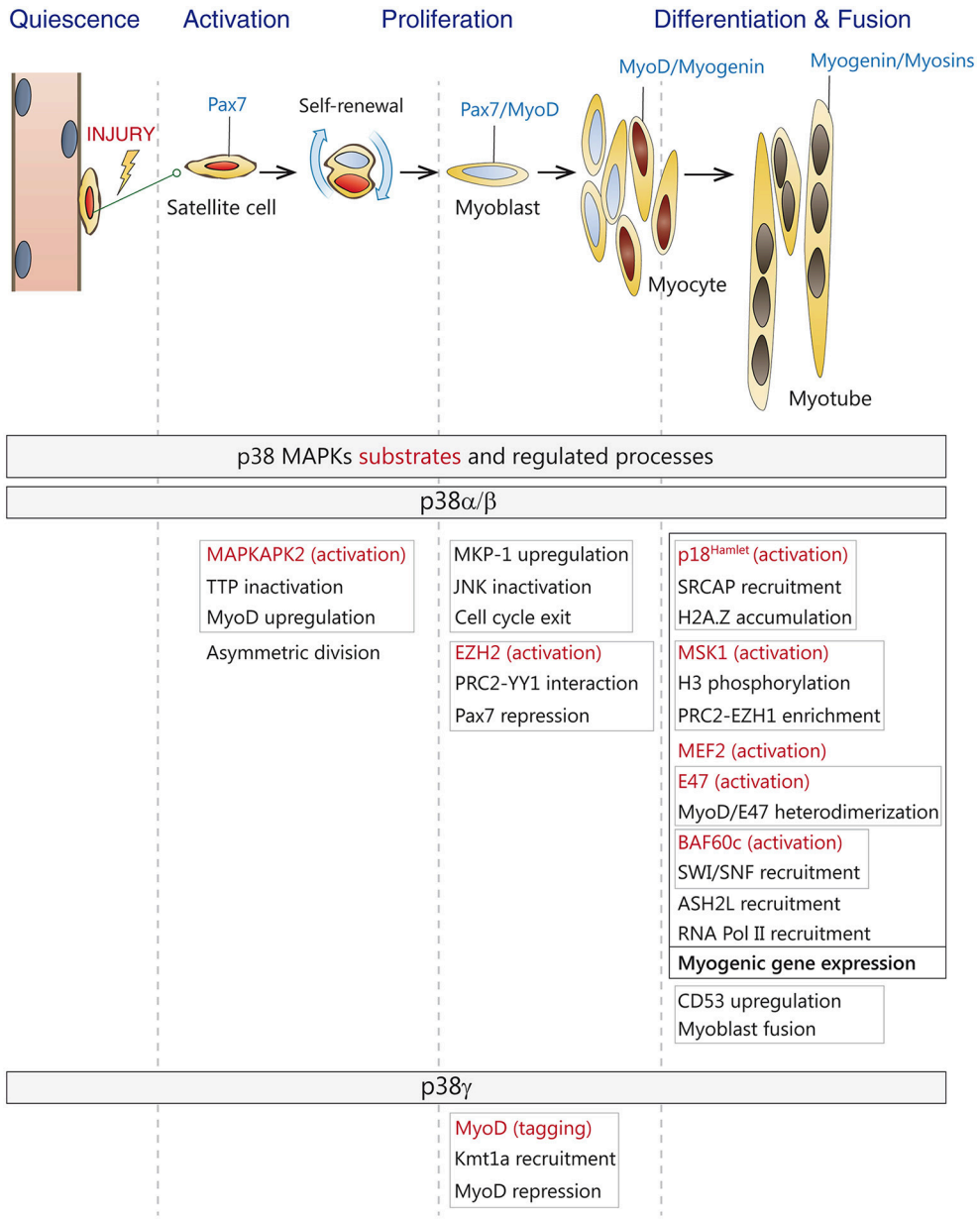

FIGURE 2 | Roles of p38 MAPKs in satellite cell-driven myogenesis. (Top) Satellite cells in adult muscles are in a quiescent state. Upon injury, satellite cells are activated, undergo asymmetric division and generate a self-renewing daughter cell and a committed progenitor or myoblast which enters the cell cycle. Myoblasts proliferate, differentiate and fuse to form myotubes and new myofibers during adult muscle regeneration. Canonical markers for each stage are indicated-**(Bottom)** Known substrates and regulated processes by p38α/β and p38γ MAPKs during the different stages of myogenesis. Direct substrates are indicated in red. Linked processes are boxed in gray or black.

et al., 2016), in agreement with previous reports for other related kinases (Bungard et al., 2010; Tiwari et al., 2012; Di Vona et al., 2015). Interestingly, p38α was also associated to promoters that were transcriptionally inactive or repressed at the onset of differentiation (Segales et al., 2016). Thus, p38α is recruited to a large set of myogenic gene promoters to facilitate their activation or repression, hence pointing to more complex regulatory mechanisms than previously anticipated. How is p38α recruited to muscle loci is not known, but it likely involves interaction with chromatin-regulatory and/or transcription factors, as demonstrated for several stress-induced genes (Ferreiro et al., 2010). Of interest, p38α-bound promoters are enriched with binding motifs for several transcription factors, principally Sp1, Tcf3/E47, Lef1, FoxO4, MyoD, and NFATc, which are known to be phosphorylation substrates of p38 MAPK (Segales et al., 2016).

p38 MAPK REGULATION OF MUSCLE STEM CELL FUNCTIONS DURING AGING

Aging is associated with an alteration of organism homeostasis and a progressive decline of tissue functions (Oh et al., 2014). Particularly, in skeletal muscle, mass and strength decline with aging (a process called sarcopenia), which is also linked to a progressive loss of muscle stem cells regenerative capacity. Age-related dysfunction of muscle regeneration has been attributed to both extrinsic alterations in the regenerative microenvironment and to satellite cell-intrinsic mechanisms (recently reviewed in Garcia-Prat et al., 2013; Blau et al., 2015; Brack and Munoz-Canoves, 2015; Sacco and Puri, 2015; Sousa-Victor et al., 2015). Indeed, whereas parabiosis (a process whereby the circulatory system of two living organisms is surgically joined), and whole muscle grafting experiments in mice proved that alterations in the regenerative environment of aged muscles regulate stem cell function (Conboy et al., 2005; Brack et al., 2007), data from recent studies using transplantation of purified satellite cells support cell-intrinsic deficits with aging (Chakkalakal et al., 2012; Bernet et al., 2014; Cosgrove et al., 2014; Price et al., 2014; Tierney et al., 2014; Sousa-Victor et al., 2014a,b; Garcia-Prat et al., 2016). These cell-intrinsic functional deficits can include DNA and oxidative damage, impaired mitochondrial function and alterations of the epigenetic profile, such as post-translational histone modifications or changes in the DNA methylation patterns. In this regard, a progressive increase in DNA methylation in aging muscle has been recently reported (Ong and Holbrook, 2014; Zykovich et al., 2014) and it has also been described that muscle stem cells isolated from aged mice show alterations both in the levels and distribution of the H3K27me3 chromatin mark (Liu et al., 2013). At the level of single genes, the promoter of *INK4a*, which encodes the cell cycle inhibitor and marker of senescence $p16^{INK4a}$, is epigenetically repressed in young quiescent satellite cells via ubiquitination of histone H2A; a mechanism deregulated in geriatric cells, which highly express $p16^{INK4a}$ and undergo a transition to a presenescent state. This conversion from quiescence to senescence is a hallmark of geriatric satellite cells, and it can be reversed through the downregulation of $p16^{INK4a}$ expression, which also restores the self-renewal capacity of satellite cells (Sousa-Victor et al., 2014a,b). In human geriatric satellite cells $p16^{INK4a}$ is upregulated, so these findings are particularly relevant for stem-cell rejuvenation in sarcopenic muscles.

Contrary to other types of stem cells, such as hematopoietic stem cells, both the function and the number of satellite cells decline with aging, compromising the recovery capacity of sarcopenic muscles in response to injury (Brack et al., 2005; Chakkalakal et al., 2012; Sousa-Victor et al., 2014b). Whether satellite cell decline might contribute to age-associated loss of muscle mass in the absence of muscle damage is a matter of active debate. By depleting satellite cells in young sedentary animals, one study showed that these cells do not contribute to the maintenance of muscle size or fiber type composition during aging, but that their loss may contribute to age-related muscle fibrosis (Fry et al., 2015). Through genetic lineage experiments, another study showed that even in the absence of injury, satellite cells contribute to myofibers in all adult muscles, although the extent and timing differed (Keefe et al., 2015). However, genetic ablation experiments in this latter study showed that satellite cells are not globally required to maintain myofiber size of uninjured adult muscle.

Several signaling pathways have been found altered in satellite cells from aged mice, among them fibroblast growth factor receptor-1 (FGFR1), p38 MAPK, and JAK/STAT, thus contributing to impaired control of quiescence and compromised self-renewal capacity (Bernet et al., 2014; Cosgrove et al., 2014; Price et al., 2014; Tierney et al., 2014).

There is clear evidence that during aging the FGFR1 signaling pathway is altered. It has been suggested that increased FGF2 signaling in aged muscle can cause the disruption of satellite cell quiescence (Chakkalakal et al., 2012). In more recent studies it has been proposed that the augmented FGF2 in the aged satellite cell niche constitutes a mechanism to compensate for the loss of FGFR1 signaling. Furthermore, alterations in FGF2 signaling, together with elevated levels of TNF observed in old muscles, have been associated to constitutive and aberrant activation of the p38 MAPK pathway, which leads to impaired self-renewal of aged muscle stem cells (Bernet et al., 2014). Indeed, it has been proved by two different groups that this aberrant activation of the p38 MAPK in aged satellite cells interferes with asymmetric division, resulting in highly reduced self-renewal and regenerative capabilities. Interestingly, pharmacological inhibition of p38α/β can partially restore the proliferative capacity and self-renewal of aged muscle stem cells as assessed in muscle transplantation experiments (Bernet et al., 2014; Cosgrove et al., 2014). Of note, the advantageous effects of p38α/β MAPKs neutralization were strongly enhanced if satellite cells treated with p38 MAPK chemical inhibitors were cultured in a hydrogel matrix, mimicking the biomechanical properties of young muscles, thus providing the basis for improving stem cell engraftment for muscle regeneration in aged individuals (Cosgrove et al., 2014).

Finally, the reduced regenerative potential of aged muscle stem cells has also been correlated with increased JAK/STAT signaling, which impairs satellite cell function by stimulating asymmetric division (Price et al., 2014). Moreover, IL-6-activated STAT3 regulates the expression of the myogenic factor MyoD, promoting differentiation of satellite cells in detriment to their expansion (Tierney et al., 2014). Thus, these studies evidence that intracellular signaling pathways, such as JAK-STAT and p38 MAPK might play distinct age-dependent roles in myogenesis.

PERSPECTIVE

As discussed in this review, an emerging concept in the field of myogenesis is that the cell-intrinsic signaling networks that are activated in response to external cues are dysregulated in satellite cells with aging. One paradigm of this age-dependent dysregulation is the p38 MAPK signaling pathway. While it is transiently required for the activation, cessation of proliferation

and initiation of the myogenic-differentiation gene program of adult satellite cells, thus playing an important positive role for muscle regeneration in the adult, its persistent activation in aged satellite cells has deleterious consequences on the functionality of these cells, thus impairing efficient regeneration with aging. Thus, identifying the epigenetic and transcription drivers and targets of the p38 MAPK pathway (and of other major myogenic signaling cascades) will be key to shed light on the complex regulation of myogenesis throughout life, and will facilitate the identification of molecular targets that can be druggable for intervening in muscle aging and disease, in order to enhance regeneration of degenerating muscle in both conditions.

AUTHOR CONTRIBUTIONS

JS, EP, and PM wrote the manuscript. All authors read and approved the final manuscript.

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REVIEW
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p38 MAPK Signaling in Osteoblast Differentiation

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The skeleton is a highly dynamic tissue whose structure relies on the balance between bone deposition and resorption. This equilibrium, which depends on osteoblast and osteoclast functions, is controlled by multiple factors that can be modulated post-translationally. Some of the modulators are Mitogen-activated kinases (MAPKs), whose role has been studied *in vivo* and *in vitro*. p38-MAPK modifies the transactivation ability of some key transcription factors in chondrocytes, osteoblasts and osteoclasts, which affects their differentiation and function. Several commercially available inhibitors have helped to determine p38 action on these processes. Although it is frequently mentioned in the literature, this chemical approach is not always as accurate as it should be. Conditional knockouts are a useful genetic tool that could unravel the role of p38 in shaping the skeleton. In this review, we will summarize the state of the art on p38 activity during osteoblast differentiation and function, and emphasize the triggers of this MAPK.

Keywords: p38, MAP kinase, bone development, bone homeostasis, osteoblast, cell signaling, cell differentiation

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INTRODUCTION

Two decades ago, the Mitogen-activated kinases (MAPKs) were revealed as key players in skeletal development and bone homeostasis that particularly affect osteoblast commitment and differentiation. Of the three classic MAPKs, scientific evidence predominantly points to p38 and ERK activities as determining and shaping the skeleton. Remarkably, there are hundreds of reports of *in vivo* and *in vitro* studies that analyse the relevant role of p38 and ERK throughout the osteoblastic commitment process, from a mesenchymal progenitor into a fully functional anabolic bone cell. This field of study has been facilitated by the availability of specific inhibitors of MAPK activity. However, these inhibitors may lead to dubious correlations between the specific causal effect of MAPK inhibition and MAPKs' role. There are several complete or cell-directed knockouts that provide a broader view of the many effects that MAPKs have on bone differentiation. The purpose of this review is to describe the precise role of p38-MAPK on osteoblast differentiation and the several upstream events that can trigger its activation, in the interests of guiding anabolic therapies for bone-related pathologies.

Bone and Its Constituents

The skeleton is a very dynamic, calcified organ whose structure is maintained by bone deposition and resorption. During the last decade, new skeletal functions beyond those associated with locomotion and organ protection have been discovered, including fertility, glucose and adipose metabolism, phosphate renal clearance and maintenance of the hematopoietic niche (Karsenty and Ferron, 2012). Structurally, bone tissue is composed of different cells and an extracellular matrix (ECM). This matrix has two components: one organic and another inorganic. The latter

is mainly formed by hydroxyapatite, which represents 99% of the body's calcium and 80% of the body's phosphate. The organic component is composed of collagen fibers, glycosaminoglycans, proteoglycans and glycoproteins. Collagen I is the most common protein in bone, accompanied by proteins such as bone sialoprotein (*IBSP*) and osteonectin (*SPARC*). Moreover, certain cytokines, such as TGF-β (transforming growth factor-β) and BMP (bone morphogenetic protein), remain bound to ECM fibers and can be freed during resorption processes (Dallas et al., 2002; Gregory et al., 2005).

Bone formation can be explained from two perspectives: embryological origin, and ossification. Embryologically, facial skeletal structures are derived from the ectoderm, while the axial and appendicular skeletons emerge from the paraxial mesoderm and the lateral mesoderm plate, respectively (Berendsen and Olsen, 2015). Ossification can be defined as endochondral or intramembranous. The latter mechanism occurs directly from mesenchymal condensation, in which mesenchymal stem cells (MSCs) differentiate into osteoblasts (Percival and Richtsmeier, 2013). Endochondral ossification is a very well-characterized process that takes place in long bones. A cartilage cast is invaded by mesenchymal progenitors, which leads to the appearance of a chondrocyte-enriched growth plate that allows longitudinal bone growth, and an osteoblast-driven ossification center in each epiphysis. Concurrently, cells in the periphery of the cartilage cast will differentiate into osteoblasts, which create the bone cortical structures (Long and Ornitz, 2013).

Once formed, several cells from different origins end up composing the skeleton. The osteoblast is considered the most anabolic bone cell, due to its ability to secrete and calcify the extracellular matrix. Osteoblasts are cuboidal cells that lie on the surface of bone matrix (Long, 2012). They are derived from MSCs that can also differentiate into adipocytes, myoblasts, chondrocytes and fibroblasts (Augello and De Bari, 2010). Differentiation into osteoblasts is a complex process, in which serial commitment landmarks are achieved sequentially (**Figure 1**). It is accepted that during the first steps of commitment, these progenitors can still express stem markers and chondrocyte markers (e.g., SOX9). Pre-osteoblasts have already lost signature genes of the chondrogenic profile, and they start to express certain specific markers associated with the osteoblast linage, although they cannot produce extracellular matrix yet. These osteoblast-specific genes include transcription factors such as *RUNX2*, *DLX5* and *SP7* (Osterix). Soon after, other markers that are related to matrix formation start to be expressed: osteocalcin (*Bglap2*), fibromodulin (*Fmod*), and bone sialoprotein (*Ibsp*), among others (Franceschi et al., 2007; Long, 2012).

Once osteoblasts have reached the bone formation phase, four outcomes may occur: (1) they get trapped in bone matrix as osteocytes; (2) they become bone-lining cells; (3) they enter apoptosis; and (4) they can trans-differentiate into cells that depose chondroid tissue (Franz-Odendaal et al., 2006; Rochefort et al., 2010). Typically, osteocytes are closed in lacunae inside calcified bone. In the beginning of this embedding process, young osteocytes still look like secretory cells and express osteoblast markers. Some of these will be lost during terminal differentiation, while mature osteocytic markers appear (Rochefort et al., 2010). Osteocytes tend to express higher levels of *FGF23* (fibroblast growth factor 23), *NPY* (neuropeptide-Y), *RLN* (reelin), *SOST* (sclerostin), *DMP1* (dentin matrix acidic phosphoprotein 1), *PHEX* (phosphate-regulating neutral endopeptidase, X-linked), *PDPN* (gp38/E11), while matrix metalloproteinases and collagen proteins are more closely related to the osteoblast state (Paic et al., 2009). Recently, relevant functions have been attributed to osteocytes, including bone remodeling and regulation of the hematopoietic stem cell niche (Asada et al., 2013). Remarkably, osteocytes show dendritic-like cytoplasmic prolongations (50–60 per cell) that form a canalicular system inside bone (Rochefort et al., 2010; Klein-Nulend et al., 2013). These structures serve for sensing and interpreting mechanical inputs like bone loading (Rochefort et al., 2010; Xu et al., 2012).

Bone resorption is the process by which the mineral extracellular matrix is degraded. This process is primarily carried out by osteoclasts, which are derived from monocytes and macrophages (Ikeda and Takeshita, 2016). They proliferate in the bone marrow, and fuse to give rise to multinuclear reabsorbing cells close to the resorption region. Osteoclast progenitors express RANK (receptor activator of nuclear factor-κB), which interacts with RANKL (receptor activator of nuclear factor-κB ligand). Several cells produce RANKL, including osteoblasts, osteocytes, stromal cells and lymphocytes (O'Brien et al., 2013; Ikeda and Takeshita, 2016). The RANK activation process can be antagonized by osteoprotegerin (OPG), a RANKL competitor that is mainly expressed by osteoblasts (Simonet et al., 1997). Other factors contribute to the activation and function of osteoclasts, such as vitamin D and SOST, which is also mainly produced by osteocytes (van Bezooijen et al., 2005).

THE MAPK SIGNALING PATHWAYS

Introduction to the MAPKs

MAPKs are a family of enzymes that are implicated in a series of processes in which extracellular stimuli (e.g., environmental stress, growth factors and cytokines) are transduced into different cellular actions. In some cases, they act as a signaling hub in which different signaling pathways converge to activate a MAPK in a given time frame or tissue. Conventional MAPK members are the extracellular signal-regulated kinases 1/2 (ERK1/2) and ERK5, c-Jun amino (N)-terminal kinases 1/2/3 (JNK1/2/3), and the p38 isoforms (p38α, p38β, p38γ, and p38δ) (reviewed in Cargnello and Roux, 2011). All of them contain a Ser/Thr kinase domain, activated by phosphorylation by other Ser/Thr kinases. Thus, MAPK signaling constitutes a series of phosphorylations, in which several elements are at play until the final substrate is targeted. Therefore, once stimuli have reached the cell, MAPKK kinases (MAP3K) are activated and phosphorylate MAPK kinases (MAP2K), which in turn phosphorylate and activate the aforementioned MAPKs. Each

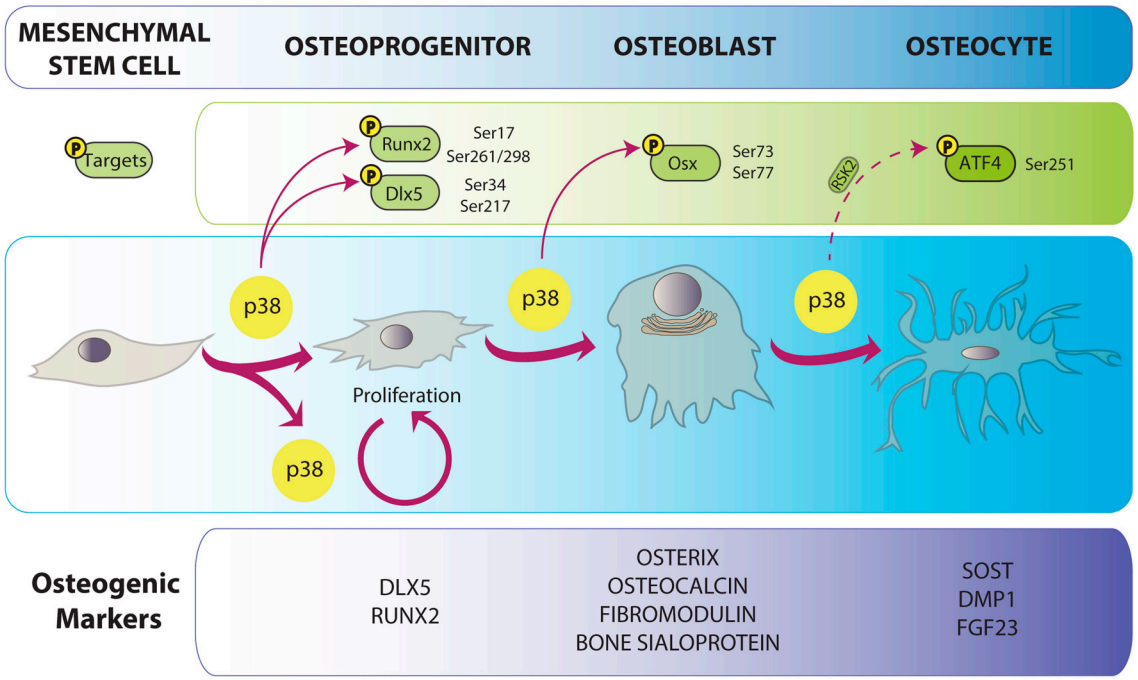

FIGURE 1 | p38 implication in osteoblast differentiation. Osteoblast differentiation is characterized by the expression of a specific cohort of proteins depicted in the lower panel. p38 is related both to differentiation and proliferation of bone progenitors. p38-mediated phosphorylation promotes progression in osteogenesis by the enhancement of the activity or expression of osteoblast-specific transcription factors genes. Direct arrows indicate direct phosphorylation while dashed arrow indicates indirect action of p38. Phosphorylated residues are specified for every target.

MAPK group has its own series of upstream activators, thus each represents a specific signaling cascade (Cuadrado and Nebreda, 2010).

JNK Signaling in Osteoblastic Differentiation

The role of JNK in osteoblastogenesis seems to be somewhat contradictory, with different functional outputs depending on the study. For instance, interleukin-1β (IL1β) and tumor necrosis factor-α (TNFα) favor JNK activation and this effect is determinant for osteoblast differentiation of human periosteal cells (Hah et al., 2013). Moreover, Liu et al. found that inhibition of the JNK route by chemical inhibitors or siRNAs led to decreased mineralization and downregulation of several osteogenic markers, while its activation by a constitutive active form favored osteogenesis. These effects were, at least in part, due to reduced SMAD6 binding to BMPRI (BMP receptor I), which allows SMAD1 to access the receptor (Liu et al., 2011). However, another effect of JNK activation might be to negatively regulate osteogenesis. JNK1 reduces RUNX2 transcriptional activity by phosphorylation at Ser104 (Huang et al., 2012). In addition, the inhibition of JNK with SP600125 in human MSC increases ALP activity induced by BMP-2 (Biver et al., 2014). This inhibitory action may also integrate SMAD1 signaling, as MAPK phosphorylation is necessary for GSK3β (Glycogen synthase kinase 3β)-induced SMAD1 degradation (Fuentealba et al., 2007; Biver et al., 2014).

ERK Signaling in Osteoblastic Differentiation

Both ERK1 and ERK2 are expressed in osteoblasts and have relevant functions in bone metabolism. There is genetic evidence of the implication of the ERK pathway in osteogenesis. First, the expression of a dominant negative form of MEK1 under the regulation of the osteocalcin promoter (mOG2:*Mek1*DN) exhibits calvarial and clavicular defects, which phenocopy the *Runx2* deficiency. The phenotype of $Runx2^{+/-}$ is recovered by crossing these mutant mice with a constitutive active form of MEK1 (mOG2:tg*Mek1*SP) (Ge et al., 2007). Matsushita and collaborators generated the double mutant $Erk1^{-/-};Erk2^{Prx1:Cre}$. They showed the positive effect of ERK1 and ERK2 on osteoblast differentiation, and the inhibitory effect of these MAPKs on chondrocyte differentiation at the perichondrium. In addition, ERK has certain effects on RANKL in these cells, which has an impact on osteoclast activation (Matsushita et al., 2009).

RUNX2 phosphorylation is the action of ERK signaling on osteoblast specification that has been most widely studied. First, use of the MEK1 inhibitor PD98059 blocks osteocalcin induction by RUNX2 (Xiao et al., 2000). U0126, another MEK1/2 inhibitor, halts the expression of osteocalcin and *Ibsp* mediated by BMP7 and ascorbic acid (Xiao et al., 2002a). In addition, FGFs activates pERK, and subsequently a phosphorylated form of RUNX2 is detected. Again, these effects can be blocked by U0126 (Xiao et al., 2002b). Finally, the same group proved the existence of four serine residues (Ser43, Ser301, Ser319, and

Ser510) targeted by ERK1/2. Ser301 and Ser319 were found to be responsible for RUNX2 activating abilities (Ge et al., 2009). The increase in pRUNX2 transcriptional activity induced by ERK could be attributed to the binding of cofactors such as CREB, CBP/p300 or vitamin D receptor (Sierra et al., 2003), as seen in p38 phosphorylation (Greenblatt et al., 2010). Related to this, an increase in cytoplasmatic pERK has been associated with an increment in nuclear RUNX2 upon overexpression of the transmembrane glycoprotein CD99, which implies that RUNX2 phosphorylation takes place in the cytoplasm (Sciandra et al., 2014).

NF1 (neurofibromatosis type I) is a GTPase-Activating Protein that turns off Ras action on ERK signaling. The inactivating mutation of *NF1* seems to have different results depending on the developmental status at which deletion takes place (Greenblatt et al., 2013). In fact, analysis of the osteoblast-specific mutant of *Nf1* shows a blunted response to BMP-2 that is overcome when ERK was inhibited by U0126 (de la Croix Ndong et al., 2015). Besides differentiation, ERK is related to the activity of cyclinD1, enhanced by PTH and PTHrP, and favoring osteoblast proliferation (Datta et al., 2007). Recently, it has been shown that ERK regulates the antiapoptotic and proliferative effects of EGF on osteoprogenitors (Chandra et al., 2013).

p38 MAPK Cascade

The p38 MAPK family is composed of four proteins: p38α (encoded by the gene *Mapk14*), p38β (*Mapk11*), p38γ (*Mapk12*), and p38δ (*Mapk13*). Their coding genes have a distinct tissue distribution and they appear differentially expressed, being *Mapk14* the most highly expressed (Cuadrado and Nebreda, 2010). p38 MAPKs are substrates for three MAP2K (MKK6, MKK3, and MKK4). The contribution of each of these MAP2K to p38 MAPKs activation depends on the stimulus and the cell type (Alonso et al., 2000; Brancho et al., 2003; Remy et al., 2010; **Figure 2**). The MAP3Ks that lead to p38 MAPKs activation are ASK1, DLK1, TAK1, TAO1, TAO2, TPL2, MLK3, MEKK3, MEKK4, and ZAK1 (Cuadrado and Nebreda, 2010). p38α can also be autophosphorylated, due to activation through ZAP70, $p56^{lck}$, and TAB1, and downregulation of Cdc47 (Cuadrado and Nebreda, 2010).

It is known that there are several scaffold complexes along this route that facilitate MAPK-MAP2K interaction or locally increase the concentration of effectors of the MAPK cascade, driving fine spatio-temporal regulation. The most commonly reported molecules related to this function belong to the protein family named JIP (from c-Jun NH2-terminal kinase-interacting protein) that can tether p38 and JNK to their upstream MAP2K. JLP, which have the isoforms JIP4 and SPAG9 (Kelkar et al., 2005; Dhanasekaran et al., 2007) and JIP2, have been implicated in regulating p38 by tethering to MKK3, MEKK3, and MKK4 (Dhanasekaran et al., 2007). The role of these scaffold proteins in regulating MAP2K action could be of special interest, as it has been shown that constitutive activation of MKK3 or MEK1 has deleterious effects on BMP-induced osteoblastogenesis (Huang et al., 2014). Apart from JIP proteins, there are other potential MAPK scaffolds: RACK1 (Arimoto et al., 2008), KSR-2 (Liu et al., 2009), Sec8 (Tanaka et al., 2014) and the osmosensing scaffold for Rac-MEKK3-MKK3 (OSM) (Dhanasekaran et al., 2007). Out of these, *Mapk8ip2* (JIP2) is detected in the developing skeleton, and *Exoc4* (Sec8 protein), which interacts with JIP4, is expressed in bone cells (www.genecards.org and www.eurexpress.org). Besides the aforementioned proteins, TAK1 and TAB1 could also be considered a sort of scaffold proteins, as they facilitate the recruitment and activation of upstream effectors of the p38 pathway. However, no MAPK scaffold proteins have been investigated in osteoblast development *in vivo* to date.

p38 MAPKs are inactivated mainly by dephosphorylation by certain phosphatases belonging to the DUSP family (Bermudez et al., 2010). Moreover, the catalytic activity of p38α is modulated according to how many and which threonine or tyrosine residues are phosphorylated in the activation loop (Zhang et al., 2008). Like any other relevant molecule, p38 is also regulated by mechanisms such as acetylation, protein degradation and stabilizing cofactors and, obviously, changes in the expression of their coding genes (reviewed in Cuadrado and Nebreda, 2010). p38α is localized in the nucleus and the cytoplasm (Raingeaud et al., 1995). Its localization depends on activation and on active transport, as p38 does not have a nuclear localization signal. It is known that DNA damage favors phosphorylation and nuclear accumulation of p38α, probably by releasing p38α from TAB1 or MK2, as they act as cytoplasmatic anchors (Wood et al., 2009).

p38 AND MESENCHYMAL DIFFERENTIATION

The p38 pathway has been implicated in the differentiation of several mesenchymal cells. Most reports on this topic have been cell-based studies in which activation or inhibition of the pathway was achieved mainly pharmacologically.

Adipocyte Differentiation

There is some controversy about the role of p38 in adipogenesis. Some authors claim that it depends on the species or on the specific cell type used *in vitro*, where different factors could be at play. The generation of a tissue-specific knockout would shed light on this theme. p38 was linked to the phosphorylation of C/EBPβ and the expression of PPARγ (peroxisome proliferator-activated receptor-γ) in 3T3-L1 cells (Engelman et al., 1998). In fact, the overexpression of an active form of MKK6 favors adipocyte differentiation (Engelman et al., 1999). (2S)-7,4′-dihydroxy-8-prenylflavan also stimulates the adipogenic program and glucose uptake via p38 (Ji et al., 2015). Contrary to the main current of knowledge regarding BMP-directed osteogenesis, this cytokine also stimulates adipogenesis of mesenchymal precursors via p38, probably involving SMAD proteins as well (Hata et al., 2003; Huang et al., 2009). Regarding adipocyte physiology, one of the few experiments performed on living animals was the administration of the p38 inhibitor FR167653. This treatment significantly reduced weight gain, fat depots and adipocyte size (Maekawa et al., 2010). p38 also seems relevant for brown adipocyte commitment, as seen with DUSP10 induction (Choi et al., 2013) or the inhibitory effects of silica nanoparticles (Son et al., 2015). Indeed, NPY seems to

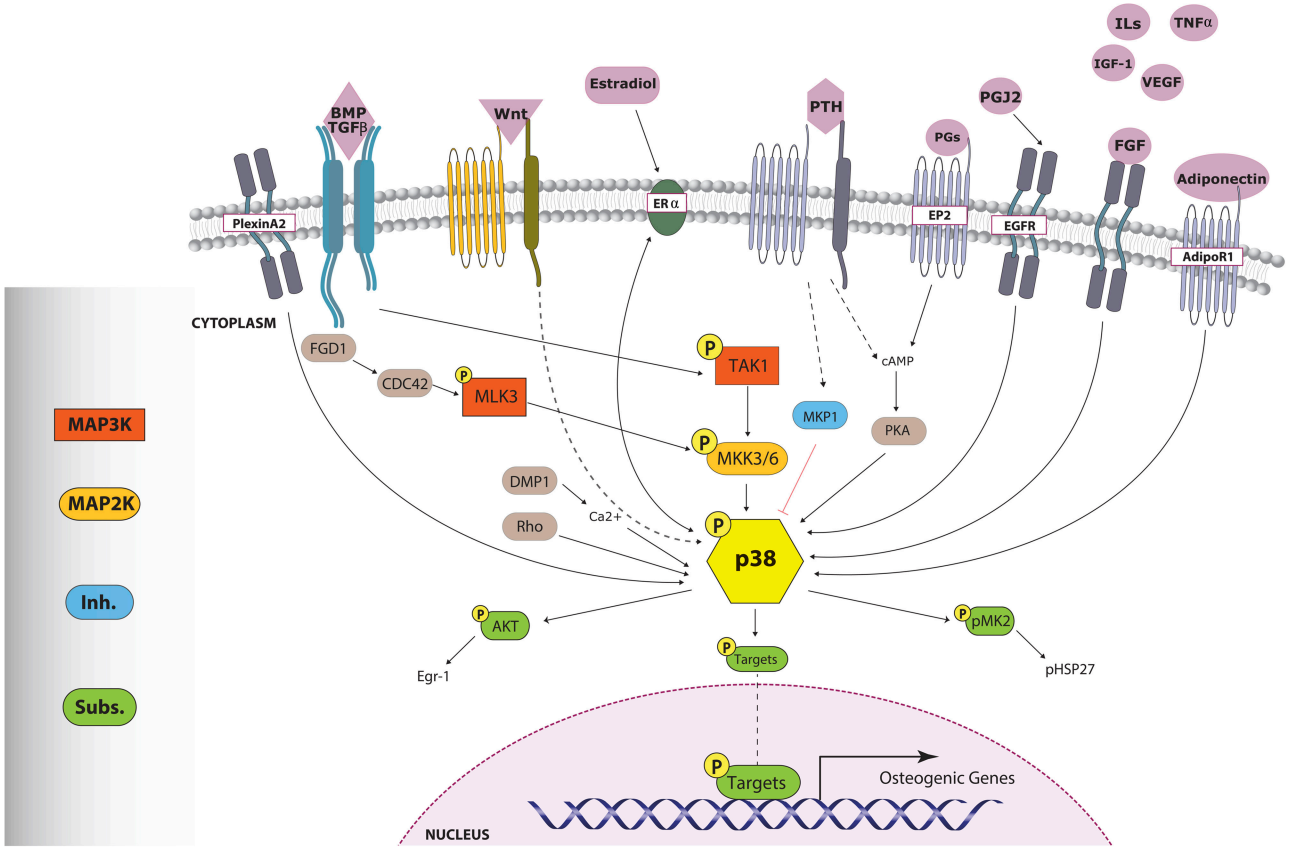

FIGURE 2 | p38 triggers involved in osteogenesis. Numerous stimuli, through distinct mechanisms, lead to p38 MAPK phosphorylation. Activated p38 phosphorylates important osteoblast-related transcription factors, which will enhance osteogenic gene expression. pp38 can also trigger other pathways through the activation of cytoplasmic-acting substrates such as AKT and pMK2. Sharp arrows indicate direct activation. Dashed arrows indicate an unknown or indirect mechanism of action while the red flat-tipped arrow denotes inhibition.

affect the brown fat gene program and reduce p38 and CREB phosphorylation (Wan et al., 2015).

Other studies describe contrary effects. For example, p38 activates CHOP, a negative regulator of C/EBPβ (Wang and Ron, 1996), and also blocks NFATc4, which is a transcription factor for *Pparg* (Yang et al., 2002). In chicken adipocytes, adiponectin prompts a cascade through p38/ATF2 that inhibited CEBPα, and affects preadipocyte differentiation (Yan et al., 2013). Adipogenesis conducted by microtubule affinity-regulating kinase 4 (Marc4) depends on JNK activation and p38 downregulation in 3T3-L1 (Feng et al., 2014). p38 activity seems to decrease during adipocyte differentiation, and inhibition of p38 augments PPARγ transactivation and other adipocyte markers. Furthermore, p38-deficient MEFs have increased expression of adipogenic markers such as adiponectin or leptin (Aouadi et al., 2006). Strikingly, the same group proposed a positive role for p38 during human adipocyte differentiation, mainly based on C/EBPβ phosphorylation (Aouadi et al., 2007). Ferguson et al. linked the role of DUSP1 phosphatase to the deactivation of p38 and ERK during the first steps of preadipocyte commitment, which establishes a framework for self-regulatory feedback (Ferguson et al., 2016).

Myoblast Differentiation

p38 MAPK isoforms have multiple roles in muscle development and homeostasis in the adult. They control self-renewal, proliferation, asymmetric division and differentiation of satellite cells (Brien et al., 2013; Bernet et al., 2014). Early studies show higher p38α, β, and γ levels during myoblast maturation, and that p38 inhibition blocks muscle-specific gene transcription (Cuenda and Cohen, 1999; Zetser et al., 1999). p38 activity is absolutely required for the transition from proliferating satellite cells into terminally differentiated myoblasts (Puri et al., 2000). This control of the muscle-specific gene program is performed through multiple steps that finally lead to activation of key myogenic transcription factors. For instance, p38 induces the transcriptional activity of MEF2 family members through direct phosphorylation at Thr293 (Wu et al., 2000; Lluis et al., 2006). In addition, p38 also phosphorylates and activates the obligate MyoD partner E47 (Lluis et al., 2005). Several reports indicate that p38 activity is required for chromatin remodeling, to allow access and stabilization of the binding of myogenic transcription factors to myogenic loci (Lluis et al., 2006). These mechanisms involve histone acetylation by PCAF and p300, and recruitment of the SWI/SNF complex (Simone et al., 2004; de la Serna et al.,

2005). Subsequently, p38 activity is important for myocyte fusion and myofibrillogenesis (Gardner et al., 2015).

More recently, the role of p38 in muscle development has been extended to the activation of satellite cells during muscle regeneration. Muscle regeneration takes place in a highly inflammatory environment, and inflammatory cytokines such as IL6 or TNFα activate p38 (Li et al., 2014c). Signaling from p38α/β is involved in the exit of satellite cells from quiescence (Jones et al., 2005; Brien et al., 2013), and arranges asymmetric division and self-renewal (Troy et al., 2012; Bernet et al., 2014). Importantly, skeletal muscle aging results in a loss of muscle mass and regenerative capacity. These defects arise because satellite cells from aged mice fail to self-renew and increase p38 activity. Since pharmacological manipulation of p38α/β activity ameliorates these age-associated defects, this could be a potential therapeutic opportunity to treat muscle wasting (Bernet et al., 2014).

Osteoblast Differentiation

One of the first studies to indicate that p38, ERK, and JNK were activated during osteoblast differentiation of human MSC was Jaiswal et al. (2000). Gallea and collaborators showed that ERK and p38 activation in C2C12 favored osteoblast determination (Gallea et al., 2001). Soon after, it was shown that the inhibitor SB203580 impairs MC3T3 pre-osteoblast differentiation and the expression of osteoblast markers such as ALP, OC, and collagen (Suzuki et al., 1999, 2002). As will be seen later, many stimuli have been assayed as triggers of p38 activation in osteoblasts, to explore their osteogenic activities.

THE STUDY OF p38 IN BONE: DIFFERENT STRATEGIES, THE SAME GOAL

Knockouts of p38

Different groups have generated germline knockout mice in which the p38 pathway is mutated. It should be stated that p38α is the most highly expressed isoform of p38-MAPK in osteoblasts (Greenblatt et al., 2010; Rodriguez-Carballo et al., 2014). Embryos with a homozygous deletion of *p38a*, as well as embryos with a double knockout of *Mkk3/Mkk6*, die during embryogenesis, while mutations of *Map2k3* (MKK3), *Map2k6* (MKK6), *Mapk11*, *Mapk12,* and *Mapk13* are viable (Cuadrado and Nebreda, 2010). Both $Map2k3^{-/-}$ and $Map2k6^{+/-}$ display severe skeletal defects in long bones, while only $Map2k3^{-/-}$ show abnormalities in craniofacial bone structures (Greenblatt et al., 2010). The embryonic lethality of *Mapk14* loss is associated with neural and cardiac defects (del Barco Barrantes et al., 2011). Initially, a lack of *Mapk11* was not implicated in severe defects (Beardmore et al., 2005), although later it has been associated with some mild bone defects (Greenblatt et al., 2010). The $Mapk13^{-/-}$ mice has reduced sensitivity to skin carcinogenesis (Schindler et al., 2009), as well as higher insulin secretion and tolerance to glucose (Sumara et al., 2009). No bone phenotype was described in the original article in which $Mapk12^{-/-}$ and $Mapk13^{-/-}$ were generated (Sabio et al., 2005), or in successive studies.

In the last 5 years, three studies reported the use of bone conditional knockouts affecting the p38 pathway in osteoblasts (reviewed in Thouverey and Caverzasio, 2015a). Greenblatt and collaborators' integral approach involved analysing the deletions of different effectors of the pathway. The conditional deletion of MAP3K TAK1 in pre-osteoblasts leads to defects in osteoblast differentiation and bone formation that affects the entire skeleton. In fact, the phenotype resembles human cleidocranial dysplasia, which is related to RUNX2 mutations. Indeed, one of the conclusions of the study is that RUNX2 is a target of p38 kinase activity, which is essential to the transactivation function of RUNX2 (Greenblatt et al., 2010). Craniofacial defects are mainly associated with the function of TAK1, MKK3, and p38α, particularly when it affects pre-osteoblasts, as with the use of an Osterix-driven recombinase (Greenblatt et al., 2010; Rodriguez-Carballo et al., 2014). No cranial phenotype was described when an osteocalcin-Cre animal model was used (Thouverey and Caverzasio, 2012). Additionally, with the latter approach, a post-natal bone acquisition defect starts only after 5 weeks of age and disturbs trabecular and cortical bone volumes of mice (Thouverey and Caverzasio, 2012). A doxycycline-induced recombinase can be used to monitor the impact of p38α deletion in osteochondroprogenitors at different time points. When the deletion starts at 3 weeks of age, the anabolic defects are mainly trabecular at 30 weeks of age, and cortical at 60 weeks. When the deletion starts in young adults (circa 8 weeks of age), only cortical defects are encountered. This means that p38α function is particularly important at different stages of osteoblast commitment throughout life (Rodriguez-Carballo et al., 2014). Interestingly, this low bone mass model was also used to hypothesize about the crosstalk between the skeleton and the adipose tissue (Rodriguez-Carballo et al., 2015).

Pharmacological Inhibitors of p38

The use of knockout models represents the most faithful, general analysis of MAPK activity on tissues. Primary osteoblast cultures from these knockout animals, or the manipulation of pathway effectors (via constitutive activation or dominant negative forms), allows molecular analysis in specific models. However, the most frequent approach is the use of selective inhibitors of the different MAPKs. The most common p38 inhibitor is the pyridinyl imidazole molecule SB203580 (Lee et al., 1994; Cuenda et al., 1995). This is considered a highly selective p38α/β inhibitor and has been widely used for more than 20 years (it had over 6000 PubMed entries up to 2015). Nevertheless, it has been shown that it can inhibit other kinases, such as GAK, CK1, RIP2, RAF, and GSK3, and the formation of ZMP from AICAR (Bain et al., 2007). The SB203580 inhibitor has been assayed in some inflammatory skeletal conditions, such as arthritis (Badger et al., 1996). BIRB0796 is even more powerful at blocking p38α/β, but at moderate doses it can also halt the activity of p38γ, p38δ, and JNK2α2 (Bain et al., 2007). SD-822 is considered a more selective p38α inhibitor (Koppelman et al., 2008) and has been used in studies of osteoarthritis to reduce the outcome of the disease (Medicherla et al., 2006). Caverzasio and colleagues showed the potential benefits of this inhibitor for the treatment

of osteoporosis, as it reduces osteoclast activity (Caverzasio et al., 2008).

p38 Osteogenic Targets

The osteogenic potential of p38 kinase is related to its capacity to phosphorylate and increase the activity of some key osteogenic transcription factors. As explained above, both ERK and p38 can phosphorylate RUNX2, boosting its transcriptional potential (Ge et al., 2009, 2012; Greenblatt et al., 2010; Artigas et al., 2014). In addition, our group has described new p38 phosphorylation targets in recent years. For instance, BMP-2 stimulus increases DLX5 transactivation of the *Sp7* (*Osx*) promoter through a feed-forward mechanism. First, *Dlx5* mRNA levels are elevated by BMP treatment (Miyama et al., 1999; Luo et al., 2001; Holleville et al., 2003; Ulsamer et al., 2008; Rodriguez-Carballo et al., 2011). Then, post-translationally, DLX5 is phosphorylated by p38 MAPK at serines Ser34 and Ser217, which facilitates the recruitment of p300 (Ulsamer et al., 2008; **Figure 1**). Another DLX member, *Dlx3*, is also induced by BMP-2. In this case, it occurs through cooperation between SMAD5 and pp38 as they translocate to the nucleus and exert their function on the *Dlx3* promoter (Yang et al., 2014). These p38-activated osteogenic events continue with the phosphorylation of Osterix at serines Ser77 and Ser33. As for DLX5, these modifications represent an increase in the transcriptional ability of OSX by helping to recruit p300 and BRG-1 (Ortuno et al., 2010, 2013). It was also shown that OSX can cooperate with RUNX2 to induce *Col1a1* (Ortuno et al., 2013). In fact, soon after, it was proven that indeed OSX and RUNX2 bind each other and cooperate to increase their transcriptional power. This interaction requires the action of p38 and ERK MAPKs, as mutation of the phosphorylation sites of RUNX2 and OSX prevented their interaction (Artigas et al., 2014).

In addition to these classic osteogenic targets, p38 can phosphorylate ATF members at threonines Thr69 and Thr71, as well as CREB at the serine Ser133. Both ATF and CREB are transcriptional co-factors and, again, these phosphorylations increase their transactivation capacity, including the recruitment of histone acetyltransferase p300 (Livingstone et al., 1995; Waas et al., 2001). More importantly, ATF4 phosphorylation by RSK2 has been shown to be absolutely required for bone development (Yang et al., 2004). Both ERK and p38 turn on RSK2 in different cell types (Siebel et al., 2013; Czaplinska et al., 2014), which then can lead to activation of ATF4 by its phosphorylation at Ser251 (Yang et al., 2004).

Signaling Interaction between MAPKs in Osteoblasts

Very few studies have focused on the degree of interaction between different MAPK pathways. The crosstalk between pathways can be both opposed and cooperative. As a rule of thumb, inhibition of one MAPK pathway activates the other. The dominant negative form of Mek (MekDN) favors the activation of p38, while the constitutively active form has the opposite effects. The same goes for ERK when Mkk6dn and Mkk6sp are used (Ge et al., 2012). Nevertheless, blocking of both pathways by SB203580 and U0126 has deleterious consequences on calvaria mineralization in organotypic cultures (Ge et al., 2012). Similarly, some reports showed that osteogenic effects require cooperation between JNK and p38 signaling (Guicheux et al., 2003).

p38 and Cell Migration

Other actions besides participation in cell proliferation and differentiation are attributed to p38 MAPK, including control of cell migration. This function is vital to understand p38 MAPK's role in physiological processes, such as chemotaxis, fracture healing and wound closure, as well as the consideration of p38 as a target to diminish the invasiveness of cancer cells. The first model in which p38 was associated with cell motility was endothelial cells. SB203580 inhibited VEGF-induced cell migration (Rousseau et al., 1997). Subsequently, p38 was shown to be essential to cell migration in a plethora of different cell types. In other studies, p38 is negatively linked to cell motility. The loss of p38δ has been associated with an increase in cell motility and proliferation of squamous cancer cells (O'Callaghan et al., 2013). In C2C12, interleukin-17 inhibits cell migration by downregulating urokinase through p38α activation (Kocic et al., 2013).

As said, the positive link between p38 and cell movement appears evident. In skeletal cells, p38 activation is positively related to cytoskeletal reorganization and stimulation of cell motility. In 2004, it was reported that PDGF stimulated the proliferation and migration of MC3T3 through different MAPKs. This migration halted when p38 was inhibited, but not when JNK or ERK were blocked (Mehrotra et al., 2004). The flavonoid quercetin impairs cell migration in osteoblastic cells, but the inhibition of ERK and p38 stops this effect (Nam et al., 2008). Our group studied the function of BMPs as chemotactic agents and as inductors of actin cytoskeletal reorganization in mesenchymal cells. These events require the activation of two pathways: PI3K/CDC42/LIMK1 and p38/MAPKAP2/HSP25. Moreover, in fibroblasts depleted for *Mapk14* or *Mapkapk2*, BMP-2 signaling could not favor actin cytoskeletal reorganization and induction of movement (Gamell et al., 2008, 2011). Another interesting aspect of p38 in osteoblastic matrix remodeling is attributed to its role in a model of collagen contraction (Parreno and Hart, 2009).

Fracture assays can be performed *in vivo* and *in vitro*. In a model of fracture healing in rat, TNFα stimulates the proliferation and migration of bone marrow MSCs to the fracture site. This effect was explained by the activation of p38 and inhibition of differentiation in this area, as shown *in vitro* (Zhou et al., 2006). These migratory effects of TNFα were corroborated *in vitro* with MSCs (Fu et al., 2009).

PTH treatment stimulates amphiregulin in osteoblasts. This osteoblast expression enhances the migration and recruitment of close-by mesenchymal progenitors, due to signaling on EGFR through PI3K and p38 (Zhu et al., 2012). SDF-1 (stromal cell-derived factor-1) is described as an inductor of migration of umbilical cord MSCs through activation of AKT, ERK and p38 (Ryu et al., 2010). In fact, shear stress stimulation of JNK and p38 signaling can also provoke SDF-1 secretion in human mesenchymal cells, which activates the CXCR4 receptor and favors migration (Yuan et al., 2012).

SIGNALING TRIGGERS p38 IN OSTEOBLAST FUNCTION

The great variety of stimuli that can activate MAPK and the extended use of specific inhibitors have facilitated the evaluation of the MAPK role in osteogenesis (**Figure 2**). These factors make it easier to design experimental studies to validate different osteogenic treatments. However, conclusions about the specific action of a given treatment that is supposed to affect MAPK signaling should be treated with caution. First, inhibition of a MAPK could favor the activation of another MAPK. Secondly, different inhibitors have associated effects that have not been characterized fully. Third, MAPK pathways are stress-activated cascades, and if there are no proper controls, osmotic stress could account for part of the related effects. And fourth, too often researchers do not ascertain which mechanism activates p38.

Osteogenic Hormones and Growth Factors

p38 Is a Non-Canonical TGFβ/BMP Signaling Pathway

The stimulation of p38 by BMP through TAK1 was first observed in the PC12 cell line (Iwasaki et al., 1999). Then, it was shown how BMP and TGFβ activate p38, ERK and JNK in different mesenchymal cells, including osteoblasts. The involvement of these MAPKs has been observed in different osteogenic *in vitro* models (Gallea et al., 2001; Viñals et al., 2002; Guicheux et al., 2003). A recent report showed that p38 was predicted to account for 20% of the phospho-residues identified after treatment of MSCs with BMP (Halcsik et al., 2013). TGF-β provokes the phosphorylation of p38, ERK and JNK in a very rapid manner in MC3T3 and primary osteoblasts (Arnott et al., 2008). p38 and ERK are needed for *Col3a1* expression in UMR cells under BMP or TGF-β treatment (Selvamurugan et al., 2004). In addition, p38 is necessary for TGF-β-induced synthesis of VEGF in MC3T3 (Tokuda et al., 2003). Although essentially independent, there is a certain level of cross-regulation between SMADs and p38. For example, in human osteoblast cells, inhibition of p38 by SB203580 seems to make SMAD1 phosphorylation and its nuclear accumulation difficult (Noth et al., 2003). In addition, both signaling pathways are needed for BMP5 induction of limb development (Zuzarte-Luís et al., 2004). Nevertheless, p38 and ERK should be inhibited by BMP4 to guarantee self-renewal and stemness (Qi et al., 2004).

BMP signal transduction through p38 has been studied in several cell models. As mentioned previously, the onset of the cascade begins, as for SMADs, at BMP receptor (BMPR) level. Both SMADs and p38 need the kinase activities of BMP receptor complexes. After receptor triggering, MAP3K TAK1 is activated. Mechanistically, it is well-established that TGF-b regulates the TAK1/p38 pathway through recruitment and ubiquitylation of TRAF6 by activated receptor complexes (Sorrentino et al., 2008; Yamashita et al., 2008; **Figure 3**). BMPRs form a complex with NRAGE, TAK1, XIAP, and TAB1, which favor p38 activation (Yamaguchi et al., 1999; Kendall et al., 2005). Interestingly, a member of the insulin/Rln family, named Relaxin, can phosphorylate TAK-1 and cooperate with BMP-2 in promoting osteogenesis by enhancing the BMP activation of the p38 signaling pathway (Moon et al., 2014).

Wnt Signaling

The Wnt pathway interacts with p38 at different levels. Wnt can activate p38 through Disheveled proteins, as the silencing of *Dvl-3* avoids Wnt3a-induced ATF2 phosphorylation (Bikkavilli et al., 2008). At the same time, p38 can reinforce the Wnt/β-catenin pathway. In COS-7 and endodermic F9 cells, p38α inactivates GSK3β by phosphorylating its Ser9, allowing β-catenin to accumulate. Indeed, blocking the p38 pathway affects the triggering of Wnt3a downstream events (Bikkavilli et al., 2008). The activation of both p38 and ERK by Wnt3a, and its effects on pre-osteoblasts' commitment without disturbing proliferation, have also been reported (Caverzasio and Manen, 2007). In dental pulp cells, BMP-2 facilitates *Lef1* expression and β-catenin nuclear accumulation. The authors claimed that this was due in part to p38, as the inhibitor SB20350 prevented these effects (Yang et al., 2015b). Very recently, Ehyai reported that p38-mediated phosphorylation on MEF2 enhances β-catenin nuclear accumulation (Ehyai et al., 2015). The convergence of both pathways has also been demonstrated at the level of the WNT receptor LRP6. In HEK 293, LRP6 phosphorylation by the MAPKs p38, ERK1/2, and JNK is key for the recruitment of the multiprotein degradation complex that includes GSK3β (Cervenka et al., 2011; **Figure 4**). This reciprocal interaction has a negative feedback loop. GSK3β inhibits the activation of p38 and JNK by binding the MAP3K MEKK4. It was proposed that GSK3β could potentially phosphorylate an N-terminal residue of MEKK4, but this has not been proven yet (Abell et al., 2007). Another indirect negative loop has been proposed in several models: the expression of Wnt inhibitor DKK-1 relies on p38 activation, as shown in primary osteoblasts (Kamiya et al., 2010; **Figure 4**).

Estrogen Receptors

Estrogen receptor (ER) effects take place through the classic or genomic pathway, in which ERα and ERβ translocate to the nucleus to bind specific responsive elements on target genes. However, faster effects need signaling through PKA, PI3K and MAPK (Honda et al., 2000; Yamakawa and Arita, 2004). Accordingly, the ER pathway can activate p38, and reciprocally p38 can act on ER. p38 is stimulated by β-estradiol in human dental papilla cells, in which both proliferation and some odonto-osteogenic markers are enhanced, depending on JNK and p38 (Li et al., 2014b). The activation of p38 by this molecule takes place through ERα, but not ERβ (Geraldes et al., 2003; Mori-Abe et al., 2003). In contrast, in human dental pulp cells, the upregulation of OPG by estradiol is reported, and SB203580 blocks this effect. Strikingly, these actions do not seem to take place through the classic ERα and ERβ receptors, as their chemical agonists do not trigger downstream effects on OPG (Manokawinchoke et al., 2016). In MC3T3, saponin diosgenin acts on ER and, as a result, stimulates the production of VEGF via the interplay of p38, PI3K, and HIF (Yen et al., 2005). In MG-63 osteoblastic cells, β-estradiol and mechanical stress can phosphorylate ERK and p38 independently, with different kinetics. By combining both

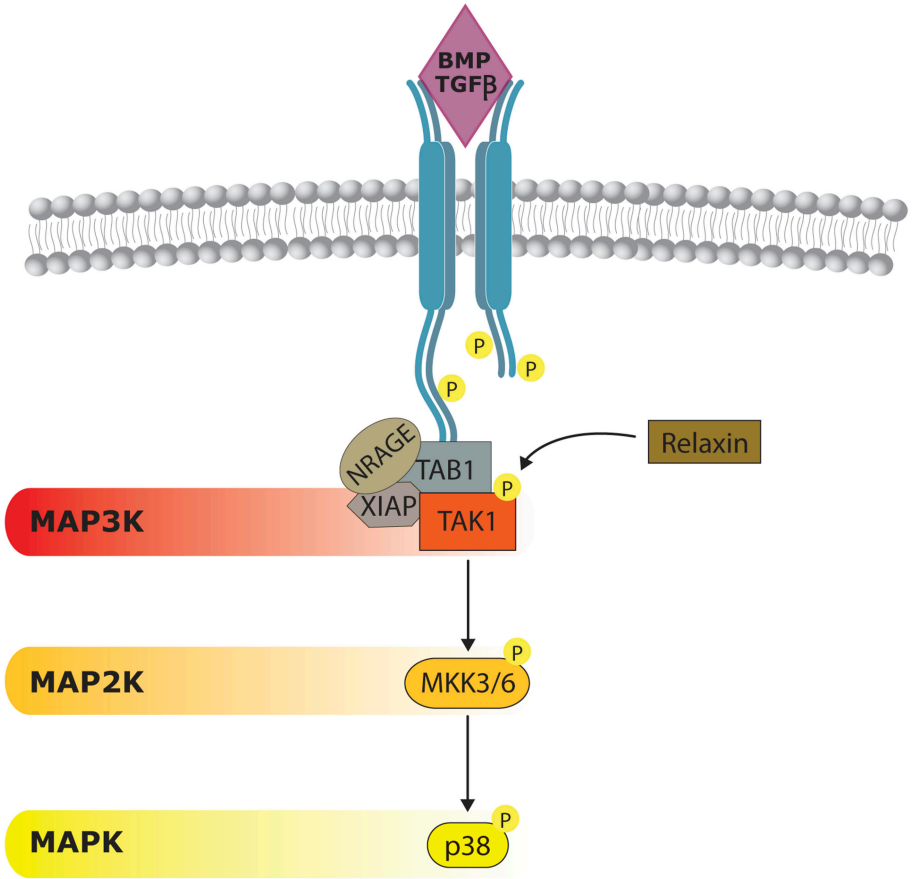

FIGURE 3 | Non-canonical BMP/TGFβ signaling. p38 activity can be triggered by a BMP-dependent mechanism. Once BMP receptors (BMPR) are stimulated they form a complex formed by NRAGE, XIAP, and TAB1, which is responsible of TAK1 activation. Relaxin can act on TAK1, cooperating on p38 activation. Although considered to be independent from SMAD activity, some cross-regulation have also been reported.

treatments, the researchers reported the synergistic expression of *Ptgs2* (encoding COX2) and *Fos* by elevating *Integrin-b1* levels (Yeh et al., 2010).

PTH

The first link of PTH with p38 in MC3T3 implicated the necessary activation of the cAMP-PKA pathway (Rey et al., 2007). The chronic or intermittent treatment of PTH on the beta-arrestin-2 (*Arrb2*) knockout show that the main signaling pathways implicated are p38 and NFκB (Bianchi and Ferrari, 2009). On the *Mkp1* knockout, the interplay between this phosphatase, p38 and PTH was investigated. The continuous treatment of PTH leads to effects on p38 signaling by acting on the phosphatase MKP-1, negatively regulating ERK and p38 (Mahalingam et al., 2013). PTH downregulates the cell cycle and apoptosis regulatory protein 1 (CARP1) via p38 in osteoblastic cells (Sharma et al., 2013). p38 was also needed for COX2 stimulation by PTH in primary osteoblasts (Park et al., 2007).

The most relevant study on intermittent treatment with PTH and the role of p38 has been published recently. As shown with the conditional knockout *Ocn-Cre*; $p38a^{f/f}$, PTH could not exert its anabolic effects *in vivo*, due to a lack of p38 in mature osteoblasts. Although the authors reported that, in these animals, PTH-induced expression of *Rankl* is supressed, still the net effect of p38 deletion is reduced ossification, due to the inability of PTH to induce osteogenic genes. In addition, the authors establish the possibility that PTH activates p38 through cAMP/PKA signaling (Thouverey and Caverzasio, 2015b). Mechanistically, it has been proposed that PTH increases both SMAD and p38 BMP signaling in MSCs (Yu et al., 2012).

FGF, IGF-1, and VEGF

The first hints of p38 activation by FGF2 in MC3T3 were reported in 1997, on behalf of MMP1 expression, even before any commercial pp38 antibody was available (Newberry et al., 1997). Subsequently, it was proven that FGF2 induces p38 phosphorylation in MC3T3 (Kozawa et al., 1999), and in immortalized human neonatal calvarial cells (Debiais et al., 2001). In another report, it was suggested that strontium ranelate plays a similar role in combination with FGF2. Moreover, MAPK induction is blocked by an FGF inhibitor

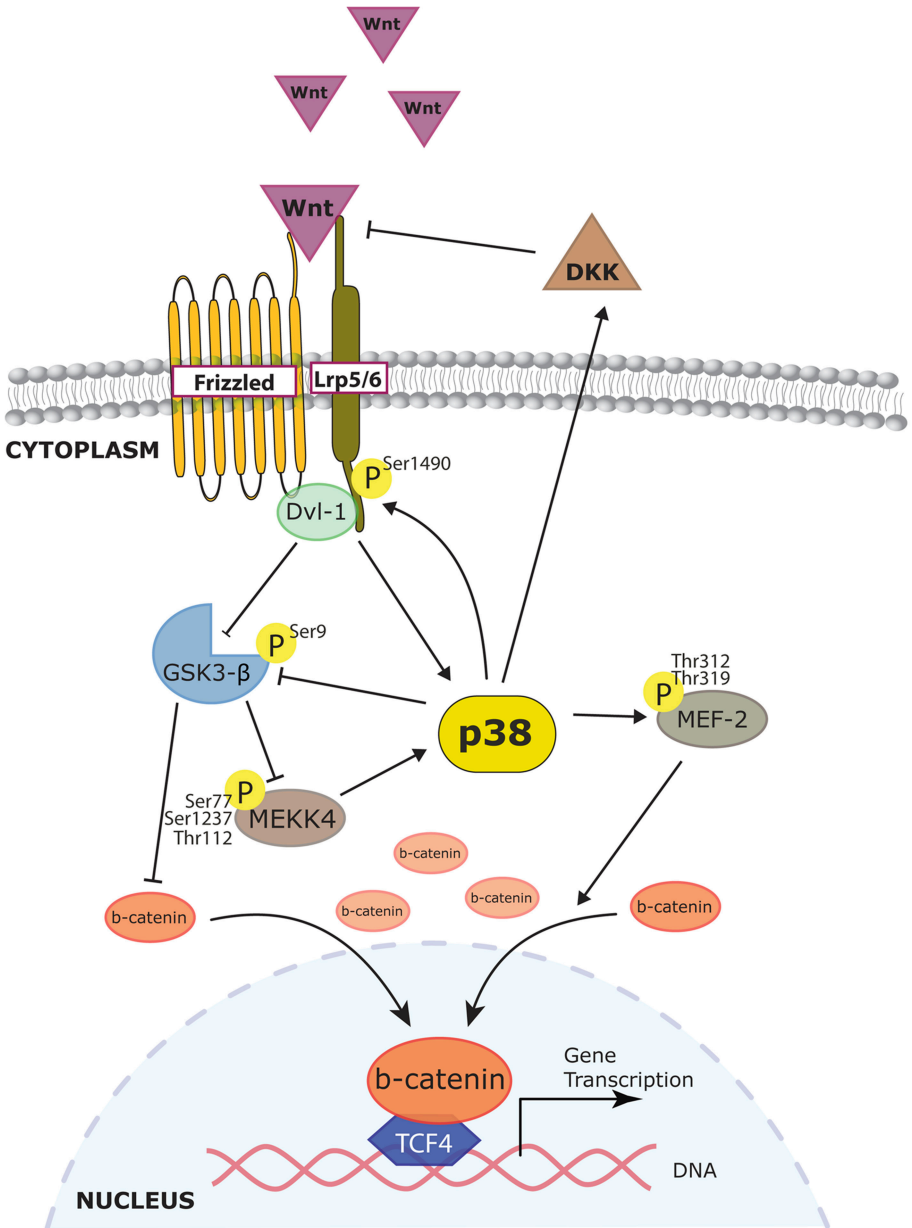

FIGURE 4 | Role of p38 MAPK in Wnt signaling. Wnt pathway activation leads to β-catenin accumulation, which enables β-catenin to translocate and initiate the transcription of target genes. p38 participates in the regulation of β-catenin accumulation by targeting different steps of the Wnt signaling. Wnt can activate p38 through Disheveled (Dvl) proteins and at the same time also inactivate GSK3β allowing β-catenin to accumulate. Activated p38 can reinforce GSK3β inhibition therefore increasing cytoplasmic β-catenin. Moreover, effects of p38 phosphorylation on MEF2 also enhance β-catenin nuclear accumulation. p38 also exerts negative effects on Wnt signaling since DKK-1 expression (a Wnt inhibitor) relies on p38 activation.

(Caverzasio and Thouverey, 2011). It should be noted that strontium ranelate alone stimulates p38 and ERK activation in C3H10T2, inducing proliferation (Caverzasio, 2008). In human MSC, IGF-1 activates p38, JNK, and ERK. These kinases are essential to the upregulation of Osterix upon IGF-1 treatment, as this is blocked by chemical inhibitors (Celil and Campbell, 2005). Although VEGF cannot activate p38 alone in human adipose mesenchymal stem cells, it can cooperate with BMP6 signaling to direct differentiation toward osteoblastic commitment, and to avoid adipogenesis. This positive collaboration is mediated via simultaneous activation of p38 and AKT inhibition (Li et al., 2015a).

AMPK/Adiponectin

Adiponectin can activate p38 and JNK by binding to its own receptor, as AdipoR1 siRNAs block p38 phosphorylation in primary trabecular osteoblasts. Interestingly, the p38 and

JNK cascades are independently and respectively associated with osteoblast activity and proliferation (Luo et al., 2005). Indeed, in C3H10/T2 cells, adiponectin induces osteogenic genes such as *Spp1, Bglap,* and *Ptgs2* via p38 through AdipoR1, but not AdipoR2. Also, adiponectin favors RUNX2 and CREB recruitment to *Bglap* and *Spp1* promoters. (Lee et al., 2009). Adiponectin is also seen as a p38 stimulus to favor *RANKL* transcription and at the same time inhibit *OPG*, thus leading to osteoclast activation (Luo et al., 2006). Intriguingly, regarding osteoblastogenesis, the relationship between adiponectin and p38 stimulation may generate different outputs depending on the cell type. In calcifying vascular smooth muscle cells, adiponectin p38-activation is related to impairment of osteoblastic differentiation (Luo et al., 2009).

Inflammatory Cytokines

Interleukins

Interleukin-1β (IL-1β) activates p38 in MG-63 and MC3T3 cells, which helps to induce IL-6 and stabilize its mRNA (Kumar et al., 2001; Patil et al., 2004). In addition, IL-1β activates both ERK and p38, but not JNK, in MG-63 cells (Lambert et al., 2007). It also accelerates osteoblastic differentiation, presumably through p38 activation, as the inhibitor SB2035820 supresses primary rat osteoblasts' mineralization (Lin et al., 2011). Conversely, one report analyzed the negative impact of IL-1β on BMP-2 osteogenesis. These effects are, at least in part, due to p38 activation by this interleukin (Huang et al., 2014). Moreover, p38 and ERK are linked to IL-1β-induced *Opg* expression, therefore presumably preventing resorption (Lambert et al., 2007).

Prostaglandins

In calvarial osteoblasts, PGE_2 activates p38 via EP2 and cAMP, while ERK is triggered via EP4/PKC, and JNK can be activated by both EP2 and EP4. Inactivation of each pathway results in decreased osteogenic markers (Minamizaki et al., 2009). In MC3T3, PGE_1 signaling through p38 and ERK increases VEGF synthesis and ALP activity. Moreover, the same authors suggested that in this cascade, p38 would be downstream of cAMP and PKA (Tokuda et al., 2001; Kakita et al., 2004). In the cell line MG-63, 15d-PGJ_2 (15-deoxy-delta-12,14-prostaglandin J2) independently activates p38 in a very rapid manner. The use of inhibitors PD169316 and PD098580 blocks 15d-PGJ_2-induced *PTGS2* transcription (Kitz et al., 2011). The same prostaglandin prevents cell death of this osteosarcoma line. The mechanism of action includes signaling via the p38/AKT/Egr-1 axis (Koyani et al., 2016).

TNFα

TNFα is recognized as an inductor of p38 and is implicated in IL6 synthesis in MSCs (Zhou et al., 2006) and in MC3T3 (Dai et al., 2006). Huang et al showed that TNFα inhibited BMP2 osteogenesis via p38. Chemical inhibition of p38 restores the expression levels of RUNX2 and other osteogenic markers triggered by BMP-2, despite the presence of TNFα (Huang et al., 2014). TNFα-mediated p38 activation has also been linked to *Rankl* early expression, but not during the late phase (Dai et al., 2006).

ECM and Adhesion

Rho-Kinase

Endothelin-1 and prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$) can activate IL-6 by acting both on Rho-kinase and p38 on MC3T3. The effects disappear after treatment with the Rho inhibitors fasudil or Y27632, or the p38 inhibitors SB2035820 and BIRB0796 (Minamitani et al., 2008). Thrombin can also directly activate ERK and JNK, and indirectly activate p38 through Rho-kinase. The actions on ERK and p38 lead to an increase of IL-6 that can be stopped by fasudil and Y27632 (Kato et al., 2011). Fluid flow forces can also activate PI3K, ERK, and p38 via RhoA-ROCK in osteoblasts (Hamamura et al., 2012). Dr. Glimcher's group found that FGD1 signals through CDC42 to the MAP3K MLK3, which in turn activates p38 and ERK, provoking RUNX2 phosphorylation. Mutations in *FGD1* are associated with faciogenital dysplasia (FGDY), which is characterized by skeletal defects that are comparable to the phenotype of $Mlk3^{-/-}$ mice (Zou et al., 2011).

Extracellular Matrix Proteins

The extracellular matrix holds a set of molecules that can directly activate the MAPK or are needed for MAPK function. For instance, calcium, one of the main constituents of bone ECM, elevates MC3T3 proliferation via ERK and p38 activation, as its chemical blockage interrupts the proliferative effect (Yamaguchi et al., 2000). CCNs are ECM proteins that can act as growth factors and are critical for osteoblast function. CCN-1 to -6 have a positive impact on *Runx2*, *Sp7*, *Col1a1*, *Alp*, and *Bglap* expression, partly due to activation of ERK and p38 (Kawaki et al., 2011). The expression of BMP4 induced by CCN3 depends on p38 and JNK function, as demonstrated with a chemical antagonist and dominant negative MAPK forms (Tan et al., 2012). Dentin matrix protein1 (DMP1) has been proven to serve as an inductor of differentiation for odontoblasts and osteoblasts. It was demonstrated that DMP-1 increases intracellular calcium levels in several osteoblastic cells, and stimulates phosphorylation and nuclear translocation of p38, which leads to activation of downstream targets such as MK2 and HSP27, and nuclear translocation of RUNX2 (Eapen et al., 2010). DDR2 (Discoidin Domain Receptor-2) is a tyrosine-kinase receptor with collagen affinity. *Ddr2* is transcriptionally activated by ATF4 and C/EBPβ, where the former is a known target of p38. At the same time, DDR2 needs p38 activity to trigger Runx2 and osteocalcin expression (Lin et al., 2010). In addition, Plexins (Plxn) are semaphorin receptors that were originally involved in cell adhesion and migration. PlxnA2 is expressed in bone, and its siRNA inhibition reduces phosphorylated levels of SMAD1, AKT, and p38, as well as Runx2 expression and mineralization. Moreover, it seems that PlxnA2 activity may be associated with binding to BMPRs (Oh et al., 2012).

Stresses and Physical Inputs

Chemical Stressors

MAPKs have been traditionally described as stress kinases that can be triggered by multiple stressors. The use of certain chemical compounds was recurrent in many early *in vitro* experiments. For example, arsenate induces p38, and this leads to an increment

in peroxiredoxin I protein levels in MC3T3, but does not affect its mRNA levels (Li et al., 2002). Another chemical stressor, cadmium, requires p38 function to induce PGE_2 in primary osteoblasts (Miyahara et al., 2004). In addition, temperature stress can also trigger p38 in MSC, which has a positive effect on their osteogenic commitment. Indeed, chemical blockage of this MAPK prevents the stimulation of proteins such as RUNX2, OPN, BSP, and collagen I (Nie et al., 2015).

Hypoxia

In MSCs, hypoxia clearly induces osteoblastic differentiation through ERK and p38 activation, particularly when they are seeded in bone-derived scaffolds (Zhou et al., 2013). Similar effects are seen in periodontal ligament cells when co-cultured with endothelial cells (Wu et al., 2013). Hypoxia has also been studied in osteoarthritic osteoblasts, where it upregulates leptin, which also involves p38 signaling (Bouvard et al., 2014).

Mechanical Inputs

Mechanical loading constantly shapes the skeleton, and some physical stimuli have been shown to activate p38 in several *in vitro* and *in vivo* experimental settings. Static stretching is an *in vitro* model that tries to reproduce the effects of bone distraction on osteoblasts. In this model, p38 and ERK are phosphorylated, which stimulates VEGF release in human MSCs. When these MAPKs are inhibited, VEGF is no longer activated (Kim et al., 2010). Using the same model, other authors showed effects of p38 on BMP-2 and BMP4 production, which in turn activate *Col1a1*, *Runx2*, *Spp1, Alp,* and *Bglap* in MC3T3 (Wang et al., 2012; Zhang et al., 2013). Interestingly, siRNAs against TAK1 dramatically soften this stretching activation in MC3T3, as well as IL-6 expression, which is also decreased by blocking p38 (Fukuno et al., 2011). ROS17/2.8 cells subjected to stretching or to microgravity show that inhibition of p38 by SB203580 extended the time expression of *Egr1* (Granet et al., 2001). Physiologically, stretching forces also play a role in the periodontal space. Indeed, several reports show that cyclic tension activates MAPK signaling and increases osteogenesis in human periodontal ligament cell (Li et al., 2014a; Suzuki et al., 2014). In this model, p38 and ERK activation, but not JNK, are related to *PTGS2* and *BMP2* transcription (Suzuki et al., 2014).

Furthermore, fluid flow shear stress can also activate p38 and ERK in bone marrow stromal cells (Kreke et al., 2008) and in MG63 (Lee et al., 2008a). The activation of these MAPKs is associated with the interplay between integrins $a_v\beta_3$ and β1 with the adaptor protein Shc. This leads to *Fos*, *Ptgs2,* and *Spp1* expression (Lee et al., 2008a). Integrins can also activate p38 by contacting other molecules. In human osteosarcoma cells, angiopoietin-like protein 2 (ANGPTL2) appears to interact with integrin $\alpha_5\beta_1$ to promote p38 activation and *MMP9* expression, which favors invasiveness (Odagiri et al., 2014). Fluid flow cell stress in MC3T3 activates ERK and p38 via Rho kinase, leading to expression of *Ptgs2*, *Spp1,* and *Per2* (Hamamura et al., 2012).

Ultrasound, Electric and Magnetic Fields and Lasers

Other osteogenic stimuli that are not related to loading mechanical forces but electromagnetic waves also have osteogenic potential via p38 (Xiao et al., 2009). Ultrasounds can stimulate MAPKs in primary osteoblasts cultures and favor the expression of metalloproteinase 13 (MMP13), which increases the binding of Fos and Jun to AP-1 elements at promoter level. These effects are supressed by treatment with p38 inhibitors SB2035820 or JNK inhibitor SP600125, but not when ERK is inhibited (Chiu et al., 2008). Previously, it was shown that p38 inhibitor SB203580 prevents ultrasound-induced COX2 and osteocalcin expression in murine cells ST2 (Naruse et al., 2003). Electromagnetic stimulation increases p38, JNK, and ERK phosphorylation levels in MC3T3. Inhibition of these MAPK is related to a reduction in electromagnetically induced osteopontin, PDGF and VEGF levels (Yumoto et al., 2015). Similarly, low frequency electromagnetic fields favor collagen accumulation, probably via p38, as the use of SB203580 diminishes this effect (Soda et al., 2008). In addition, high frequency fields raise p38 mRNA levels in C3H10T2 (Teven et al., 2012). Biphasic electric current has also been applied in an *in vitro* osteogenic model with human stromal cells. This stimulus activates both ERK and p38 MAPK pathways, leading to increased proliferation and induction of VEGF and HIF-α, which can be supressed by chemical inhibition (Kim et al., 2009).

Pharmacological Compounds

Bisphosphonates were found to induce ERK and p38 pathways, halt proliferation, and favor apoptosis in sarcoma cell lines (Kubo et al., 2008). Statins, therapeutic drugs to fight hypercholesterolemia, can have a positive effect on bone formation. Simvastatin induces HSP27 specifically via p38 signaling in MC3T3, and SB203580 blocks this effect (Wang et al., 2003). Generally, statins and bisphosphonates can have a dual action on bone cells: they can inhibit the ERK pathway, and accentuate p38 signaling. ERK and p38 seem to be reciprocally inhibited. The activation of p38 favors OPG overexpression, while the inhibition of ERK downregulates CD-1, RANKL, and MCSF. Thus, the net result of bisphosphonate and statin treatment would be inhibition of osteoblast-induced osteoclast maturation (Tsubaki et al., 2012). Thiazolidinediones (TZD), a drug for diabetes type 2 treatment, can also stimulate ERK and p38 via GPR40 and Ras activation. Apparently, this pathway, which is independent of PPARγ activity, leads to TZD-induced osteocyte apoptosis (Mieczkowska et al., 2012).

In traditional Asian medicine, a myriad of herb and plant compounds have been used historically to treat bone diseases such as osteoporosis or fractures. Many of these compounds contain remarkable doses of pro-estrogenic chemicals, antioxidants or anti-inflammatory agents, and have been linked to p38 function. Phytoestrogens (isoflavonoids) group non-steroidal compounds of plant origin that resemble human estrogens. Some of them have been studied as bone anabolic therapies, as they increment the levels of several osteogenic markers. They include genistein (which involve a p38-RUNX2), 8-prenylkaempferol, sesamin and caffeic acid 3,4-dihydroxy-phenethyl ester (CADPE) (Chiou et al., 2011; Wanachewin et al., 2012; Liao et al., 2014; Schilling et al., 2014). Another important group of natural substances is the flavonoid family. Flavonoids are present in fruit, vegetables and

seeds, and have long been known for their beneficial effects. Naringin, ugonin K, neobavaisoflavone, puerarin, quercetin, icariin and apigenin have shown anabolic abilities in some osteogenic models both *in vivo* and *in vitro* (Ming et al., 2013; Welch and Hardcastle, 2014; Li et al., 2015b; Zhang et al., 2015; An et al., 2016). The saponins family also has proven *in vitro* osteogenic actions that affect both osteoblast proliferation and differentiation (Jeong et al., 2010; Niu et al., 2011; Zhou et al., 2015) and osteoclast activation (Zhou et al., 2015). Other substances, such as coumarinic compounds, have only been associated with osteoblast commitment (Tang et al., 2008). Lactone derivatives affect both p38 and ERK pathways and are linked to osteoblast function (Lee and Choi, 2011) and osteoclast impairment (Zhai et al., 2014). Isoquinolines, quinones and lignans can increase ALP activity and *Bmp2* expression, as well as upstream effectors like RUNX2, in pre-osteoblasts and mesenchymal cells and *in vivo* models (Lee et al., 2008b; Kim et al., 2014; Moon et al., 2015; Yang et al., 2015a). In addition, phenolic compounds are associated with positive effects on osteogenesis. A diet enriched in blueberries seems to ameliorate bone marker levels and diminish bone resorption in rats. These changes are related to p38 activation and high levels of beta-catenin (Chen et al., 2010). Xanthonoids in gambogic acid are linked to decreased osteoclastogenesis and bone loss (Ma et al., 2015).

CONCLUDING REMARKS

p38 MAPK plays a pivotal role in different steps of osteoblast differentiation. *In vivo*, p38 deletion hampers osteoblast terminal differentiation and the appearance of osteocytes, which directly affects bone composition and maintenance. Taking into account the growing number of triggers of p38 activity and the key action of MAPK in bone development and homeostasis, we should consider p38 as a central hub for signaling convergence toward osteoblastogenesis. As seen in multiple *in vitro* cell-based models, p38 integrates inputs from different stimuli. The latter range from mechanical loading to signaling molecules like cytokines, of which BMP/TGFβ and Wnt pathways have been the most widely studied. The *in vivo* evidence reinforces this hypothesis. As seen in specific knockouts, deleting some key effectors of the p38-MAPK pathway affects osteoblast development at different moments during differentiation. Therefore, disturbing p38 affects a necessary integrator of various signaling inputs.

Matrix deposition and shaping is a local, fine-tuned event affected by multiple factors. We know that MAPKs such as p38, ERK, and JNK are rapidly activated, and are thus convenient for an anabolic cell like the osteoblast to respond to different local spurs. Bearing all this in mind, the p38 cascade could be a good target for anabolic bone therapies. However, the large number of activators and the different levels of self- and cross-regulation make it difficult to specifically target it therapeutically. On another front, assessing p38 activity could inform clinicians about the ability of bone to respond to anabolic therapies.

AUTHOR CONTRIBUTIONS

ER, BG, and FV conceived, analyzed and discussed the manuscript. ER and FV wrote the manuscript. ER and BG draw the figures.

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Mitogen-Activated Protein Kinases and Mitogen Kinase Phosphatase 1: A Critical Interplay in Macrophage Biology

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Macrophages are necessary in multiple processes during the immune response or inflammation. This review emphasizes the critical role of the mitogen-activated protein kinases (MAPKs) and mitogen kinase phosphatase-1 (MKP-1) in the functional activities of macrophages. While the phosphorylation of MAPKs is required for macrophage activation or proliferation, MKP-1 dephosphorylates these kinases, thus playing a balancing role in the control of macrophage behavior. MKP-1 is a nuclear-localized dual-specificity phosphatase whose expression is regulated at multiple levels, including at the transcriptional and post-transcriptional level. The regulatory role of MKP-1 in the interplay between MAPK phosphorylation/dephosphorylation makes this molecule a critical regulator of macrophage biology and inflammation.

Keywords: kinases, phosphatases, inflammation, macrophages, immune response, signal transduction

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MACROPHAGES A KEY ELEMENT OF THE IMMUNE SYSTEM

Monocytes are the precursors of macrophages and they originate from undifferentiated stem cells in the bone marrow. Differentiation is induced in response to growth factors such as M-CSF, IL-3, and GM-CSF. M-CSF being the only specific one because the M-CSF receptor (CSF1R, M-CSF-R, or CD115 is encoded by the *c-fms* proto-oncogene) is present only in the monocyte/macrophage lineage. Monocytes reach all the tissues in the body via the blood and then they become macrophages (Epelman et al., 2014). In response to various factors, including cytokines, cell-cell contacts, and extracellular matrix interactions, macrophages differentiate to more specialized cells, depending on the tissue. For example, in response to RANKL, these cells differentiate to osteoclasts in the bone (Edwards and Mundy, 2011), to Kupffer cells in the liver, to Langerhans cells in the skin, to bone marrow macrophages in the bone, and to crypt macrophages in the intestine. The activities of differentiated macrophages vary in function of the tissue. The interaction of macrophages with factors such as GM-CSF, IL-4, and TGF-β induces differentiation to dendritic cells. These cells are key elements in antigen presentation in the lymph node a process that induces T cell activation and the acquired immune response. The immune system responds to stress induced by chemical, physical, and infectious agents by producing inflammation. In this process, macrophages play a key role, initially through their capacity to remove bacteria or parasites. This activity is achieved through many components that can indirectly damage the surrounding tissues. After this pro-inflammatory activity (classical activation or M1), macrophages remove all the damaged tissues and start the reconstruction process. In this anti-inflammatory phase (alternative activation or M2), macrophages prompt the synthesis of the extracellular matrix and cell growth (Dey et al., 2014).

Under physiological conditions, monocytes show the Ly6C^{-} and CD43^{++} phenotype in mice and CD14^{-} and CD16^{++} phenotype in humans (Ziegler-Heitbrock et al., 2010). Localized around blood vessels, these cells serve to monitor healthy tissues. When homeostasis is altered, they enter the affected tissues to initiate an early immune response. During the first 24 h of this response, the inflammatory loci are invaded first by neutrophils and then by monocytes carrying the Ly6C^{++} CD43^{-} phenotype in mice and the CD14^{++} CD16^{-} phenotype in humans. These monocytes trigger the pro- and anti-inflammatory activities mentioned earlier. The same macrophages that are polarized to pro-inflammatory action become anti-inflammatory a few days later (Arnold et al., 2007; Takeuch and Akira, 2011). Interestingly, pro-inflammatory activity by agents such IFN-γ or LPS block macrophage proliferation (Xaus et al., 1999). However, during anti-inflammatory activation, tissue macrophages proliferate locally (Arnold et al., 2007) in a process mediated by IL-4 (Jenkins et al., 2011).

MAPKs AND MACROPHAGE BIOLOGY

MAPKs have been conserved over evolution; however, the subcellular compartmentalization and the kinetics of MAPK activation are cell-type specific, and these kinases orchestrate a range of cellular responses. One of the critical issues in macrophage biology, as in most cell types, is their capacity to respond to stimuli and to proliferate. Although M-CSF, GM-CSF, and IL-3 induce macrophage proliferation, M-CSF is the only specific grow factor for these cells. M-CSF interacts with its receptor-specific receptor CSF1R on the cell surface and induces the activation of signal transduction. The interaction is followed by receptor dimerization, which leads to the autophosphorylation of tyrosine residues in the intracellular domain and the recruitment of signaling molecules (Yu et al., 2012). Macrophage proliferation required the stimulation of the MAPK signaling pathway (Pixley and Stanley, 2004). These serine/threonine kinases are induced by external signals, and they play a critical role in regulating the growth, activation, differentiation, and apoptosis of various types of cells. MAPK activation calls for phosphorylation on the threonine and tyrosine residues situated in the activation loop. Upon phosphorylation, these kinases regulate transcription factors such as Ets-1, Elk/TCF, and AP-1, all of which are involved in immediate, early and late gene expression (Yoon and Seger, 2006), and also in protein expression by regulating the stability, transport or translocation of mRNA species that contain AU-rich elements (Wang and Liu, 2007). In response to growth factors, ERK-1/2, c-Jun NH2-terminal kinase 1 (JNK-1), and p38 are activated in macrophages. However, for the proliferation of these cells, only ERK-1/2 phosphorylation is required (Jaworowski et al., 1999; Valledor et al., 2000a; Sanchez-Tillo et al., 2007). The activation/phosphorylation of Ras is necessary to phosphorylate ERK-1/2 and the downstream targets such as the ribosomal S6 kinases and mitogen- and stress-activated kinases 1 and 2 (Murphy and Blenis, 2006). Activated ERK-1/2 translocate to the nucleus and, by means of phosphorylation, activate a number of transcription factors that regulate genes involved in proliferation. Remarkably, M-CSF triggers other pathways independent of MAPKs to induce macrophage survival through the phosphorylation of phosphoinositide 3-kinase which prompts the translocation and activation of Akt (Comalada et al., 2004).

Lipopolysaccharide (LPS) is a powerful inflammatory signal present in the membrane of Gram-negative bacteria. It regulates the expression of many genes, including TNF-α, which causes septic shock when present in large amounts. ERK-1/2 becomes phosphorylated through the interaction of LPS with LPS binding protein (a serum protein), CD14, and TLR4 (membrane proteins in macrophages; Valledor et al., 2000b). The inhibition of this phosphorylation blocks the production of TNF-α and IL-1β. This observation serves to demonstrate the role of these two molecules in macrophage activation (Valledor et al., 2000a).

IFN-γ, a major endogenous macrophage activator, not only requires Stat1 for signaling but also MAPKs. At early times (30 min), p38 is activated strongly, but ERK-1/2 and JNK-1 become phosphorylated between 2 and 5 h. Although p38 and ERK-1/2 regulate the expression of genes of the innate response, such as chemokines, TNF-α, and inducible NO synthase, the genes involved in antigen presentation are regulated by JNK-1 (Valledor et al., 2008b). These results emphasize the diversity of MAPK kinetics and activity in macrophages.

The kinetics of MAPK phosphorylation with respect to the proliferation or functional activation of macrophages may be associated with various factors, such as cell-surface receptor concentration and the different pathways used after ligand-receptor engagement (Murphy and Blenis, 2006). The induction of ERK-1/2 phosphorylation by M-CSF or LPS involves the same molecules, namely Ras, Raf, and MEK 1/2 (Casals-Casas et al., 2009). However, the early steps differ. While M-CSF receptor encloses a tyrosine kinase domain in the intra-cytoplasmic region, thus inducing rapid phosphorylation, LPS interacts with LPS-binding protein CD14, and finally TLR4 and requires more time to initiate the phosphorylation (Kolch, 2005).

THE MKP-1 PHOSPHATASE AND ITS REGULATION

MKP-1 phosphatase, also called dual specificity phosphatase 1 (DUSP1), is expressed ubiquitously. However, it is regulated in a cell-context manner and is able to dephosphorylate tyrosine or serine/threonine residues. MKPs dephosphorylate and consequently inactivate ERK-1/2 (Pouyssegur and Lenormand, 2003), p38, MAPKs (Kaiser et al., 2004), and JNKs (Sanchez-Perez et al., 1998). At least 11 MKP family members of these dual phosphatases have been found to differ in cellular specificity, subcellular localization, and substrate specificity (Farooq and Zhou, 2004).

MKP-1 is composed of two domains, one is bound the MAPK in the NH2-terminus and the other is the dual-specificity phosphatase domain located in the COOH terminus (Camps et al., 2000). This phosphatase localizes exclusively to the nucleus through a NH2-terminus LXXLL motif (Wu

et al., 2005). The catalytic activity is regulated through interaction with MAPKs mediated by a kinase interaction motif (Doddareddy et al., 2012).

In most cases, the induction of phosphatases is a negative response mechanism of the kinases that triggers the expression or activation of the phosphatases. In macrophages, MPK-1 induction yields conflicting results. In primary cultures of bone marrow-derived macrophages, LPS-induced MKP-1 expression is partially blocked by co-administration of inhibitors of ERK-1/2 and p38 (Ananieva et al., 2008) or when macrophage from a p38 KO mouse are used (Kim et al., 2008). In other studies using the same cell type, the inhibition of the MEK pathway required for M-CSF- or LPS-induced ERK-1/2 phosphorylation was not found to affect MKP-1 expression, thereby showing that this expression is independent of kinase activation (Valledor et al., 1999, 2000b).

The early steps in signal transduction to induce MKP-1 involves the phosphorylation of PKCε (Valledor et al., 1999, 2000b). Interestingly, the expression of MKP-1 induced by either M-CSF or LPS also requires Raf-1 activation, as does the phosphorylation of ERK-1/2 (Sanchez-Tillo et al., 2006). Another mediator of MKP-1 stimulation is JNK-1, and inactivation of this kinase results in prolonged ERK phosphorylation (Sanchez-Tillo et al., 2007). Finally, inhibition of mTOR induces MKP-1 expression, thereby suggesting that the repression of immunity is mediated through MKP-1 (Rastogi et al., 2013).

MKP-1 is not expressed under basal conditions; however, in response to various stimuli MKP-1 is an immediate early gene that requires transcriptional activation, although its regulation is also mediated at several other levels. In the MKP-1 promoter, a region containing a CREB/AP-1 box is essential for the induction of transcription through a variety of different stimuli (Arthur et al., 2004; Ananieva et al., 2008; Cho et al., 2008; Kim et al., 2008; Casals-Casas et al., 2009). Upon activation by M-CSF or LPS, this box is occupied by phosphorylated CREB and c-Jun. It has been reported that the phosphorylation of CREB is mediated by p38 (Kamata et al., 2005) and by ERK-1/2. However, this phosphorylation is not direct but mediated through MSK1/2 activated by either ERK-1/2 or p38 (Ananieva et al., 2008; Kim et al., 2008). However, it has been reported that the inhibition of ERK does not block MKP-1 induction by M-CSF and LPS (Valledor et al., 1999, 2000b). Other studies have described that CREB phosphorylation is mediated by M-CSF-induced PI-3K (Kanagasundaram et al., 1999) or by LPS-activated PKA (Zhong et al., 1997). Vitamin D induces MKP-1, thus inhibiting pro-inflammatory cytokine production, and the binding of the vitamin D receptor at the vitamin D response element in the MKP-1 promoter is increased (Zhang et al., 2012). In the context of the proliferation vs. activation issue, IFN-γ, the major endogenous activator of macrophages, inhibits M-CSF-induced MKP-1 expression (Valledor et al., 2008a). The inhibition is mediated through STAT1, but no such repressive activity has been reported for this transcription factor. Transcription, in turn, calls for epigenetic modifications or chromatin remodeling on the MKP-1 promoter region. Through phosphorylation and acetylation of histone H3, chromatin is modified, thereby increasing the binding of RNA polymerase II and inducing MKP-1 transcription (Li et al., 2001; **Figure 1**).

MKP-1 mRNA in macrophages has a very short half-life (Valledor et al., 1999). In other cellular models, the short mRNA half-life has been related to AU-rich 3′ untranslated regions and the RNA binding proteins HuR and NF90 (Kuwano et al., 2008). It seems that NF90 represses the translation of mRNAs bearing the AU-rich signature sequence without affecting their half-lives (Kuwano et al., 2010). Tristetraprolin binds to these AU-rich sequences of MKP-1 and thus forms part of a negative feedback loop to limit the mRNA half-life (Emmons et al., 2008). In fact, tristetraprolin expression is suppressed by MKP-1 through inhibition of p38 (Huotari et al., 2012).

The activity of MKP-1 can be modified by stabilization of his on protein, which is determined by its ERK-mediated phosphorylation through a degradation pathway independent of polyubiquitination (Crowell et al., 2014). If Ser359 and Ser364 become phosphorylated, the protein is stabilized (Brondello et al., 1999); however, if phosphorylation occurs in Ser298 and Ser323, then proteosomal degradation is facilitated (Lin et al., 2003). We should mention that glucocorticoids not only induce the transcription of MKP-1 but also attenuate their proteasomal degradation (Kassel et al., 2001; **Figure 1**).

Metabolic stress in monocytes and macrophages induces Nox4, an inducible NADPH oxidase that promotes MKP-1-S-glutathionylation, resulting in MKP1 inactivation and subsequent proteasomal degradation (Kim et al., 2012). This observation shows that a redox-regulated mechanism links oxidative stress directly to metabolic disorders and macrophage hyperactivity.

The repressive cytokines IL-10 and TGF-β block most immune responses and increase LPS-dependent MKP-1 induction, thereby enhancing the degree and duration of the phosphatase and consequently dephosphorylating MAPKs with the consequent inhibition of inflammatory cytokine production (Jono et al., 2003; Hammer et al., 2005). Micro-RNA-101 (miR-101) and miR-210 transfections reduce LPS-dependent induction of MKP-1 and also prolong the phosphorylation of p38 and JNK (Zhu et al., 2010; Jin et al., 2014).

Glucocorticoids are one of the major families of inhibitors of the immune response used in clinical practice. Remarkably, these drugs increase the expression and decrease the degradation of MKP-1. Consequently, MKP-1 dephosphorylates MAPKs, thus decreasing the activity of these kinases, as well as reducing macrophage activation (Kassel et al., 2001; Shipp et al., 2010). However, the observation that MKP-1 KO mice with enhanced cytokine expression remain sensitive to glucocorticoids suggests that the drug targets molecules other than MKP-1 (Maier et al., 2007; Wang et al., 2008).

In response to LPS, MKP-1 is acetylated by p300/CBP on lysine 57 within its substrate-binding domain, thus increasing its interaction and dephosphorylation of p38 (Cao et al., 2008). This observation reveals another level of regulation of the functional activity mediated by histone deacetylase isoforms (HDAC1, HDAC2, and HDAC3; Jeong et al., 2014).

Therefore, the mechanisms regulating MKP-1 expression are critical in determining the duration of MAPK phosphorylation

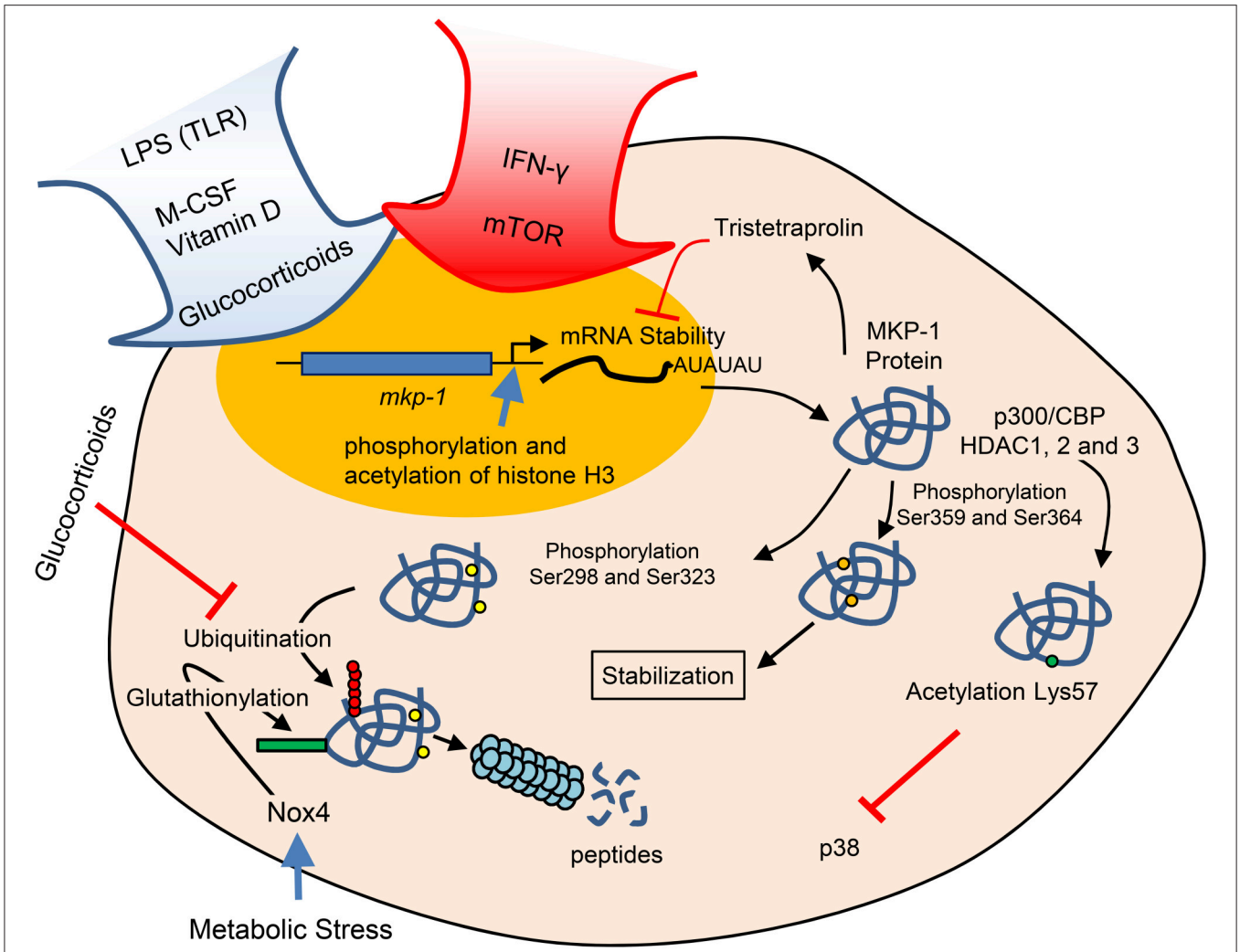

FIGURE 1 | The regulation of MKP-1 arises by multiple mechanisms, including transcription, mRNA stability, and protein acetylation and degradation. This last depends of different sites of phosphorylation.

and the length of the immune response (Manetsch et al., 2012; Tomida et al., 2015).

THE SWITCH BETWEEN PROLIFERATION AND ACTIVATION IS MEDIATED BY MKP-1

The activation and proliferation of macrophages are reciprocally exclusive processes (Xaus et al., 1999); however, both require ERK-1/2 phosphorylation, although with distinct kinetics. For macrophage proliferation, ERK-1/2 phosphorylation occurs with a prompt peak around 5 min, while for activation the phosphorylation takes place later, around 15 min (Valledor et al., 2000a; Suzu et al., 2007; **Figure 2**). The time course of ERK-1/2 dephosphorylation differs for growth factors and activating molecules, occurring 30 min after phosphorylation for M-CSF-induced proliferation and 90 min for LPS-induced functional activation. MAPK phosphorylation is regulated by the dominant action of protein phosphatases, as evidenced by phosphorylation being reversible even in the continued presence of activating stimuli.

Both stimuli triggering proliferation and functional activation induce nuclear MKP-1 (Valledor et al., 1999, 2000b). The transcription of MKP-1 follows the same kinetics by M-CSF or LPS, producing early or late mRNA, depending on the stimuli. Regardless of whether macrophages are treated with M-CSF or LPS, ERK-1/2 dephosphorylation is associated only with the induction of MKP-1 (Valledor et al., 2008a; **Figure 2**). This induction requires the binding and phosphorylation of CREB and c-Jun, and the kinetics of these two molecules correlates to the different times courses of MKP-1 expression. The distinct kinetics of dephosphorylation suggests that the critical factor that allows LPS or IFN-γ to inhibit macrophage proliferation is related to the duration of ERK phosphorylation. This hypothesis was tested by inhibiting MKP-1 expression. However, Mkp-1 knockout (KO)

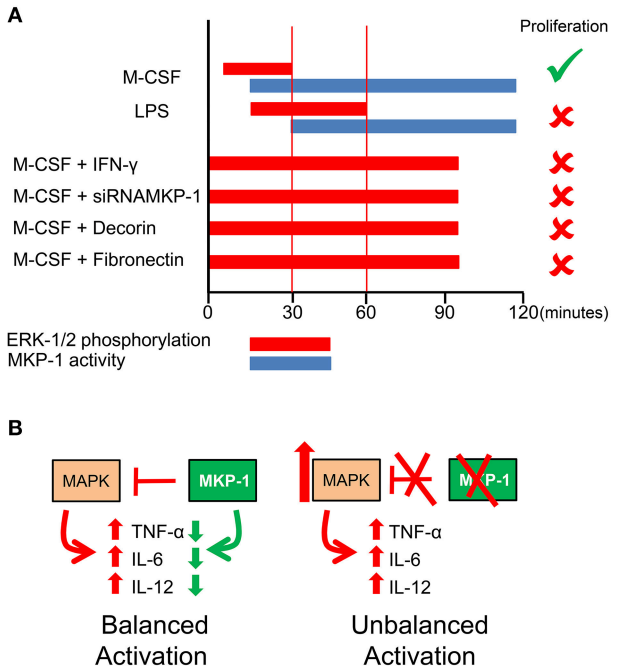

FIGURE 2 | Interplay between MAPKs and MKP-1. (A) The kinetics of MKP-1 induction determines the different time courses of ERK phosphorylation governing the proliferation or the functional activation of macrophages. The repression of MKP-1 expression prolongs ERK-1/2 phosphorylation and blocks proliferation (Valledor et al., 1999, 2000a,b, 2008a; Xaus et al., 2001; Sanchez-Tillo et al., 2007). **(B)** For balanced activation MKP-1 is required. The absence of MKP-1 induces an excess of activation.

mice cannot be used for this purpose because compensatory mechanisms replace the role of MKP-1 in proliferation. In fact, in contrast to wild-type cells in which MKP-4 is not induced by M-CSF, in MKP-1 deficient macrophages, there is an early MKP-4 response to M-CSF. By using siRNA, it was observed that the inhibition of MKP-1 expression extends ERK-1/2 phosphorylation and blocks M-CSF-dependent proliferation (Valledor et al., 2008a).

The inhibition of proliferation induced by IFN-γ stimulation correlates with extended ERK-1/2 phosphorylation induced by M-CSF and is due to the inhibition of MKP-1 expression (Valledor et al., 2008a). Interestingly, this inhibition is mediated by STAT1. This observation would suggest that this transcription factor represses some genes.

The M-CSF-dependent induction of MKP-1 expression can be altered by other agents. MKP-1 expression can be inhibited by signaling through extracellular matrix proteins, such as decorin and fibrinogen (Xaus et al., 2001). As result of such inhibition, ERK phosphorylation is extended, and macrophage proliferation is inhibited (**Figure 2**).

OTHER ACTIVITIES REGULATED BY MKP-1

MKP-1 KO mice show extended MAPK phosphorylation that correlates with an increased expression of cytokines such as TNF-α, IL-6, and IL-12, all of which increase inflammation (mice exhibit kidney failure, severe hypotension, inflammatory infiltrates in the lung and other tissues, and impaired circulation compared with wild-type mice) and cause higher susceptibility to endotoxic shock (Chi et al., 2006; Hammer et al., 2006, 2010; Salojin et al., 2006; Zhao et al., 2006; Frazier et al., 2009; Rodriguez et al., 2010). In a microarray of 14,000 genes from mkp-$1^{-/-}$ mouse spleen cells, 608 genes were found to be upregulated (Hammer et al., 2006). The absence of MKP-1 produces the longest p38 phosphorylation, which in turn increases C/EBPβ phosphorylation, a critical process in several LPS-induced genes (Serrat et al., 2014). Another mechanism to by which to increase the gene expression of natural immunity in the absence of MKP-1 may be related to histones such as H3, which is a substrate of MKP-1 (Kinney et al., 2009). The loss of histone dephosphorylation may affect the transcription of several genes.

Interestingly, the half-lives of mRNAs of several cytokines, including IL-6, IL-10, and TNF-α, seem to be controlled by MKP-1 through translocation of RNA binding proteins from the nucleus to the cytosol (Yu et al., 2011). Recent data shows that MKP-1 modulates the activity of the cytokine mRNA by destabilizing the phosphorylation status of tristetraprolin, an RNA-destabilizing protein (Smallie et al., 2015).

In conclusion, in this review we have addressed the critical issue of the duration of ERK-1/2 kinase phosphorylation and its role in mediating macrophage proliferation or functional activation. The duration of the phosphorylation state is determined by the induction of MKP-1, thus making MKP-1 one of the critical regulators of macrophage biology.

AUTHOR CONTRIBUTIONS

JL: review literature, discuss with the other collaborators, made part of the original data and draw the graphics. LV, JT, and TV: review literature, discuss with the other collaborators and made part of the original data. AC: review literature, discuss with the other collaborators, made the original data and wrote the manuscript.

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Emerging Roles of the Mitogen and Stress Activated Kinases MSK1 and MSK2

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Mitogen- and stress-activated kinases (MSK) 1 and 2 are nuclear proteins activated downstream of the ERK1/2 or p38 MAPK pathways. MSKs phosphorylate multiple substrates, including CREB and Histone H3, and their major role is the regulation of specific subsets of Immediate Early genes (IEG). While MSKs are expressed in multiple tissues, their levels are high in immune and neuronal cells and it is in these systems most is known about their function. In immunity, MSKs have predominantly anti-inflammatory roles and help regulate production of the anti-inflammatory cytokine IL-10. In the CNS they are implicated in neuronal proliferation and synaptic plasticity. In this review we will focus on recent advances in understanding the roles of MSKs in the innate immune system and neuronal function.

Keywords: MSK1, MSK2, CREB, MAPK, p38, IL-10, innate immunity, synaptic plasticity

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INTRODUCTION

MSK1 and 2 were first identified in 1998 as proteins that shared homology to the RSK kinase family (Deak et al., 1998; New et al., 1998; Pierrat et al., 1998). MSKs contain 2 kinase domains—an N-terminal kinase domain (NTKD) in the AGC kinase family and a C-terminal kinase domain (CTKD) from the calmodulin kinase family (Caenepeel et al., 2004). MSKs are activated by the ERK1/2 or p38 MAPK pathways. ERK1/2 and/or p38 phosphorylates 3 sites on MSKs, which activates the CTKD. This causes autophosphorylation and activation of the NTKD, which in turn phosphorylates MSK substrates (Deak et al., 1998; McCoy et al., 2005, 2007). MSKs exist in all vertebrate species examined, with the exception of the lamprey. The chordates *Ciona intestinalis*, *Ciona savignyi,* and *Branchiostoma floridae* possess a single MSK homolog indicating that MSKs diverged from RSK before the onset of vertebrate evolution. MSK1 and 2 probably derive from a duplication event early in vertebrate evolution; orthologs of both MSK1 and 2 exist in the elephant shark, *Callorhinchus milii* (Venkatesh et al., 2014) and in bony fish. In cells, MSK1 and MSK2 are functionally redundant (Wiggin et al., 2002). Perhaps because of this, not all vertebrates have retained both genes. In the Ensembl database, MSK2 is absent in 5 bird genomes while 11 out of the 38 mammalian genomes appear to have lost MSK isoforms. Orthologs of MSKs have been identified in nematodes and insects, although of these only the *Drosophila* protein Jil-1 has been studied in detail (Jin et al., 1999; Wang et al., 2001). Interestingly the main area of homology for Jil-1 with MSKs is in the NTKD. Unlike the chordate and nematode MSKs, the CTKD of Jil-1 lacks the classical MAPK phosphorylation sites.

MSKs are predominantly localized to the nucleus and this is reflected in their known substrates. The best characterized MSK substrates are Histone H3 and the related transcription factors CREB and ATF1. These have been validated via both pharmacological inhibition and mouse genetics

(reviewed in Arthur, 2008). The majority of the genes regulated by MSKs are also targets of CREB. The detailed molecular mechanism by which MSKs regulate CREB is however unclear. MSKs phosphorylate CREB on Ser133, a site that is also targeted by other kinases including protein kinase A (PKA). Phosphorylation of CREB by PKA creates a binding site for the co-activator proteins CBP and p300 (Gonzalez and Montminy, 1989; Chrivia et al., 1993; Cardinaux et al., 2000; Mayr and Montminy, 2001), whose recruitment promotes the efficient transcription of CREB target genes (Yamamoto et al., 1988; Gonzalez and Montminy, 1989). Several studies have demonstrated that CREB phosphorylation downstream of MAPK signaling does not lead to CBP or p300 recruitment (Brindle et al., 1995; Mayr and Montminy, 2001; Mayr et al., 2001; Kasper et al., 2011), leading to the suggestion that MAPKs, and by inference MSKs, do not activate CREB-dependent transcription. Furthermore, PKA- but not MSK-mediated CREB phosphorylation leads to efficient CBP or p300 recruitment to endogenous CREB-dependent promoters as judged by chromatin immunoprecipitation (ChIP) (Naqvi et al., 2014). Despite this, mutation of the Ser133 site to alanine in the endogenous CREB gene actually had a bigger impact on CREB target genes in response to MSK activating stimuli compared to PKA activating stimuli. How MSK-mediated CREB phosphorylation activates CREB is however unresolved (Naqvi et al., 2014). A number of other substrates for MSKs, including NFκB, HMG-14, RAR-related orphan receptor alpha (RORα), KDM3A, Trim7, and Trim28 have been proposed, although their overall importance to MSK function is currently less clear (Soloaga et al., 2003; Vermeulen et al., 2003; Bruck et al., 2009; Cheng et al., 2014; Chakraborty et al., 2015; Singh et al., 2015). While MSKs are expressed in many tissues, their function, as discussed below, has been best studied in the innate immune system and brain.

MSKs in Innate Immunity

p38α MAPK has been extensively studied in innate immunity and regulates the production of pro-inflammatory cytokines in innate immune cells. As a result p38 inhibitors have been developed as potential anti-inflammatory drugs, although none have progressed in the clinic. More recently, anti-inflammatory roles for p38 have emerged which may contribute to the lack of efficacy of p38 inhibitors in the clinic (reviewed in Arthur and Ley, 2013; Salgado et al., 2014). A key question therefore was whether MSKs regulated pro- or anti-inflammatory functions downstream of p38α. MSK1/2 knockout does not result in an overt phenotype in unchallenged mice (Wiggin et al., 2002), however these mice are sensitized to lipopolysaccharide (LPS)-induced endotoxic shock, indicating that MSK activation has an anti-inflammatory effect (Ananieva et al., 2008). MSK1/2 knockout also resulted in elevated levels of TNF, IL-6, and IL-12 production downstream of LPS stimulation, but decreased production of the anti-inflammatory cytokine IL-10 (Ananieva et al., 2008; Kim et al., 2008). IL-10 is known to repress pro-inflammatory cytokine production, and neutralization of IL-10 in wildtype isolated macrophages increases the production of IL-6 and IL-12, similar to that found in MSK1/2 knockouts with decreased endogenous IL-10. However, additional neutralization of IL-10 in MSK1/2 knockouts did not further affect pro-inflammatory cytokine production in isolated macrophages (Ananieva et al., 2008). Interestingly the effect on TNF was partial, suggesting additional IL-10 independent mechanisms of TNF regulation by MSKs.

MSKs regulate IL-10 by controlling its transcription in macrophages and dendritic cells (Ananieva et al., 2008; Elcombe et al., 2013). In addition to IL-10, several other anti-inflammatory genes are regulated by MSKs in macrophages, including dual specificity protein phosphatase 1 (DUSP1), tristetraprolin (TTP) and IL-1 receptor agonist (IL-1ra) (Brook et al., 2006; Ananieva et al., 2008; Darragh et al., 2010) (**Figure 1**). DUSP1 is a phosphatase that inactivates the p38 and JNK MAPKs, and like MSK1/2 knockout, DUSP1 knockout sensitizes mice to endotoxic shock (reviewed in Lang et al., 2006; Wang and Liu, 2007). TTP is able to bind to AU rich elements in the 3′ UTR of multiple cytokines including TNF, inhibiting translation and promoting mRNA degradation (Brooks and Blackshear, 2013). IL-1ra is a member of the IL-1 family that binds to the IL-1 receptor but cannot activate signaling, thus acting as an inhibitor of IL-1 *in vivo* (Garlanda et al., 2013).

TTP knockout gives rise to multiple inflammatory phenotypes leading to early mortality (Taylor et al., 1996). Loss of IL-10 in mice or humans results in the development of colitis (Kühn et al., 1993; Shah et al., 2012) while mutation of IL-1ra in humans gives rise to an early onset auto-inflammatory condition, DIRA (Aksentijevich et al., 2009). Given the phenotypes associated with its target genes, it is surprising that MSK knockout does not give a more overt phenotype. This may be because other pathways compensate for MSK *in vivo*. Related to this, CREB is phosphorylated by other kinases in addition to MSKs including PKA (Johannessen et al., 2004). Prostaglandin E2 (PGE_2) activates PKA in macrophages and acts synergistically with TLR agonists to induce IL-10. This effect is dependent on CREB, but does not require PKA-mediated CREB phosphorylation (MacKenzie et al., 2013b). Instead PKA phosphorylates salt-inducible kinase 2 (SIK2) thus inhibiting the ability of SIK2 to phosphorylate CRTC3. As a result CRTC3 becomes dephosphorylated and translocates to the nucleus where it acts as a co-activator for CREB on the IL-10 promoter (Clark et al., 2012; MacKenzie et al., 2013b).

MSKs also have complex roles in regulating prostaglandin production. *Ptgs2*, the rate-limiting enzyme in prostaglandin production, is a CREB regulated gene. Early *Ptgs2* transcription downstream of TLR agonists is positively regulated by MSKs, suggesting that MSK inhibition might reduce prostaglandin production. IL-10 can however suppress *Ptgs2* mRNA induction. As MSK knockout reduces IL-10 production, at later time points after LPS stimulation MSK1/2 knockout macrophages actually show increased *Ptgs2* induction and elevated prostaglandin production (MacKenzie et al., 2013a).

In addition to their anti-inflammatory roles, various pro-inflammatory roles have also been suggested for MSKs. In human neutrophils LPS can induce MSK-dependent CREB phosphorylation. Both CREB phosphorylation and induction of

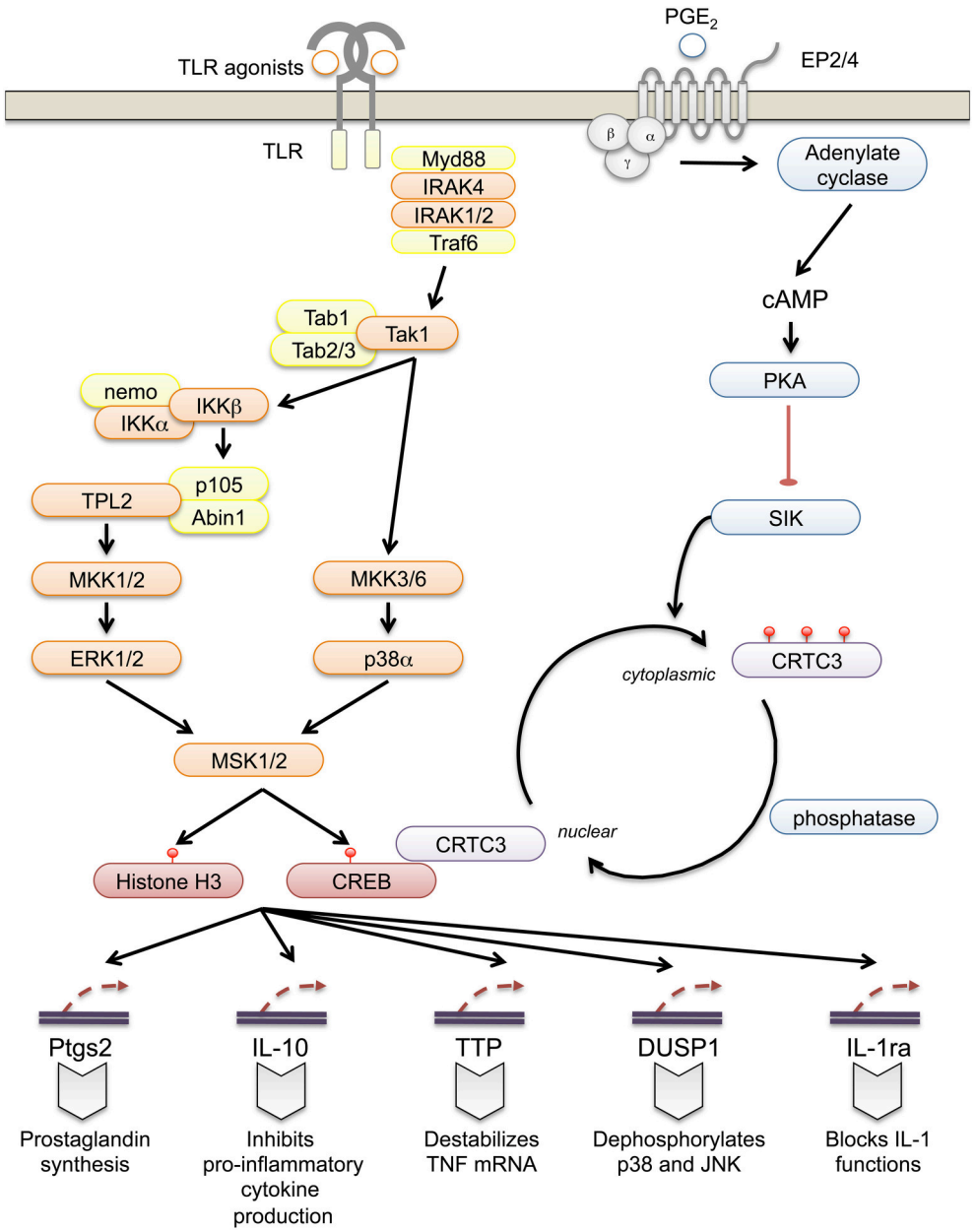

FIGURE 1 | Regulation of innate immune function by MSK1 and 2. In innate immune cells such as macrophages and dendritic cells TLRs, with the exception of TLR3, can activate downstream signaling via Myd88. Upon ligand binding, the TLR recruits Myd88 resulting in the formation of a Myd88osome that also contains IRAK4 and IRAK1 and/or 2. This leads to the recruitment of Traf6 and the formation of K63 and M1 ubiquitin chains that help mediate the activation of Tak1. Tak1 then activates the p38 MAPK cascade and indirectly activates ERK1/2 via signaling to the IKK-mediated activation of Tpl2 through p105. ERK1/2 and p38 are then both able to activate MSK1 and 2, which in turn phosphorylate CREB and Histone H3. This leads to the induction of a number of genes with potential anti-inflammatory roles including *IL-10*, *IL-1ra*, *TTP*, *DUSP1,* and *Ptgs2*. Ptgs2 is the rate-limiting enzyme in the production of prostaglandins, small lipids that can have both pro- and anti-inflammatory roles. In the context of macrophages, prostaglandin E can boost IL-10 production and suppress pro-inflammatory cytokines following TLR stimulation. It acts via the G-protein coupled receptors EP2 and EP3 to elevate cAMP levels and activate PKA. PKA phosphorylates SIK2 and inhibits its ability to phosphorylate CRTC3. This allows CRTC3 to be dephosphorylated and translocated to the nucleus where it will act as a co-activator for CREB.

TNF, IL-8 (CXCL8), CCL3 and CCL4 were reduced by Ro318220, an inhibitor that targets MSKs amongst other AGC kinases. In support of a role for CREB, transfection of PLB-985 cells, a neutrophil-like cell line, with a dominant negative CREB reduced IL-8 and TNF induction (Mayer et al., 2013). MSK1 has also been linked to IL-8 production in human keratinocytes and airway smooth muscle cells, where siRNA-mediated knockdown of MSK1 reduced IL-8 production (Funding et al., 2006; Rahman et al., 2014). Interestingly both MSK1 and 2 activity are increased in human lesional psoriatic skin (Funding et al., 2006, 2007),

suggesting a potential involvement of MSKs in the pathology of this disease. Related to this, DMF is used as a treatment for psoriasis and it has been proposed that its efficacy may be in part due to MSK inhibition (Gesser et al., 2007; Peng et al., 2012). In mice however MSK1/2 knockout increases skin inflammation following PMA treatment or in oxazolone-induced allergic contact dermatitis (Ananieva et al., 2008; Bertelsen et al., 2011). This may reflect a difference between human and mouse skin; if in human keratinocytes MSK acts predominantly to regulate inflammation via IL-8, these differences may relate to the lack of a direct IL-8 homolog in mice.

Herpesviridae are a family of double stranded DNA viruses some of which, including Kaposi's sarcoma-associated herpes virus (KSHV) and human cytomegalovirus (HCMV), cause human disease. Upon infection these viruses can either enter a lytic replication cycle to produce further virions or enter a latent phase (Roizman and Baines, 1991). Following infection of Human Umbilical Vein Endothelial Cells (HUVEC) with KSHV, MSK is activated and phosphorylates CREB. siRNA-mediated knockdown of either CREB or MSK1 and 2 did not prevent infection, but did reduce the production of infectious virions. This correlated with a drop in the levels of the viral genes involved in lytic replication (Cheng et al., 2015). HCMV can be reactivated in cells with a latent infection via a process stimulated by IL-6 (Hargett and Shenk, 2010; Reeves and Compton, 2011). This process correlates with CREB phosphorylation and furthermore CREB binding can be demonstrated on the viral MIEP during reactivation (Kew et al., 2014). This process was blocked by inhibition of the ERK1/2 pathway, suggesting a role for either MSK or RSK. The RSK inhibitor DI-D1870 did not affect this process while H89, a compound that targets several kinases including MSKs, reduced transcription from the MIEP promoter (Kew et al., 2014).

MSKs and Neuronal Function

Both MSK1 and MSK2 are expressed in the brain; however MSK1 is the major isoform in most brain structures (Arthur et al., 2004). While MSK1/2 knockout mice do not exhibit gross defects in CNS development, under some circumstances MSKs play roles in neuronal proliferation or survival. Following pilocarpine-induced seizure, proliferation of neuronal progenitors in the subgranular zone (SGZ) of the dentate gyrus of mice was reduced by knockout of MSK1 and 2. In addition reduced neurite arborization was also observed in immature neurons in this region, suggesting that MSKs were helping drive proliferation and the maturation of new neurons (Choi et al., 2012). Similar results were also reported in the SGZ in a model of cerebral ischemia (Karelina et al., 2015).

Several reports have also suggested roles for MSKs in neurodegenerative diseases. Spinocerebellar ataxia type 1 (SCA1), a condition resulting from the expansion of a polyglutamine tract in ataxin-1, results in neurodegeneration in the cerebellum and brain stem (Manto, 2005). Ataxin-1 can be phosphorylated by MSKs on Ser766 and lead to stabilization of mutant forms of the protein. Loss of one or more MSK alleles was protective in a mouse model of SCA1 driven by an $Atxn^{154Q}$ mutation, raising the possibility that MSK inhibitors may be useful for treating this disease (Park et al., 2013). Huntington's disease results from the expansion of a CAG motif in the Htt gene giving rise to a polyglutamine repeat (Walker, 2007). While it affects many areas of the brain, the striatum is particularly sensitive to damage. Decreased MSK1 expression was observed in the caudate nucleus from the striatum of Huntington's patients (Roze et al., 2008). In the R6/2 transgenic mouse model of Huntington's, both MSK1 and Histone H3 Ser10 phosphorylation were decreased (Roze et al., 2008). Furthermore, MSK1 overexpression can promote expression of PGC-1α, a gene that is neuro-protective in Huntington's and MSK1 knockout mice showed evidence of striatal degeneration upon aging (Martin et al., 2011). Parkinson's disease is associated with reduced dopamine levels. Dopamine therapy, while beneficial for Parkinson's can result in Levodopa-induced dyskinesia (LID). In animal models this correlates with increased δFosB expression in the striatum. Two studies showed that MSK knockout decreased δFosB expression in LID, however while in one study LID intensity was attenuated in the other it was not (Brami-Cherrier et al., 2005; Alcacer et al., 2014; Feyder et al., 2016).

The striatum is also involved in addiction. MSK1 regulates glutamate-stimulated Histone H3 phosphorylation in cultured striatal neurons and cocaine administration activated MSK1 in the striatum *in vivo* (Brami-Cherrier et al., 2007). MSK1 knockout mice showed increased response to low doses of cocaine in placed preference tests, while following repeated injections of cocaine locomotor sensitization was decreased (Brami-Cherrier et al., 2005).

The hippocampus plays important roles in encoding memory and is involved in rodent models of spatial memory and contextual fear conditioning. CREB is implicated in synaptic plasticity and memory (Shaywitz and Greenberg, 1999; Lonze and Ginty, 2002; Carlezon et al., 2005; Benito and Barco, 2010; Sakamoto et al., 2011) while the importance of histone modifications, including phosphorylation, have also been recognized (Day and Sweatt, 2011; Mifsud et al., 2011; Kandel et al., 2014; Alberini and Kandel, 2015). The MAPK pathway has been also found to be a critical component of consolidation of memory in the hippocampus (Besnard et al., 2014) and ERK1/2-stimulated histone phosphorylation and acetylation in hippocampal CA1 neurons is associated with memory consolidation (Levenson et al., 2004; Chwang et al., 2006). The involvement of both the upstream activators of MSK and its substrates in these processes suggest the involvement of MSKs in memory.

In primary cortical neuronal cultures stimulated with the neurotrophin BDNF, MSK1 was the critical isoform for both CREB phosphorylation and the induction of CREB-dependent IEGs (Arthur et al., 2004). The effect of MSK1 knockout has now been reported in several hippocampal-dependent learning models. In the forced swim test, mice repeatedly placed in a pool with no ability to escape will display a learned helplessness (De Pablo et al., 1989; West, 1990; Korte, 2001). These behavioral changes are accompanied by an increase in phosphorylation and acetylation of Histone H3 in dentate gyrus granule neurons (Bilang-Bleuel et al., 2005), and knockout of MSK1/2 restricted

this behavioral response and correlated with decreased Histone H3 phosphorylation (Chandramohan et al., 2008).

In contextual fear conditioning mice learn to associate a specific context with an aversive stimulus. MSK1 activation has been shown to occur during this process (Sindreu et al., 2007), which is also associated with CREB and Histone H3 phosphorylation (Impey et al., 1998; Taubenfeld et al., 1999). MSK1 knockout resulted in a mild deficit in contextual fear conditioning and decreased CREB and Histone H3 phosphorylation (Chwang et al., 2007). MSK1 knockout also resulted in a mild impairment in the Morris water maze (Chwang et al., 2007), a widely used model for spatial memory (D'Hooge and De Deyn, 2001). Long term potentiation (LTP), which allows the strengthening of specific synapses in response to high frequency stimulation, is a form of synaptic plasticity considered to provide a molecular model for encoding memory in the hippocampus (Martin et al., 2000). The role of MSKs have not been directly examined in LTP and it would be of interest to address this in the future. Related to this, in response to neurotrophins, MSKs control the production of two closely related miRNAs, miR-132, and miR-212 that have been implicated in synaptic function (Wayman et al., 2008; Remenyi et al., 2010). mir-132/miR-212 knockouts showed normal LTP in the hippocampus but impaired neocortical theta burst-induced LTP (Remenyi et al., 2013).

Although the role of MSKs has not been reported in LTP, their role has been investigated in homeostatic scaling. This is a form of non-Hebbian plasticity that allows a neuron to co-ordinately regulate the strengths of all its synaptic inputs in order to maintain its firing rate within physiological boundaries (Davis, 2006), thus allowing protection of neural networks from excessive or inhibitive stimuli. This scaling process has been linked to levels of BDNF (Rutherford et al., 1998; Turrigiano et al., 1998). Homeostatic scaling can be modeled in culture by globally blocking axon potentials using TTX, which blocks voltag- gated sodium channels. Cultured hippocampal neurons from MSK1 kinase-dead mice failed to show homeostatic scaling of synaptic transmission in response to TTX (Corrêa et al., 2012). BDNF is also involved in the synaptic scaling and remodeling that occurs in response to environmental enrichment *in vivo* (Baroncelli et al., 2010; Cowansage et al., 2010). A failure to upregulate synaptic strength during environmental enrichment has been reported in MSK1 kinase dead knockin (Corrêa et al., 2012) and MSK1 knockout mice (Sakamoto et al., 2011). Given the role of MSKs in response to environmental enrichment it will be especially interesting to look at the effect of MSK knockout on LTP and memory in animals raised in enriched conditions.

CONCLUSION

This review highlights the critical importance of MSKs in limiting inflammation in innate immunity and their role in inflammatory disease, however their role in the adaptive immune responses remain uncharacterized. MSKs are also implicated in neurodegeneration and synaptic plasticity. Currently, much more work remains to uncover their precise roles in these processes of inflammation and neurodegenerative disease. Outside these systems, work has recently suggested further roles for MSKs. For example MSKs may be involved in skin tumor formation (Chang et al., 2011; Dong et al., 2014; Liu et al., 2014) and have been linked to cellular transformation (Raab-Traub, 2002; Pérez-Cadahía et al., 2011; Reyes et al., 2014). Future research will help unravel the exact mechanisms underlying these enzymes *in vivo* in health and disease.

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All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Induction of Macrophage Function in Human THP-1 Cells Is Associated with Rewiring of MAPK Signaling and Activation of MAP3K7 (TAK1) Protein Kinase

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Macrophages represent the primary human host response to pathogen infection and link the immediate defense to the adaptive immune system. Mature tissue macrophages convert from circulating monocyte precursor cells by terminal differentiation in a process that is not fully understood. Here, we analyzed the protein kinases of the human monocytic cell line THP-1 before and after induction of macrophage differentiation by using kinomics and phosphoproteomics. When comparing the macrophage-like state with the monocytic precursor, 50% of the kinome was altered in expression and even 71% of covered kinase phosphorylation sites were affected. Kinome rearrangements are for example characterized by a shift of overrepresented cyclin-dependent kinases associated with cell cycle control in monocytes to calmodulin-dependent kinases and kinases involved in proinflammatory signaling. Eventually, we show that monocyte-to-macrophage differentiation is associated with major rewiring of mitogen-activated protein kinase signaling networks and demonstrate that protein kinase MAP3K7 (TAK1) acts as the key signaling hub in bacterial killing, chemokine production and differentiation. Our study proves the fundamental role of protein kinases and cellular signaling as major drivers of macrophage differentiation and function. The finding that MAP3K7 is central to macrophage function suggests MAP3K7 and its networking partners as promising targets in host-directed therapy for macrophage-associated disease.

Keywords: chemical proteomics, kinomics, phosphoproteome, monocyte-to-macrophage differentiation, kinome, signaling, THP-1 cells

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INTRODUCTION

Cellular differentiation is a fundamental process in development that is triggered by internal or external stimuli resulting in cells with increased degree of specialization with regard to its respective progenitors. On the molecular level cell transformation is founded on signaling

Abbreviations: 5Z, (5Z)-7-Oxozeaenol; CFU, colony forming units; ELISA, enzyme linked immunosorbent assay; GFP, green fluorescent protein; IPA, Ingenuity pathway analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen activated protein kinase; MPS, mononuclear phagocyte system; PMA, phorbol-12-myristate 13-acetate; SILAC, stable isotope labeling by amino acids in cell culture; SMAC, small molecule affinity chromatography; TAK1, Transforming growth factor β-activated kinase; LC-MS/MS, Liquid chromatography–mass spectrometry.

networks that transduce stimuli from the cell surface to modulators by protein phosphorylation and dephosphorylation as the most important posttranslational modification in transmission and integration of signals in cellular networks.

The mononuclear phagocyte system (MPS) represents an impressive example of how cell differentiation generates functional and phenotypic diversity. The common origin of cells of the MPS are hematopoietic stem cells located in the bone marrow that transform through different myeloid progenitor intermediates to monocytes that enter circulation, migrate into tissue and terminally differentiate to replenish resident dendritic cells and macrophages (Geissmann et al., 2010a). Macrophages differ from their monocytic precursors morphologically and are characterized by elevated lysosomal and mitochondrial content as well as in their responsiveness to pathogen-associated molecular patterns—small molecular motifs conserved within a class of microbes (Cohn, 1968; Ross and Auger, 2002). Furthermore, monocytes and macrophages are both critical effectors and regulators of inflammation and the innate immune response e.g., by producing growth factors and cytokines (Geissmann et al., 2010b). The differential degree of functional specialization and adaptive capabilities are attributable to molecular structures that are significantly co-determined by cell signaling components. We hypothesize that major differences exist between the kinomes of monocytes and macrophages and that this has a determining influence in shaping the functional cellular characteristics. Comparative profiling of the kinomes of both cell types could thus increase our understanding in the signal transduction capabilities and assist identification of kinases that are associated with cell type-specific functions.

Analysis of monocyte function and differentiation is still a challenging task. Monocytes permanently sense their environment and react with rapid alteration in phenotype and behavior already during isolation and processing (Auffray et al., 2009). Moreover, limited quantities and heterogeneity of the monocyte population in terms of differentiation stages hinder their analysis. To overcome these drawbacks, a number of human leukemia model cell lines with monocytic characteristics have been established that represent a relative homogenous population and can easily be expanded *in vitro*. These cell lines are blocked at different stages of differentiation but can be released for further progression using specific stimuli (Auwerx, 1991). The human cell line THP-1 is one of the most frequently used model system with monocytic properties (Tsuchiya et al., 1980). THP-1 cells can be differentiated into macrophage-like cells that resemble properties of mature macrophages by activation of protein kinase C (PKC) with phorbol-12-myristate-13-acetate (PMA), ultimately resulting in cells with increased adherence and loss of proliferative activity (Schwende et al., 1996).

Several recent studies employed proteomic techniques that revealed changes in the proteome signature that are associated with differentiation of monocytes to macrophages. Accordingly, increased expression of proteins involved in the suppression of NF-κB including superoxide dismutase 2 (SOD2), the sodium pump subunit alpha-1 (ATP1A1) and a serine peptidase inhibitor (SERPINB2) was highlighted during differentiation of primary human monocytes (Kraft-Terry and Gendelman, 2011). In addition, PMA-induced differentiation of U937 cells is associated with elevated expression of proteins involved in carbohydrate metabolism, antioxidant defense and actin filament rearrangement (Sintiprungrat et al., 2010). Although, these studies shed light on the functional rearrangement on the proteome level, cellular signaling nodes including kinases and phosphatases as the driving forces behind cellular differentiation are underrepresented and hence their impact on monocyte and macrophage properties is not well understood.

Here, we applied a quantitative chemical proteomics strategy for the systematic analysis of the kinome following PMA-triggered differentiation of THP-1 monocytes. We reveal major kinome rearrangement following monocyte differentiation and demonstrate that the macrophage-associated kinase MAP3K7/TAK1 is a central hub in the signal transduction involved in bacterial killing, chemokine production and the differentiation process itself.

MATERIALS AND METHODS

Cell Culture, SILAC, and Differentiation

THP-1 monocytes were purchased from the German collection of microorganisms and cell cultures (DSMZ). Stable isotope labeling with amino acids in cell culture (SILAC, Ong et al., 2002, 2003) was achieved by propagating cells in RPMI 1640 free of L-lysine, L-arginine and L-glutamine (PAA Laboratories) supplemented with 220 μM L-lysine and 144 μM L-arginine in either their light (Lys-0 and Arg-0) or heavy isotope-labeled forms ($^{13}C_6^{15}N_2$-L-lysine/Lys-8 and $^{13}C_6^{15}N_4$-L-arginine/Arg-10) (Silantes), 2 mM L-glutamine (Sigma-Aldrich), 10% heat-inactivated dialyzed fetal calf serum (FCS) (Sigma-Aldrich) and the antibiotics penicillin and streptomycin (Biochrom) at concentrations of 100 units/ml and 100 μg/ml respectively. Cell culturing was performed at 37°C and 5% CO_2 in a humidified atmosphere. Cells were grown for at least six cell doublings to ensure complete incorporation of labeled amino acids. For differentiation, phorbol-12-myristate 13-acetate (PMA) (Sigma-Aldrich) was added to a final concentration of 100 nM. After 3 days, the PMA supplemented media was removed, cells were washed with PBS and rested in fresh PMA-free media for further 24 h in order to obtain phenotypic characteristics of macrophages (Daigneault et al., 2010).

Immobilization of Kinase Inhibitors

Affinity beads were prepared with modifications as described elsewhere (Bantscheff et al., 2007; Wissing et al., 2007). Carbodiimid chemistry was used in order to immobilize the kinases inhibitors SU6668 (Tocris), Purvalanol B (Tocris), and VI16832 (Evotec) to either ECH-Sepharose or EAH-Sepharose (GE) beads. Inhibitors were dissolved in coupling buffer (50/50 sodium phosphate pH 7.0/dimethylformamide) and beads were prepared according to manufacturer's recommendations. Crosslinking was performed in coupling buffer for 24 h at 4°C using N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC, Sigma-Aldrich) as crosslinker. Beads were extensively washed with coupling buffer and blocked with ethanolamine for 24 h.

Preparation of Cell Lysates and Enrichment of Protein Kinases by Small Molecule Affinity Chromatography (SMAC)

Undifferentiated THP-1 cells were harvested by centrifugation for 5 min and 130 × g at 4°C and washed twice with phosphate-buffered saline (PBS). Differentiated and undifferentiated THP-1 cells were lysed in buffer containing 50 mM HEPES pH 7.5, 2 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% (v/v) TritonX-100, 1 mM PMSF, 50 ng/ml calyculin A, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 mM NaF, and 2.5 mM Na_3VO_4. Cell lysates were prepared by freeze thawing, followed by sonication. Cell debris was removed by centrifugation for 30 min at 8700 × g at 4°C followed by passing through a 0.45 μm cellulose acetate filter (VWR). Protein concentrations of clarified lysates were determined by Bradford Assay (BioRad).

Heavy and light SILAC lysates with a total protein content of 50 mg were mixed and applied to affinity columns casted from 500 μl of a 50% bead suspension carrying Purvalanol B and SU6668 using gravity flow columns (Pierce) and incubated for 1 h at 4°C on a rotator. The flow through was collected and applied on a second affinity column casted from 500 μl bead suspension with immobilized VI16832 and further incubated for 1 h. Columns were washed with 20 column volumes (CV) cell lysis buffer followed by a wash with 20 CV cell lysis buffer with 150 mM NaCl instead of initial 1 M NaCl and a final wash with 20 CV 50 mM HEPES pH 7.5. Bound proteins were eluted with 20 CV prewarmed 0.5% (m/v) SDS and 5 mM DTT in 1 ml fractions. Collected fractions were pooled and lyophilized. Experiments were carried out in three independent biological replicates.

Protein Digest and Phosphopeptide Enrichment

Lyophilized material was dissolved in water, immediately followed by addition of 4 volumes of pre-chilled acetone and incubation at −20°C overnight. Precipitated proteins were pelleted by centrifugation (8700 × g, 30 min), dissolved in denaturing buffer (8 M urea, 20 mM HEPES, pH 8.0), alkylated by addition of iodoacetamide to a final concentration of 5 mM for 20 min at room temperature. Samples were separated into 10 gel slices by one-dimensional SDS-PAGE using pre-casted tris-glycine 4–15% gradient gels (Biorad). Tryptic peptides were obtained by in-gel digestion followed by peptide extraction. Of the resulting peptide solutions, 10% were vacuum dried and stored at −80°C until mass spectrometry analysis. The residual 90% were vacuum-dried and peptides were dissolved in 1 ml TiO_2-binding buffer (73% (v/v) acetonitrile, 10% (v/v) lactic acid, 2% (v/v) TFA) for phosphopeptide enrichment. 50 μl from a TiO_2-stock solution (30 mg/ml Titansphere TiO_2 bulk material (GL sciences) in 100% acetonitrile) were added and incubated for 20 min at room temperature. After centrifugation (1000 x g, 3 min), TiO_2-beads were washed 4 times with 80% (v/v) acetonitrile, 2% (v/v) TFA. Phosphopeptides were sequentially eluted with 5% (v/v) NH_4OH and 30% (v/v) acetonitrile, vacuum-dried, dissolved in 0.1% (v/v) TFA and purified with C18 StageTips (Thermo Scientific). The eluated phosphopetides were again vacuum-dried and stored at −80°C.

Mass Spectrometry Analysis

LC-MS/MS analyses were carried out by an EASY-nLCII HPLC system directly coupled to a LTQ Orbitrap Velos Pro hybrid mass spectrometer (Thermo Scientific) via a nano-electrospray ion source. All lyophilized (phospho)peptide samples were dissolved in 5% (v/v) acetonitrile / 0.1% (v/v) acetic acid and applied to a 20 cm-long in-house packed C18 (Aeris Peptide 3.6 μm, pore size 100 Å; Phenomenex) analytical column. Elution of peptides was carried out with binary linear gradient from 1% (v/v) acetonitrile/0.1% (v/v) acetic acid to 75% (v/v) acetonitrile/0.1% (v/v) over a period of 46 min at a flow rate of 300 nl/min. MS was operated in data-dependent mode, each full MS scan mode (300 m/z–1700 m/z; resolution 30,000). Ions with charge states of one or unassigned charge state were excluded for MS/MS scans. Fragmentation was performed either in the linear ion trap using collision induced dissociation (CID) for the most 20 intense ions with an AGC target value of 5×10^3 ions and a normalized collision energy of 35% or in the Orbitrap mass analyzer of the most 10 intense ions using higher-energy collisional dissociation (HCD) with a target value of 5×10^4 ions and 40% normalized collision energy. Precursor ions selected for MS/MS analysis were dynamically excluded for repeated fragmentation for a period of 20 s.

Processing of Data Obtained by Mass Spectrometry

Obtained .raw files were analyzed using the MaxQuant software package (version 1.3.0.5) with the integrated Andromeda search engine (Cox et al., 2011). Files were collectively searched against the reviewed human proteome deposited in UniProtKB/Swiss-Prot containing 20,252 protein entries (UniProt release 2013_04). For peptide and protein identification and quantification, the following settings were used: The variable modifications were set to methionine oxidation, phosphorylation of serine, threonine and tyrosine, and amino-terminal acetylation. Carbamidomethylation was set as fixed modification. A maximum of two missed cleavages were allowed. For peptide identification, a mass tolerance of 0.5 Da and 20 ppm was allowed for the linear ion trap and the Orbitrap mass analyzer respectively. The false discovery rate (FDR) was set to 0.01. Protein quantification was based on razor and unique peptides. Protein kinases with two independent peptide identifications including at least one unique peptide were considered. Combined log_2-transformed SILAC ratios were taken from the MaxQuant output files and considered as reliable when they were based on values from at least two independent replicates. In rare cases were a combined SILAC ratio was only calculated based on one replicate because of the absence of a signal in the unstimulated or stimulated cell state in the other replicates, this ratio was only considered if the heavy/light intensities of at least one additional replicate indicated the same direction of regulation. The assignment of kinases to groups was according to Manning et al. (2002).

IPA, KEGG Pathway, and GO Analysis

For the upstream regulator analysis, protein kinases with corresponding fold changes were imported in the Ingenuity

Pathway Analysis (IPA) tool (Qiagen) and the core pathway analysis was performed. Predicted upstream regulators with $p < 0.05$ and z-scores ≤ -2 (inactive) and ≥ 2 (active) were considered and used for network generation.

Kinases with at least two-fold abundance difference between monocytic and macrophage-like THP-1 cells were subjected to statistical enrichment analysis of KEGG pathways and GO terms in Molecular Function (MF) or Biological Processes (BP) with DAVID Bioinformatics Resources 6.7 (Huang da et al., 2009).

Immunoblotting

Proteins were separated by one-dimensional gel electrophoresis using precasted 4–15% TRX gradient gels (Bio-Rad) followed by transfer on PVDF membranes (Merck-Millipore). Blocking of membranes was carried out for 1.5 h with 5% (m/v) milk in PBS supplemented with 0.05% (v/v) Tween-20. Membranes were probed with primary antibodies overnight at 4°C under gentle agitation. The following primary antibodies were used: anti-c-Abl (abcam #ab16903), anti-LIMK1 (CST #3842), anti-ERK1/2 (CST #9102), anti-MEK1/2 (CST #9122), anti-MEK3 (Santa Cruz #sc-961), anti-MEK4 (Santa Cruz #sc-837), anti-CaMK1 (abcam #ab68234), anti-MerTK (Santa Cruz #sc-365499), anti-CDK1 (abcam # ab18). Bound primary antibodies were detected using fluorophore-conjugated secondary antibodies (either IRDye680RD or IRDye800CW (LI-COR)) and fluorescence readout was performed using an Odyssey infrared imaging system (LI-COR).

Phase Contrast Microscopy

Phase contrast micrographs were obtained by using an Olympus CKX41 fluorescence microscope.

Cell Adhesion Assay

9×10^5 undifferentiated THP-1 cells per well were seeded in 6-well plates (TPP) in RPMI 1640 (Sigma-Aldrich) medium supplemented with 10% FCS (Sigma). Cells were treated with 0.25, 1 or 5 μM (5Z)-7-Oxozeaenol (5Z, Tocris) or DMSO for 1 h. PMA to a final concentration of 100 nM was added and cells present in the cell culture supernatant or adhered to the cell culture plastic were separately counted. Cells present in the supernatant were collected by centrifugation and resuspended in RPMI 1640 medium. An aliquot was mixed with trypan blue solution (LifeTechnologies) and cell viability and cell count were determined by using and automated cell counter (Countess, LifeTechnologies). Adhered cells were detached by incubation with a 5-fold concentrated ready-made trypsin solution (Biochrom) for 5 min. Trypsinization was quenched by addition of RPMI 1640 medium following by cell harvest and counting as described for cells in the supernatant.

Cultivation and Processing of *Staphylococcus aureus* for Infection-Related Experiments

Staphylococcus aureus HG001 (Herbert et al., 2010) was grown in LB to an optical density (OD_{540}) of 0.5 at 37°C under agitation. 30 ml of the culture were centrifuged for 5 min at 8700 × g and the resulting cell pellet was washed twice and finally resuspended in RPMI 1640 medium yielding in a concentration of 5×10^7 cells/ml.

Gentamycin Protection Assay

1.8×10^5 THP-1 monocytes were seeded in a 24-well plate (TPP) and differentiated as described above. *Staphylococcus aureus* HG001 was applied to THP-1 macrophage-like cells at a multiplicity of infection (MOI) of 25 in conditioned RPMI 1640 medium supplemented with phenol red and 10% FCS. After various infection durations, medium was removed, cells were washed with PBS and extracellular staphylococci were killed by addition of conditioned RPMI 1640 medium (supplemented with phenol red and 10% FCS) containing 100 μg/ml gentamicin (Sigma-Aldrich) and 20 μg/ml lysosthaphin (Sigma-Aldrich) for 10 min. Cells were washed twice with PBS and subsequently lysed by addition of 1% (v/v) Triton-X 100 (Roth) in PBS. Intracellular staphylococci were spread on LB agar plates and colony forming units (CFU) were determined following incubation for 24 h at 37°C.

Flow Cytometry

9×10^5 differentiated THP-1 cells were either one-time treated with 1 μM 5Z or vehicle for 1 h followed by infection with a GFP-expressing isogenic mutant of *S. aureus* HG001 pCgfp at an MOI of 25 for 2 h. Cells were subsequently washed twice with PBS and non-ingested bacteria were eradicated by lysostaphin and gentamicin treatment for 10 min with final concentrations of 20 μg/ml and 100 μg/ml, respectively. Cells were washed with PBS and detached with trypsin. Digestion was quenched by addition of RPMI 1640 supplemented with 1% (v/v) FBS. Cells were pelleted by centrifugation, washed twice with PBS, resuspended in PBS buffer containing 1% (v/v) FBS and 3.8 mM sodium azide and finally analyzed for green fluorescence on an Attune Acoustic Focusing Cytometer (LifeTechnologies).

Quantification of Secreted Chemokines Triggered by Heat-Inactivated *S. aureus*

9×10^5 differentiated THP-1 cells were seeded in 6-well plates as described above. Prior of experiments, medium was exchanged with RPMI 1640 without phenol red and supplemented with 1% FCS. Cells were pretreated with 1 μM 5Z for 1 h prior addition of heat-inactivated *S.aureus* HG001. *S. aureus* cells were grown and harvested as described above. *S.aureus* cells were heat-inactivated for 1 h at 60°C and resuspended in RPMI 1640 without phenol red and supplemented with 1% FCS and either 1 μM 5Z or an appropriate amount of DMSO for control cells. *S. aureus* was applied to THP-1 cells at a MOI of 25. Following various incubation times, cell culture supernatants were harvested, passed through a 0.2 μm nitrocellulose filter (VWR) and stored at −80°C. Quantification of a selected set of chemokines from cell culture supernatants after 6 h and 48 h following addition of heat-inactivated *S. aureus* was performed using a custom ELISA Array kit (Qiagen) according to the manufacturer's recommendations. The kinetics of secretion for IL-8, GROa, MIP-1a and MIP-1b were analyzed using ELISA kits (Qiagen) according to the instruction by the manufacturer.

RESULTS AND DISCUSSION

Monocyte-to-Macrophage Differentiation is Accompanied by Major Restructuring of the Kinome, Increase of the General Kinome Phosphorylation Status and Changes in the Basal Activation of Individual Kinases

Cellular signaling mediated by protein kinases regulates virtually any cellular function, showing the vital role of this enzyme class for cell physiology. Despite their striking impact, protein kinases are commonly low abundant proteins and kinome characterization requires reduction of sample complexity i.e., kinase enrichment when using mass-spectrometry based proteomic strategies. Our approach to perform a comparative and system-wide kinome profiling of the human model cell line THP-1 before and after PMA-mediated induction of differentiation into the macrophage-like state is depicted in **Figure 1A**. We used the ATP-competitive small molecule protein kinase inhibitors Purvalanol B, SU6668 and VI16832 immobilized to sepharose beads for protein kinase family-specific pre-enrichment in combination with stable isotope labeling of amino acids in cell culture (SILAC). The inhibitors were previously demonstrated to be efficient kinase purification tools with binding of distinct but overlapping sets of protein

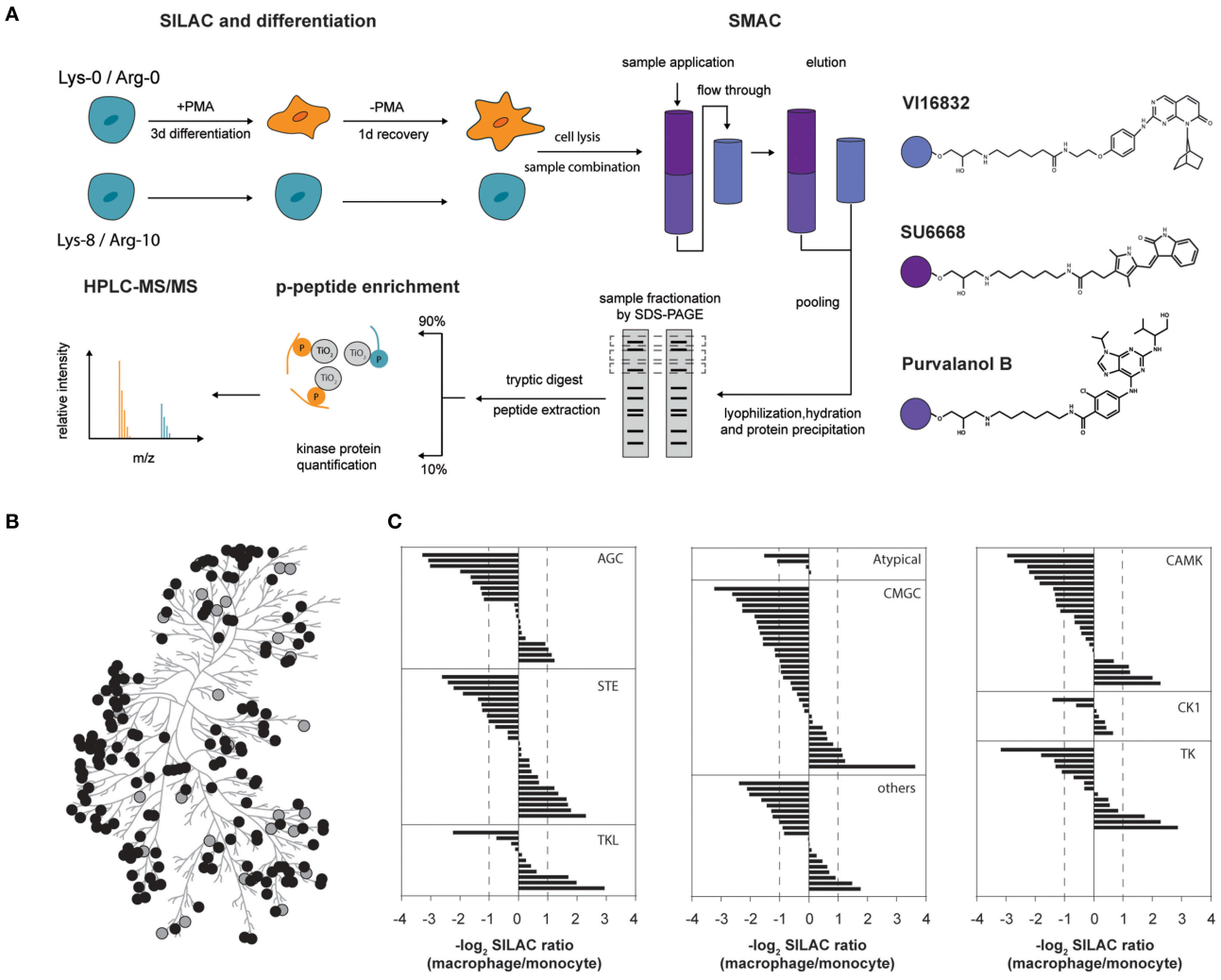

FIGURE 1 | (A) Experimental outline for comparative kinome profiling of undifferentiated (monocytic) and differentiated (macrophage-like) THP-1 cells. Cells were metabolically labeled (SILAC) and differentiation was induced by addition of phorbol-12-myristate 13-acetate (PMA) for 3 days followed by a period of resting for 1 day in PMA-free medium. Cells were lysed and lysates from both THP-1 differentiation stages were combined equally. Small molecule affinity chromatography (SMAC) utilizing broadband kinase inhibitors SU6668, Purvalanol B and VI16832 was used for protein kinase purification. Eluted kinases were lyophilized, re-hydrated and precipitated by acetone. Gel fractionated proteins were digested by trypsin and resulting peptides were extracted. Peptides were separated whereas 90% were used for TiO_2-based phosphopeptide enrichment prior to mass spectrometric analysis. The remainder was directly analyzed by mass spectrometry for quantification of protein kinases. **(B)** Phylogenetic kinase relationship (Manning et al., 2002). Protein kinases identified and quantified in our study are indicated as gray and black circles, respectively. **(C)** Assignment of log_2-SILAC abundance ratios of quantified protein kinases to their corresponding kinase groups.

kinases allowing a broad coverage of the kinome by their combined usage (Wissing et al., 2007; Daub et al., 2008; Oppermann et al., 2009; Zhang et al., 2013). In addition, kinase binding to mixed-inhibitor beads has recently been shown to be largely independent of kinase activity (Ruprecht et al., 2015). The enriched protein kinases were analyzed by liquid chromatography–mass spectrometry (LC-MS/MS) at the level of protein expression and site-specific protein phosphorylation, after applying an additional phosphopeptide enrichment.

Our workflow resulted in the identification of 199 protein kinases from at least two independent peptide sequences (Supplemental Table 1). Of these, 163 kinases fulfilled the criteria for a quantitative comparison between the monocytic and macrophage-like state (**Figure 1B**). Twenty-six protein kinases were increased at the protein level in macrophage-like cells, whereas 60 were observed with significantly higher protein amounts in the monocytic state (fold-change cut-off of 2.0). The observed changes at the level of protein affected kinases of nearly all groups of the human protein kinase-family including mitogen-activated protein kinases such as MAPK13, MAP2K1, MAP2K3, MAP2K4, MAP3K7, and MAP3K2, calcium/calmodulin-activated protein kinases e.g., CAMK1, CAMKK1, CAMK2A and CAMK2B, and Src-family kinases (FGR, HCK, SRC, YES; **Figure 1C**, Supplemental Table 1). The SILAC ratios of selected protein kinases with unchanged or differential expression were validated by western blots using independent protein extracts (Supplemental Figure 1).

Within the identified kinome, we mapped and quantified 311 phosphorylation sites (222 phosphoserine, 55 phosphothreonine, and 34 phosphotyrosine sites) in 118 protein kinases (Supplemental Table 1). Importantly, most sites have so far only a comparatively low number of records in which the modification was determined using site-specific methods or by proteomic discovery-mode mass spectrometry indicating that the enrichment strategy increased the sensitivity of detection of modified kinase-derived peptides with mass spectrometry (Hornbeck et al., 2012). For example, at Ser212 of the non-receptor tyrosine kinase Src, we mapped a novel phosphorylation site, located within its SH2-domain suggesting a potential regulatory role in the interaction with tyrosine phosphorylated Src interactors (**Figures 2A,B**). Two hundred twenty-three phosphorylation sites were considered as significantly changed following PMA-mediated differentiation when we applied the same two-fold cut-off as for the differential protein expression. Changes in phosphorylations indicated an overall increased phosphorylation status in macrophage-like cells with 142 phosphosites found increased following differentiation compared to 81 phosphosites found decreased following differentiation (Supplemental Table 1).

To assess whether the observed changes in kinase phosphorylation directly correlated with changes at the kinase expression level, we normalized the SILAC-based peptide-phosphorylation ratios of 245 phosphosites to their corresponding changes in protein expression (**Figure 2C**). More than half of the phosphosites still showed changes of at least two-fold after normalization proving higher or lower phosphorylation that exceeds the changes at the level of protein expression. In detail, 100 phosphosites in 52 kinases appeared as truly upregulated in the macrophage-like state and 38 phosphosites in 28 kinases were found as upregulated

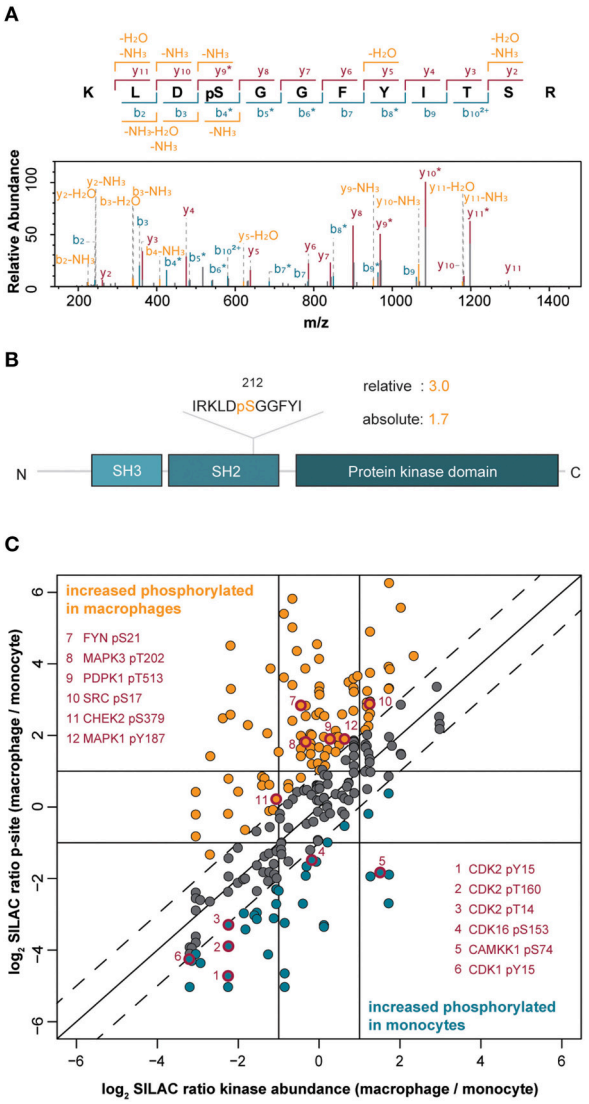

FIGURE 2 | (A) Annotated MS/MS spectrum of the peptide from the tyrosine kinase Src spanning amino acids 209–220 with phosphorylation at Ser212. **(B)** Protein domain organization of Src indicating the pSer212 position in the SH2 domain. The difference in the level of phosphorylation of Ser212 between macrophage-like vs. monocytic THP-1 cells is indicated (relative change) and normalized to changes in the Src protein amount (absolute change). **(C)** Correlation of quantified phosphorylation sites with corresponding protein kinase abundance. The solid diagonal line indicates perfect correlation between changes in the amount of phosphorylations and changes in the corresponding protein kinase amounts, i.e., differences in phosphorylations are only attributed to changes in the protein amounts. The dashed lines indicate two-fold changes of phosphorylations (in both directions), i.e., changes in phosphorylations are not exclusively attributed to changes in protein abundance. Dots highlighted in orange indicate increased phosphorylation in macrophages whereas blue dots indicate increased phosphorylation in monocytes. Red-rimmed dots highlight kinase phosphorylation sites implicated in the induction of kinase activity.

in monocytic THP-1 cells, further supporting a general trend toward a higher phosphorylation status following differentiation (Supplemental Table 1, **Figure 2C**). A comparison with the PhosphoSitePlus database (Hornbeck et al., 2012) revealed a potential functional implication for 21 of the regulated phosphosites (Supplemental Table 1). For example, in the macrophage-like cells phosphorylated Tyr687 in the receptor tyrosine kinase RET (17.3-fold up) recruits the protein-tyrosine phosphatase SHP2 and thereby contributes to activation of the PI3K/AKT pathway (Perrinjaquet et al., 2010), the monitored sites in FYN (Ser21; 9.8-fold), Src (Ser17; 2.5-fold), MAPK3 (ERK1, pT202; 4.6-fold), and MAPK1 (ERK2, pY187; 2.4-fold) are direct inducers of kinase activity (Payne et al., 1991; Schmitt and Stork, 2002; Yeo et al., 2011) suggesting an increased basal activity of these kinases, their upstream modulators as well as associated downstream pathways after cell differentiation (**Figure 2C**). This effect is likely independent of PMA stimulation and indeed relevant for the differentiation status, because PMA was removed and cells were rested in PMA-free media for 1 day before analysis.

In summary, our results show that monocyte to macrophage differentiation is accompanied by major rearrangements of the kinome, at the level of protein kinase expression as well as at the level of kinase phosphorylation.

Identification of Upstream Regulators Associated to Kinome Changes in Monocyte Differentiation

To predict potential upstream regulators implicated in switching the monocyte/macrophage kinome at the level of gene expression, we performed an upstream regulator analysis of the quantified protein kinases with the Ingenuity Pathway Analysis (IPA) software. Twelve transcriptional upstream regulators with partly mutual regulation and overlapping downstream targets could be highlighted that are likely critical in the differentiation process ($p \leq 0.05$, **Figures 3A,B**). Out of these, eight are predicted to be active (z-score ≥ 2) and four as inhibited (z-score ≤ -2). Naturally, PMA used as trigger for THP-1 cell differentiation was found among the top active regulators with a direct correlation to increased amounts of many protein kinases including MAP2K1, IRAK1, SRC, and CDK1. Among the group of transcription factors, MYC and TBX2 were predicted as inhibited with an overlap in the assigned depletion of kinases such as AURKA, AURKB, PLK1, and CDK1. Moreover, the transcriptional regulator TP53 (p53) was assigned to be active and was predicted to be involved in the transcriptional regulation of kinases including repression of DYRK1A (Zhang et al., 2011b), WEE1 (Lezina et al., 2013), PKC-alpha (Zhan et al., 2005), and induction of SGK1 (You et al., 2004). However, since expression of functional p53 has not been demonstrated for THP-1 cells so far (Sugimoto et al., 1992; Durland-Busbice and Reisman, 2002), this finding might be indicative for activation of a transcriptional regulator with a target gene pattern similar to p53. Finally, a potential significant implication for three regulatory micro-RNAs in the process of THP-1 differentiation was found, namely

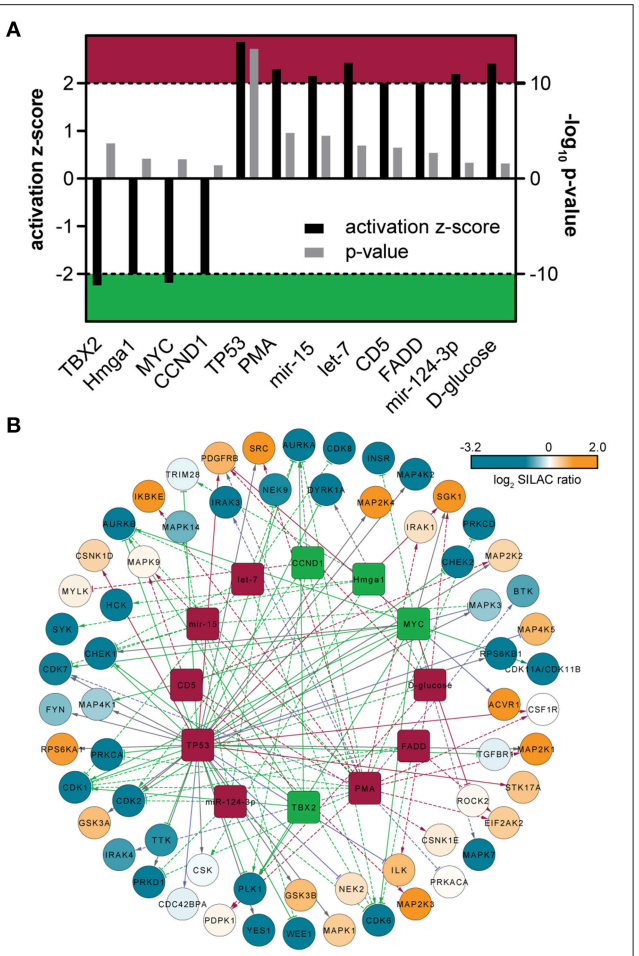

FIGURE 3 | (A) Predicted upstream-regulators implicated in kinase expression changes. Bar chart includes activation z-score and *p*-value derived from IPA. Z-scores ≤ -2 indicate inactivity (green area), whereas z-score ≥ 2 indicate active regulators (red area). **(B)** Upstream-regulator network as predicted by IPA. The outer circle is composed of quantified protein kinases, which are color-coded according to their corresponding SILAC ratios. The inner circle is composed of predicted upstream-regulators that might be involved in the regulation of kinase expression. The upstream regulators are colored according to (A). Solid lines indicate direct molecular interaction, whereas dashed lines indicate indirect molecular interaction. Coloration of lines in orange, blue, gray, and purple indicates the predicted relationship of upstream-regulators with downstream protein kinases as activation, inactivation, no prediction and inconsistency with the state of downstream molecule, respectively.

mir-124-3p, mir-15, and let-7, with the latter two involved in inhibition of transcripts of several cell-cycle-associated kinases including CHECK1, CDK1, CDK6, as well as AURKA, and AURKB.

Most of the suggested transcriptional regulators were not highlighted in a global transcriptome study of THP-1 cell differentiation (Consortium et al., 2009) suggesting a potential more significant relevance for kinase-coding genes that should be followed-up in future studies. However, MYC and p53, e.g., are frequently deregulated in cancer and THP-1 cells are derived from a patient with acute monocytic leukemia (Tsuchiya et al., 1980). Thus, translation of the results to primary

human monocytes has to be done with care. Moreover, besides transcription, protein synthesis rates were recently determined as additional primary drivers for global protein expression changes in differentiating THP-1 cells (Kristensen et al., 2013).

THP-1 Macrophages are Characterized by Kinases that are Related to Calcium/Calmodulin Signaling and Actin Dynamics

Protein kinases increased in the macrophage state point to a prominent role of these kinases in the regulation of macrophage-specific differentiation and function and are predictors of relevant intracellular signaling pathways and downstream effects. We identified LIMK1, TNIK, MERTK, FGR, ACVR1, and STK10 within the top 15 protein kinases with highest protein ratios in macrophages vs. monocytes (12.5-fold to 3.1-fold, Supplemental Figure 1). Remarkably, the majority of these kinases collectively share functional implications in signaling pathways of actin dynamics. LIMK1 is the regulatory kinase of the actin-binding factors cofilin and destrin. The actin-depolymerizing factor (ADF)/cofilin family of proteins is essential for the dynamic changes in the actin cytoskeleton that occur during cell locomotion or other processes including phagocytosis (Arber et al., 1998; Amano et al., 2001; Bierne et al., 2001; Matsui et al., 2002). TRAF2 and NCK-interacting protein kinase TNIK regulates the c-Jun N-terminal kinase pathways, which is an essential and specific activator of Wnt target genes and regulates actin rearrangements and cell spreading (Fu et al., 1999; Taira et al., 2004; Mahmoudi et al., 2009). The tyrosine kinase Mer belongs to the unique family of TAM - Tyro3, Axl, and Mer—receptors which together with their ligands Gas6 and Protein S are essential for the efficient phagocytosis of apoptotic cells and debris and act as pleiotropic inhibitors of the innate inflammatory response to pathogens—an important prevention mechanism of chronic inflammation and autoimmunity (Scott et al., 2001; Rothlin et al., 2007). STK10/LOK has recently been demonstrated to co-localize and phosphorylate ERM (ezrin-radixin-moesin) proteins in lymphocytes, which crosslink actin filaments with plasma membranes, and is thereby important in the regulation of cell shape and migration (Belkina et al., 2009). In addition to the observed increase in protein expression, several phosphorylation sites in the aforementioned kinases showed regulation independent of the changes in protein expression (Supplemental Table 1). Whereas a single upregulated site was identified in TNIK and Fgr, STK10 was found with four up- and three downregulated sites. The upstream kinases or implication of these sites in kinase function, however, is so far unknown.

Collectively, the identified kinases indicate an increased capacity in the regulation of the actin cytoskeleton in macrophages, which is important in the control of immune responses including motility and chemotaxis, phagocytosis, and antigen presentation (May and Machesky, 2001; Van Haastert and Devreotes, 2004; Stradal et al., 2006). Severe immunodeficiency has been linked to mutations that affect cytoskeletal dynamics in macrophages and many macrophage-infecting pathogens manipulate actin remodeling to support their intracellular lifestyle (Linder et al., 2000; Krachler et al., 2011; Roy et al., 2014).

To extend our study, we evaluated the macrophage-specific kinome by performing bioinformatic enrichment analysis including gene ontology (GO) classification and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (Dennis et al., 2003; Supplemental Table 2). This approach naturally revealed the overrepresentation of kinases in individual GO-molecular functions, -biological processes and KEGG pathways. Consequently, we became aware of the significant enrichment of protein kinases (Benjamini-Hochberg corrected $p \leq 0.001$) in terms that are associated with calmodulin-dependent protein kinase activity (GO:0004683) as well as Toll-like receptor and associated MAPK signaling (GO:0000165, hsa04620). The association of our data set to calcium/calmodulin-dependent protein kinase activity is due to the increased expression of CAMK1 (3.3-fold), CAMKK1 (2.8-fold), and CAMK2A/2B (4.9-fold/7.3-fold) when the macrophage-like state is compared to the monocytic precursor. In addition, several phosphosites were detected with changes in calmodulin-dependent protein kinases (Supplemental Table 2). Interestingly, CAMKK2, which was found with decreased expression, showed upregulated phosphorylation at Ser495 and Ser511 (4.8 and 4.0-fold) indicating increased upstream phosphorylation activity, for the latter site likely by the death-associated protein kinase (DAPK) (Schumacher et al., 2004). Components of calcium/calmodulin-dependent protein kinase cascades CAMKs operate in a variety of cellular functions including regulation of transcription activators, cell cycle, hormone production, cell differentiation, actin filament organization, and neurite outgrowth. In macrophages, CAMK1 has recently been demonstrated to be integral to the inflammatory response to sepsis (Zhang et al., 2011a) and CAMK2 has a role in antimicrobial activities via regulation of phagosome maturation (Malik et al., 2001) and is involved in the activation of the NLRP3 inflammasome, leading to cytokine production and the activation of the immune system (Okada et al., 2014). The observed overrepresentation of kinases in Toll-like receptor signaling was mainly based on MAPK cascade kinases [e.g., MAPK13 (12.5-fold), MAP3K7 (3.3-fold), MAP2K4 (2.6-fold), and MAP3K2 (2.3-fold)] reflecting their important role for the transduction of pathogen-associated signals to adequate immune responses in macrophages. The MAPK signaling-associated kinases, however, are shared by many other pathways. Interestingly, whereas the essential upstream kinases for TLR-signaling interleukin 1 receptor associated kinases IRAK 1 and 4 were below our two-fold significance cut-off, the negative regulator IRAK3 is 4.7-fold decreased in macrophage-like cells.

THP-1 Monocytes Show Increased Expression of Kinases Associated with Cell Cycle and DNA Repair

We then addressed which kinases are at higher protein levels in the monocyte state, i.e., before PMA treatment of THP-1 cells. Functional annotation analyses of the monocyte-associated kinome revealed an overrepresentation of kinases within biological processes and molecular functions related to

cyclin-dependent protein kinase activity, cell cycle, MAP kinases and magnesium ion binding (Supplemental Table 2, **Figure 4A**). This implicates a pronounced requirement for protein kinases in the control of the corresponding signaling pathways in the monocytic cell state. In fact, the loss of proliferative activity of terminally differentiated macrophages is a hallmark of THP-1 differentiation (Auwerx, 1991). In our hands, monocyte-to-macrophage differentiation is accompanied by a pronounced decrease in the abundance of key regulatory kinases implicated in entry and progression through different cell cycle phases including the cyclin-dependent kinases CDK1, CDK2, CDK6, AURKA, AURKB, and PLK1 as well as the checkpoint kinases CHEK1 and CHEK2 (Supplemental Table 1, **Figures 4A,B**). In addition to the cell cycle-related kinases, several kinases involved in regulation of DNA repair mechanisms were found in significant higher amounts in the monocytic state, among them the cyclin-dependent kinase CDK9 as well as DNA-dependent protein kinase catalytic subunit PRKDC (Yu et al., 2010; Jiang et al., 2015). Several phosphosites with known functional implications, mainly in enzymatic activity, were found to be regulated in the cell cycle- and DNA repair-associated kinases [CDK2 Tyr15 and Thr160 (5.5-fold and 3.1-fold, respectively, down), CDK9 Ser464 (2.5-fold), CHEK1 Ser286 (2.2-fold) and CDK1 Tyr15 (2.1-fold)]. Phosphorylation of Thr160 in CDK2, e.g., is critical in inducing kinase activity that promotes G1/S transition and progression through S phase following binding to Cyclin E and Cyclin A respectively (Girard et al., 1991; Gu et al., 1992; Ohtsubo et al., 1995).

Our data suggest that the consequent stop of proliferation following PMA-triggered THP-1 monocyte differentiation involves depletion of cell cycle-controlling kinases in the macrophage-like cell in comparison to the monocyte precursor. The decreased relative phosphorylation level in macrophages additionally indicates downregulated upstream signaling that likely advances the proliferation arrest. Since THP-1 cells are derived from a patient with acute monocytic leukemia (Tsuchiya et al., 1980), the increased expression of cell cycle and DNA repair-associated protein kinases can be most likely linked to their oncogenic profile.

Similar to the macrophage-like characteristic kinome, we again observed an overrepresentation of kinases from MAP signaling cascades in the monocytic state, which is based on the higher protein level of a different set of members of this group including TAOK3 and MAP3K1.

THP-1 Differentiation Results in Rewiring of MAPK Signaling Networks

The noticeable overrepresentation of different sets of MAP kinases and upstream MAPK kinases prompted us to a more detailed inspection. Notably, at least 30 kinases directly associated with MAP kinase signaling cascades were identified by our approach and many showed differing amounts in THP-1 monocytes vs. macrophages (**Figure 5**). The diverging protein level of such a high number of MAP kinases and associates signifies the key position of this network in monocyte/macrophage differentiation and function. Differences in protein amounts of at least two-fold were observed for two MAP kinases, two MAP kinase activated protein kinases (MAPKAPKs) as well as 11 upstream MAP kinases spanning MAP4Ks, MAP3Ks, and MAP2Ks. The latter are involved in activation of MAPKs including ERK, p38alpha and JNK or NF-kappaB signaling via phosphorylation of IkappaB kinases (Matsuda et al., 1992; Yang et al., 1997; Wang et al., 2002). The MAPKs MAPK3/ERK1 and MAPK1/ERK2, MAPK8/JNK1, and MAPK9/JNK2 as well as MAPK14/p38alpha were observed at equal protein amounts and in fact only MAPK13/p38delta was found in approximately 12-fold higher amounts in macrophage-like cells, whereas ERK5 was 6.1-fold increased in monocytic THP-1 cells (**Figure 5**, Supplemental Table 1). However, corresponding phosphorylation profiles suggest increased basal activity for ERK1/2 (see above) but also increased activity for ERK-activated ribosomal s6 kinases (RSKs) in macrophages (**Figure 5**). Aside from several upstream MAPK kinases with increased expression levels (GCK, MEKK1, TAOK1/2/3, PAK4, ERK5), the mitogen- and stress-activated protein kinases MSK 1 and 2 were found in pronounced higher levels in unstimulated monocytes suggesting a more prominent function in the monocytic cell state.

Our results indicate that the differentiation of macrophage from their monocytic progenitors is accompanied by extensive rewiring of the MAPK-signaling cascades. Obviously, MAP signaling is not organized as one-way connections from one cell surface receptor via strictly separated kinase cascades to specific responsive elements but forms highly interconnected networks with overlapping hubs. For instance, p38-alpha-related signaling with downstream MSK1/2 seems more prevalent in THP-1 monocytes, whereas ERK/RSK-mediated signaling is likely more associated with the macrophage-like cell state. RSKs are multifunctional ERK effectors that regulate diverse cellular processes via cooperative regulation of many substrates including activation of the transcription factor FOS (Hauge and Frodin, 2006). MSK, on the other hand, is mainly known for its role in gene expression by phosphorylation of transcription factors such as CREB (Hauge and Frodin, 2006). In agreement with our kinase data, FOS and CREB activity is increased and decreased, respectively, following THP-1 differentiation (Consortium et al., 2009). Our data thus shed new light on how an adapted phenotype and altered signal responsiveness following differentiation can be achieved besides changing the expression of cell surface receptors or transcription factors but by fine-tuning the expression and/or activation of intermediate signaling nodes.

MAP3K7 (TAK1) is a Central Signaling Hub in Bacterial Killing, Chemokine Production, and Differentiation Activity

As a result of PMA-induced transition from the monocyte to the macrophage state, several kinases associated with the MAPK signaling network including MAP3K2 and 7 as well as MAP2K1,3 and 4, and MAPK13 were more than two-fold increased. We selected the protein kinase MAP3K7 (Transforming growth factor β-activated kinase, TAK1) for functional analysis based on its up-stream activator position of MAP2K3/4 and MAPK13 as

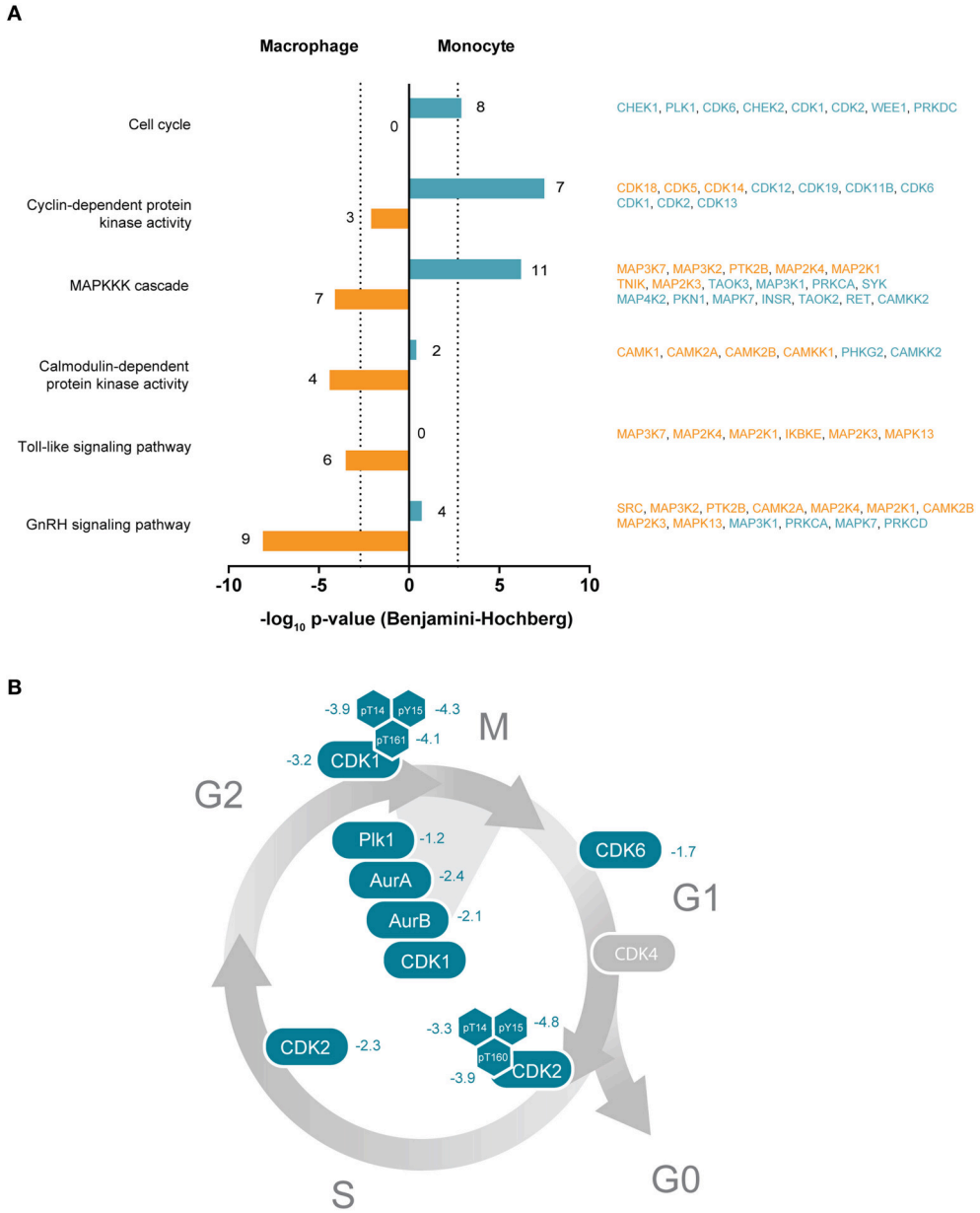

FIGURE 4 | (A) Selected significantly enriched GO terms or KEGG pathways of protein kinases enriched either in THP-1 macrophages or monocytes. Bars indicate Benjamini-Hochberg corrected *p*-values of ≤0.001 (dotted line). Denoted numbers indicate the number of protein kinases assigned to the respective term or pathway. Protein kinase names are provided on the right and colored according to their affiliation to the macrophage-like (orange) or monocytic (blue) cell state. **(B)** Schematic representation of the cell cycle with important protein kinases required for cell cycle progression and mitosis. Numbers indicate determined log_2-SILAC ratios of protein kinases and phosphorylation sites with regulatory function.

well as the possibility to inhibit TAK1 activity by pharmacological intervention. Activation of TAK1 is triggered by various stimuli, including cytokines as well as ligands of Toll-like-, B cell- and T cell receptors, and a key signaling component of NF-κB and MAPK signaling pathways that exerts cell type-specific functions (Ninomiya-Tsuji et al., 1999; Wang et al., 2001; Wan et al., 2006; Schuman et al., 2009).

From our kinomic data, we conclude TAK1 to be more active in macrophage-like cells as indicated by the increased phosphorylation of the down-stream targets MAPK1/3. Moreover, phosphorylation of MAPK1/3 (pT202/pY204) and the additional downstream targets MAPK14 (p38alpha; pT180pY182) and HDAC4 at S264 (Dequiedt et al., 2006) in short-term PMA-stimulated cells was abolished when TAK1 activity was abrogated by pre-treatment with the TAK1-selective inhibitor (5Z)-7-Oxozeaenol (5Z) (Ninomiya-Tsuji et al., 2003; **Figure 6A**).

To address the role of TAK1 in regulation of macrophage-associated functions, we inhibited TAK1 in differentiated THP-1 cells with a one-time treatment of 5Z and screened for effects

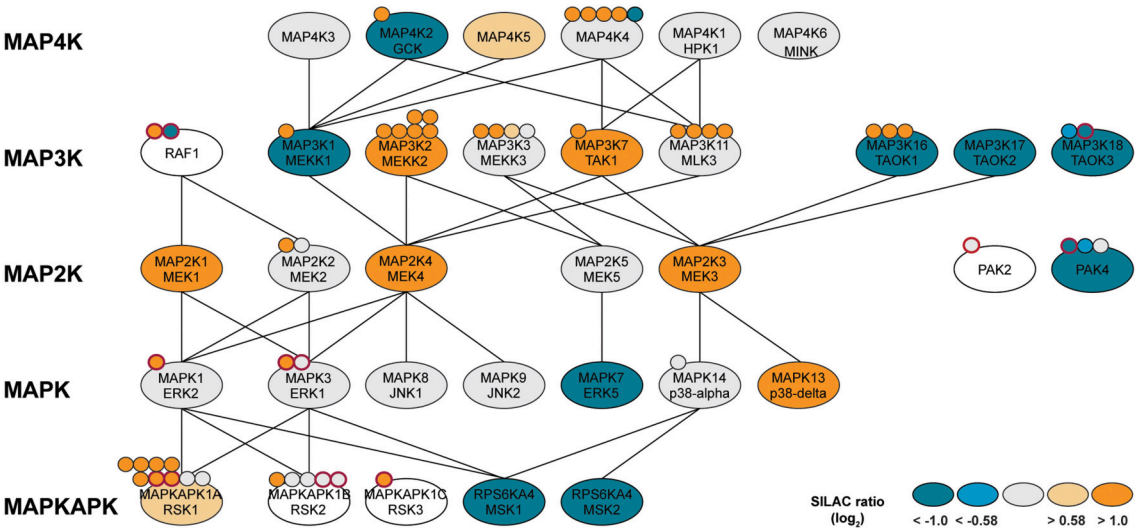

FIGURE 5 | MAP kinase network with identified protein kinases (ellipses) or phosphorylation sites (attached dots). Log_2-SILAC ratios of macrophage-like and monocytic THP-1 cells are provided as color scheme. Phosphorylation sites with known functional implications are highlighted with a red border. Lines indicate kinase-substrate relationships obtained from the literature and online resources.

on the efficacy in bacterial killing as well as the production of chemokines. First, we performed a gentamicin protection assay to compare the number of recovered staphylococci from infected THP-1 macrophages with and without inhibition of the TAK1 activity. Already 15 min. post infection, a prominent decrease in extracted vital *S. aureus* colony forming units derived from TAK1-inhibited THP-1 macrophages became apparent (**Figure 6B**). This finding of decreased surviving bacteria became significant during the course of the infection reaching a maximum of 40% 105 min post infection. Considering that recovery of *S. aureus* cells was performed only from attached THP-1 cells, we counted THP-1 cells present in the supernatant of the infection experiment to exclude that the observed strong drop in intracellular *S. aureus* is due to TAK1 inhibition-mediated increase in macrophage detachment from the cell culture surface (**Figure 6C**). TAK1 inhibition did not lead to significant differences in the number of cells in the suspension compared to non-TAK1-inhibited macrophages. Moreover, flow cytometric analyses with GFP-expressing *S. aureus* revealed a comparable bacterial load independent of TAK1 inhibition (**Figure 6D**) indicating that the efficiency of THP-1 macrophages to internalize bacteria was not altered by TAK1 activity. Collectively, the results of the infection assays thus point to a critical link of TAK1 signaling to phagocytic killing of intracellular microbes.

Next, to characterize the importance of TAK1 activity in chemokine production of macrophages, we pre-screened the secretion of an array of 12 chemokines following interaction with heat-inactivated *S. aureus* using ELISAs (Supplemental Figure 2). For seven chemokines we were able to detect a meaningful signal. While TGF-beta and MDC were secreted approximately in equal amounts in control cells and *S. aureus*-treated cells with and without 5Z, RANTES showed pronounced higher secretion in response to heat-inactivated *S. aureus* but no reduction with accompanying TAK1 inhibition. In contrast, we observed TAK1-dependent secretion of IL-8, MIP-1A, MIP-1B, and GROa which lead us to investigate kinetics of their secretion (**Figure 6E**). Strikingly, inhibition of TAK1 activity abolished the secretion of all four chemokines in response to *S. aureus*. The results thus indicate a pivotal role of TAK1 in the pathogen-induced production and/or release of specific chemokines following macrophage interaction with *S. aureus*.

TAK1 has recently been demonstrated to be essential for osteoclast differentiation (Lamothe et al., 2013). Eventually, we therefore asked if TAK1 has an implication in the differentiation of macrophages in the THP-1 background. For this purpose, monocytic THP-1 cells were one-time treated with 5Z prior to stimulation with PMA and monitored for indicators of differentiation including the ability to adhere to surfaces and to form characteristic morphological features. Inhibition of TAK1 activity in THP-1 monocytes by 5Z prevented typical cell elongation and formation of pseudopodia (**Figure 6F**), significantly reduced cellular adherence efficacy to the surface of cell culture material after PMA stimulation (**Figure 6G**) and caused abrogation of PMA-induced arrest in cell proliferation (Supplemental Figure 3). Cell viability, on the other hand, was not altered by TAK1 inhibition (Supplemental Figure 3).

Taken together, the obtained results highlight TAK1 as a central signaling hub involved in macrophage-associated bacterial killing and pathogen-induced chemokine production and development of a macrophage-like phenotype. Its character as central signaling component is most likely mediated through its well-known role in downstream activation of ERK, JNK, p38, and NF-kappaB signaling pathways (Tang et al., 2008; Lamothe et al., 2012; Mihaly et al., 2014). The observed prevention of PMA-induced phosphorylation of direct and indirect TAK1

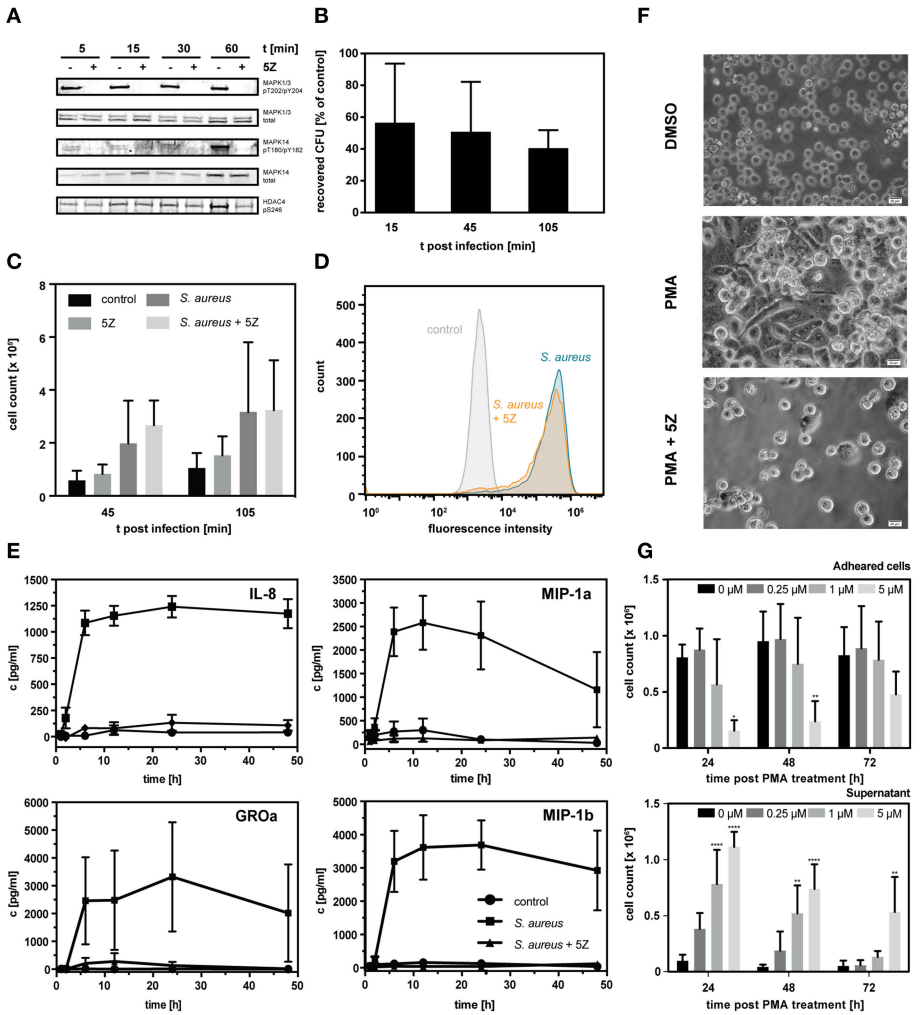

FIGURE 6 | (A) Western blot analysis of known downstream targets of TAK1. THP-1 monocytes were treated with 1 μM 5Z or vehicle for 1 h followed by stimulation with PMA for the indicated time periods. **(B)** Percentage of recovered colony forming units (CFU) of *S. aureus* infected THP-1 cells pretreated with 1 μM 5Z normalized to untreated THP-1 cells. Data is represented as mean ± S.D. ($n = 3$). **(C)** Count of THP-1 cells present in the cell culture supernatant in uninfected controls, following infection with *S. aureus* either pretreated with 1 μM 5Z or not, and uninfected controls pretreated with 1 μM 5Z. Data is represented as mean ± S.D. ($n = 3$). **(D)** Fluorescence distribution of PMA-differentiated adherent THP-1 cells infected with GFP-expressing *S. aureus* either pretreated with 1 μM 5Z or not and uninfected control cells as analyzed by flow cytometry. **(E)** ELISA-based quantification of secreted chemokines IL-8, MIP-1a, MIP-1b, and GROa following interaction with heat-inactivated *S. aureus* either pretreated with 1 μM 5Z or not compared to control THP-1 cells. **(F)** Light micrographs of THP-1 cells 3 days post PMA stimulation either with or without pretreatment with 1 μM 5Z. **(G)** Cell counts of THP-1 cells present either in the cell culture supernatant fraction (upper chart) or adhered fraction (lower chart) monitored over 72 h post PMA treatment in the absence and presence of different concentrations of 5Z. Data is represented as mean ± S.D. ($n = 3$). Statistical analysis of variance was performed by two-way ANOVA with Bonferroni post tests. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

downstream targets and the accompanying differentiation defect in the presence of 5Z implicates a functional cooperation of TAK1 and PKC, because PMA is an inducer of PKC activity which is essential for PMA-triggered THP-1 differentiation (Bazzi and Nelsestuen, 1989; Schwende et al., 1996). A PKC-dependent activation pathway of TAK1 has been described in lymphocytes. B cell or T cell receptor stimulation induces activation of protein kinase C-isoforms, which leads to phosphorylation of CARD11/CARMA1. A complex of CARD11/CARMA1, BCL10, and MALT1 then interacts with TRAF6 ubiquitin ligase, which in turn activates TAK1 via polyubiquitination of the TAK1 protein kinase complex composed of its binding partners, TAB1, TAB2, or TAB3 (Sato et al., 2005; Sommer et al., 2005; Schuman et al., 2009).

We also demonstrated stimulation of chemokines by the human pathogen *S. aureus* with a dependency on TAK1 activity. Our results indicate the activation of TAK1 via toll-like receptors that recognize *S. aureus*-associated molecular patterns. Different from PMA, TLRs activate TAK1 via MyD88 and recruitment of IRAK1 and IRAK4, which in turn activate the TRAF6 ubiquitin ligase leading to TAK1 activation (Shim et al., 2005).TLR2 has been shown to be the key sensor for

recognition of *S. aureus* (Iwaki et al., 2002) suggesting a direct function in TAK1 activation. This, however, has not yet been experimentally demonstrated. Eventually, TAK1 inhibition let to an improved intracellular killing of *S. aureus.* This suggests subcellular alterations that increase bactericidal activity and a potential implication of reactive oxygen species (ROS) such as hydrogen peroxide. Accumulation of ROS in a non-functional TAK1 background has recently been demonstrated in different models including murine keratinocytes and intestinal epithelium as well as cells of the myeloid lineage (Omori et al., 2008, 2012; Wang et al., 2015). Increased ROS levels within phagosomes of TAK1-inhibited THP-1 macrophages could not be demonstrated thus far. From our experimental setup it cannot be excluded that TAK1 plays a similar role in stimulated monocytes. Moreover, since TAK1 is at the crossroad of multiple signaling pathways additional processes in phagosomal maturation or clearance might be affected and need to be considered in future studies addressing the specific role of TAK1-signaling in phagocytic destruction of microbes. Finally, although 5Z is a highly potent TAK1 inhibitor and selective over a set of other kinases within the MAP3K pathway, off-targets cannot be fully excluded (Kilty et al., 2013).

CONCLUSION

Human monocytic THP-1 cells differentiate into macrophage-like cells with increased adherence and loss of proliferative activity by PMA treatment. We found that half of the kinome was altered at the level of protein expression and even 71% of all covered kinase phosphorylation sites were significantly changed at the level of protein phosphorylation showing a massive rearrangement of the macrophage-specific kinome in comparison to its monocytic precursor counterpart. Our analysis of the kinomic data furthermore highlights cell state-specific kinase subsets such as cyclin-dependent kinases associated with cell cycle control at higher levels in THP-1-monocytes and calmodulin-dependent kinases and kinases involved in proinflammatory signaling more expressed in macrophages. Our kinomic approach eventually revealed protein kinase MAP3K7/TAK1 as a master regulator for macrophage-associated functions. Application of functional assays allowed us to identify that TAK1 kinase activity is essentially associated with the PMA-induced arrest in cell proliferation and cellular differentiation. Moreover, we could demonstrate that TAK1 activity is associated with bacterial killing and is essential for the secretion of several chemokines including IL-8 as the primary inducer of chemotaxis in neutrophils and other granulocytes. In conclusion, we suggest the kinome rearrangement and the MAPK rewiring as a hallmark of monocyte-to-macrophage differentiation and we consider protein kinase TAK1 as a key signaling hub and master regulator for macrophage function. For future research applications, we anticipate that focus to TAK1 and its signaling network may result in the understanding of macrophage-associated diseases and may also serve as starting point for novel therapeutic targets.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: FH, ER. Performed the experiments: ER, MH, KV. Analyzed the data: FH, MH, ER, JM. Wrote the paper: FH, ER, JM.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcell.2016.00021

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The Complexity of the ERK/MAP-Kinase Pathway and the Treatment of Melanoma Skin Cancer

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The central role played by the ERK/MAPK pathway downstream of RAS in human neoplasias is best exemplified in the context of melanoma skin cancer. Signaling through the MAPK pathway is crucial for the proliferation of melanocytes, the healthy pigment cells that give rise to melanoma. However, hyper-activation of the MAPK-pathway is found in over 90% of melanomas with approximately 50% of all patients displaying mutations in the kinase BRAF, and approximately 28% of all patients harboring mutations in the MAPK-pathway up-stream regulator NRAS. This finding has led to the development of BRAF and MEK inhibitors whose application in the clinic has shown unprecedented survival responses. Unfortunately the responses to MAPK pathway inhibitors are transient with most patients progressing within a year and a median progression free survival of 7–10 months. The disease progression is due to the development of drug-resistance based on various mechanisms, many of them involving a rewiring of the MAPK pathway. In this article we will review the complexity of MAPK signaling in melanocytic cells as well as the mechanisms of action of different MAPK-pathway inhibitors and their correlation with clinical response. We will reflect on mechanisms of innate and acquired resistance that limit patient's response, with a focus on the MAPK signaling network. Because of the resurgence of antibody-based immune-therapies there is a growing feeling of failure in the targeted therapy camp. However, recent studies have revealed new windows of therapeutic opportunity for melanoma sufferers treated with drugs targeting the MAPK pathway, and these opportunities will be discussed.

Keywords: BRAF, MEK, ERK, melanoma, melanocytes, therapy, resistance, MITF

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THE ERK/MAP-KINASE PATHWAY IS A CRUCIAL REGULATOR OF MELANOCYTE PROLIFERATION AND DIFFERENTIATION

Cutaneous melanoma originates from melanocytes, neural-crest derived pigment-producing cells located in the epidermis, where their major function is to protect keratinocytes from UV-induced DNA damage (Abdel-Malek et al., 2010). Under basal conditions and in response to UV the physiology of a melanocyte is modulated by keratinocytes, which secret specific paracrine acting factors (Hirobe, 2011). These secreted factors stimulate a broad spectrum of intracellular signaling. However, a crucial downstream event triggered by almost all of the extracellular factors is the activation of the ERK/MAP-kinase (MAPK)-pathway, which plays a major role in coordinating the balance between melanocyte differentiation and proliferation (see **Figure 1A**).

Up-regulation of intracellular cAMP levels, which induces the differentiation process (Busca and Ballotti, 2000), triggers a very transient ($\leq$ 60 min) and weak activation of ERK (Wellbrock et al., 2002b). On the other hand, activation of the MAPK-pathway by the synergistic action of factors like SCF, FGF, or HGF stimulates strong sustained ERK activation, which triggers melanocyte proliferation (Bohm et al., 1995).

At the center of this transient vs. sustained ERK activation is MITF (**Figure 1A**), a tissue specific bHLH-Zip transcription factor and fate regulator of the melanocyte lineage, which is a target of ERK phosphorylation (Hemesath et al., 1998; **Figure 1A**). MITF regulates the expression of genes controlling differentiation (e.g., *TYR*) proliferation (e.g., *CDK2*) and survival (e.g., *BCL2*, *BCL2A1*) (Wellbrock and Arozarena, 2015). As ERK phosphorylation can increase MITF's transcriptional activity toward *TYR* (Hemesath et al., 1998), transient ERK activation can favor differentiation, and in the context of cAMP signaling this is paralleled by a strong transcriptional up-regulation of the MITF transcript (Price et al., 1998). However, ERK phosphorylation can also trigger ubiquitin-mediated degradation (Wu et al., 2000), and as a result of sustained ERK activation MITF protein levels are reduced, a situation that is compatible with proliferation (Wellbrock and Marais, 2005). Nevertheless, because MITF is crucial for cell survival, its expression in proliferative cells is ensured through the ERK induced transcription factor BRN2 (Wellbrock et al., 2008). In summary, the MAPK-pathway has stringent control over the melanocyte/melanoma fate-decision regulator MITF, which might explain why this pathway is so particularly critical in the biology of a melanocytic cell and hence in melanoma.

THE DISCOVERY OF THE RELEVANCE OF ERK/MAP-KINASE SIGNALING FOR MELANOMA

Melanoma is not one of the cancers with the highest incidences when compared to breast, lung or colon cancer and therefore historically not much attention was given to the research directed toward a better understanding of this skin cancer. However, this changed dramatically in 2002, when the Cancer Genome Project/Sanger Institute identified oncogenic mutations in the MEK-upstream kinase *BRAF* in over 50% of melanoma (Davies et al., 2002). This discovery led to an explosion in published work on the relevance of the MAPK-pathway in melanoma; as such research into melanoma can be divided in the pre- and post-2002 era.

The development of the MEK inhibitors PD908059 and U0126 in the pre-2002 era resulted in the first studies demonstrating a role for MEK in human melanoma cell proliferation, survival and invasion (Kortylewski et al., 2001; Li et al., 2001). The first indication for an *in vivo* relevance of MAPK signaling in this disease came however from Xiphophorus, a genetically controlled vertebrate model for melanoma first described in 1928 (Wellbrock et al., 2002a). In these animals strong constitutive MAPK activation occurs already in benign nevus-like lesions (Wellbrock and Schartl, 1999), suggesting an involvement of MAPK-signaling in the early steps of pigment-cell transformation (**Figure 1B**). In 2002, Cohen et al. reported constitutive ERK-phosphorylation in >20% of benign nevi and >80% of primary melanoma, and hence confirmed activation of MAPK-signaling as an early event in human melanoma development (Cohen et al., 2002).

Since the first description of *BRAF* mutations in melanoma (Davies et al., 2002) $BRAF^{V600E}$, the most predominant mutant, has been shown to constitutively activate ERK in melanocytes, and to transform *p16/INK4A* deficient melanocytes (Wellbrock et al., 2004). $BRAF^{V600E}$ induces melanoma in mice, where this can be accelerated by the absence of *p16/INK4A* or the PI3K-antagonist *PTEN,* or by UV exposure (Dankort et al., 2009; Dhomen et al., 2009; Viros et al., 2014). In line with what has been observed in humans, in zebrafish $BRAF^{V600E}$ only triggers the formation of benign nevi (Patton et al., 2005). However, in zebrafish mutants where a temperature shift lowers levels of functional MITF (possibly compatible with proliferation), $BRAF^{V600E}$ efficiently induces melanoma (Lister et al., 2014; Zeng et al., 2015). This further emphasizes the relevance of the BRAF/MITF connection for melanoma development.

In humans, $BRAF^{V600E}$ mutations are found in benign nevi (Pollock et al., 2003), clonal populations of senescent melanocytes (Gray-Schopfer et al., 2006). $BRAF^{V600E}$ can stimulate senescence in human melanocytes *in vitro* (Michaloglou et al., 2005). Hence, nevi might represent the result of oncogene-induced senescence. Nevertheless, formation of a nevus requires an initial pulse of melanocyte proliferation, and MAPK signaling appears to be essential for this step. This has been very elegantly shown in zebrafish that develop invasive melanoma induced by mutant RAS, which is however completely abolished when RAS is rendered incapable of activating MAPK signaling (Michailidou et al., 2009). Importantly, these fish do not even develop nevi, clearly demonstrating that no melanocyte proliferation had occurred in the absence of constitutive MAPK signaling, and tumor initiation was completely blocked (Michailidou et al., 2009).

During the last 14 years numerous studies have established the relevance of $BRAF^{V600E}$-induced MAPK signaling for most aspects of human melanoma development and progression; this includes proliferation, survival, hypoxia, invasion, and angiogenesis (Huntington et al., 2004; Karasarides et al., 2004; Gaggioli et al., 2007; Kumar et al., 2007; Klein et al., 2008; Johansson et al., 2009).

THE COMPLEXITY OF ERK/MAP-KINASE SIGNALING IN MELANOMA

The relevance of the MAPK-pathway for melanoma is reflected in the overall rate of mutations leading to deregulation of the pathway. These include not only the ~50% of BRAF mutations, but also >25% *NRAS* mutations and ~14% of melanomas with mutations in the RAS suppressor *NF1* (CancerGenomeAtlasNetwork 2015).

In contrast to *BRAF*, mutations in the other isoforms, *CRAF* and *ARAF* are rare. This is thought to be due to the more

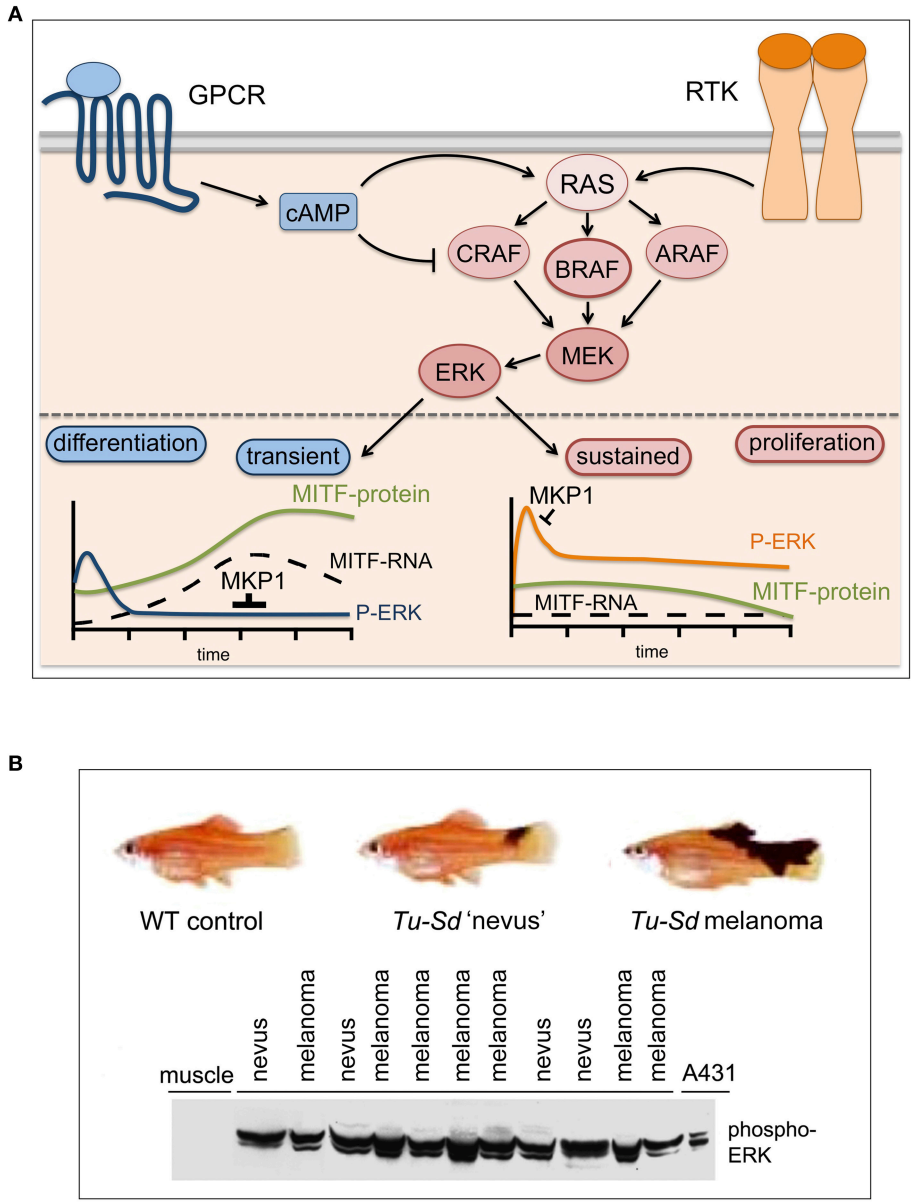

FIGURE 1 | MAPK pathway signaling: transient, sustained, and constitutive. (A) In normal melanocytes the ERK/MAPK pathway is governed by G-protein couple receptor (GPCR) induced cAMP signaling (i.e., MC1R) and receptor tyrosine kinase (RTK) signaling. MC1R signals mainly through BRAF, while CRAF is inhibited (Busca et al., 2000; Dumaz et al., 2006). All receptors lead to ERK activation, but through the differential induction of the ERK phosphatase DUSP1 (MKP1) this results in either transient or sustained ERK activation (Wellbrock et al., 2002b). The melanocyte fate regulator MITF is an ERK target and sustained ERK phosphorylation stimulates its degradation (Wu et al., 2000), which keeps MITF levels low- a state competitive with proliferation. However, cAMP signaling induces MITF expression and in the absence of active ERK this leads to MITF up-regulation, which triggers differentiation. **(B)** In *Xiphophorus* hybrids harboring the macromelanophore locus *Tu-Sd* (Tumor-spotted dorsal pattern) in which melanoma development is driven by the melanocyte specific overexpression of the EGFR homolog Xmrk, ERK is constitutively activated in early benign "nevus"-like lesions and its activation is maintained in malignant melanomas. A phospho-ERK Western blot of lysates (100 μg total protein) from individual fish carrying either nevi ($n = 4$) or malignant melanomas ($n = 7$) is shown. Lysates from A431 cancer cells and from muscle tissue served as positive and negative control respectively (adapted from Wellbrock and Schartl, 1999).

complex mechanisms underlying activation of these isoforms (Emuss et al., 2005). As a consequence activation of ARAF or CRAF would require at least two mutation events, while the BRAF kinase can be rendered active by one mutation event. The majority of these mutations affect the phosphate-binding loop (P-loop) or the activation loop (A-loop) in the kinase domain (Davies et al., 2002). The most common V600E substitution mimics phosphorylation of the A-loop, inducing an

active conformation of the kinase (Wan et al., 2004; Garnett et al., 2005).

Surprisingly, other mutations were found to render BRAF inactive (Davies et al., 2002). The biochemical analysis of these "kinase-impaired" mutations revealed that although they reduce BRAF's enzymatic activity, BRAF still activates MEK through dimerization with CRAF in a RAS dependent manner (Wan et al., 2004; Garnett et al., 2005). Confirming earlier studies (Weber et al., 2001), it is now well established that RAF kinases homo and hetero-dimerize partly in a RAS dependent manner. Importantly, these interactions can impact on the response to inhibitors of BRAF. Thereby, inhibitor-binding triggers dimerization and in the presence of (hyper)-active RAS, instead of pathway-inhibition, this leads to the so called "paradoxical" pathway-activation through CRAF (Wan et al., 2004; Garnett et al., 2005; Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2011). Elucidation of this complex mechanism has proven valuable in the understanding of some of the side effects that BRAF inhibitors produce in patients (see below).

The MAPK-pathway is not linear but part of a complex network containing scaffold proteins and feedback loops. The scaffold protein KSR competes with CRAF for inhibitor-induced BRAF-binding and can counteract the "paradoxical activation" of ERK (McKay et al., 2011), but another scaffold protein, IQGAP promotes ERK activation and the targeted interruption of its interaction with ERK1/2 can contribute to MAPK-pathway inhibition (Jameson et al., 2013). Furthermore, complex feedback loops are induced through the expression of phosphatases (e.g., DUSP6) or adaptor proteins (e.g., SPROUTY) (Hanafusa et al., 2002; Owens and Keyse, 2007). This is also crucial in the context of BRAF inhibition in patients, where these negative feedback mechanisms are relieved with the subsequent up-regulation of other MEK up-stream regulators allowing MAPK pathway activation without BRAF involvement (Lito et al., 2013).

THE DEVELOPMENT OF BRAF, MEK, AND ERK INHIBITORS

First attempts to inhibit $BRAF^{V600E}$ in patients using sorafenib (BAY 43-9006), a broadband kinase inhibitor originally designed to inhibit CRAF, were rather disappointing (Eisen et al., 2006). However, between 2011 and 2014 the FDA and the EMA have approved the use of vemurafenib (PLX4032) and dabrafenib (GSK2118436) for the treatment of BRAF mutant melanoma patients. Both, vemurafenib and dabrafenib bind to the active site in the kinase domain in its "DGF-in" (active) conformation, thereby blocking the access to ATP, and both inhibitors display similar potency for $BRAF^{V600E}$ and CRAF and selectivity against many other kinases (Bollag et al., 2010; Waizenegger et al., 2016). Phase I to III trials using these drugs showed impressive, unprecedented clinical responses in the field of targeted therapies with overall responses of 80%, median progression free survival between 6 and 9 months and median overall survival rates between 13 and 19 months (Flaherty et al., 2010; Chapman et al., 2011; Hauschild et al., 2012; Long et al., 2012; Sosman et al., 2012). Of note, up to 30% of patients treated with BRAF inhibitors develop RAS driven cancers such as squamous cell carcinomas, colon cancer or leukemia (Flaherty et al., 2010; Chapman et al., 2011; Callahan et al., 2012). These "side-effects" are most likely due to the "paradoxical" activation of CRAF in RAF dimers upon inhibitor-binding to wild-type BRAF. As mentioned before, paradoxical activation of CRAF depends on active RAS and is thus favored in cells that signal through RAS (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2011).

In parallel, inhibitors targeting MEK (e.g., selumetinib, trametinib, cobimetinib) have been developed. BRAF's unique effector is MEK, and pre-clinical studies have shown that BRAF mutant cells are significantly more sensitive to MEK inhibition inhibitors than RAS mutant cells (Solit et al., 2006), probably due to RAS activating other pathways such as the PI3K-cascade to promote cell survival (Haass et al., 2008). Despite drug related toxicities limiting the use of MEK inhibitors, recently developed highly potent inhibitors show efficacy in patients (Flaherty et al., 2012; Kirkwood et al., 2012; Ascierto et al., 2013).

Recently, the attention has also moved to ERK and the first ERK inhibitors that are effective in both, BRAF and NRAS mutant as well as cells that have developed resistance to MEK inhibitors have been described (Hatzivassiliou et al., 2012; Morris et al., 2013). Trials testing SCH772984 and GDC-0994 are currently ongoing.

MECHANISMS OF RESISTANCE TO BRAF AND MEK INHIBITORS

Despite the outstanding responses obtained with BRAF inhibitors, in the majority of patients clinical responses are transient. The analysis of melanomas from patients relapsed on BRAF inhibitor treatment revealed the vast complexity of the MAPK signaling network and over the last years a plethora of mechanisms have been identified that allow cells to bypass BRAF inhibition by activating other signaling nodes eventually re-establishing MEK activity and hence reactivation of ERK [for a detailed review see Lito et al. (2013)], which is thought to occur in >70% of patients (Shi et al., 2014; Van Allen et al., 2014).

Some of these mechanisms (**Figure 2**) involve activating *NRAS* mutations or loss of the RAS suppressor *NF1* (Whittaker et al., 2013), *BRAF* amplification or alternative splicing leading to BRAF truncations (Poulikakos et al., 2011; Shi et al., 2012) and overexpression or mutation of the MEK activators CRAF, COT/TPL2/MAP3K8 or MLKs (Montagut et al., 2008; Johannessen et al., 2010; Marusiak et al., 2014). BRAF inhibitor action can also be overcome by mutations in MEK itself, and while some of these mutations increase the basal kinase activity of MEK, others render the kinase insensitive to MEK inhibitors (Emery et al., 2009; Wagle et al., 2011).

Intriguingly, increased receptor tyrosine kinase (RTK) signaling through for instance IGF-1R, PDGFR, or EGFR is also frequently found in relapsed melanomas (Nazarian et al., 2010; Villanueva et al., 2010; Girotti et al., 2013; Sun et al., 2014), and this can lead to ERK activation via classical pathway activation through RAS and CRAF (**Figure 2**). Moreover, RTK signaling

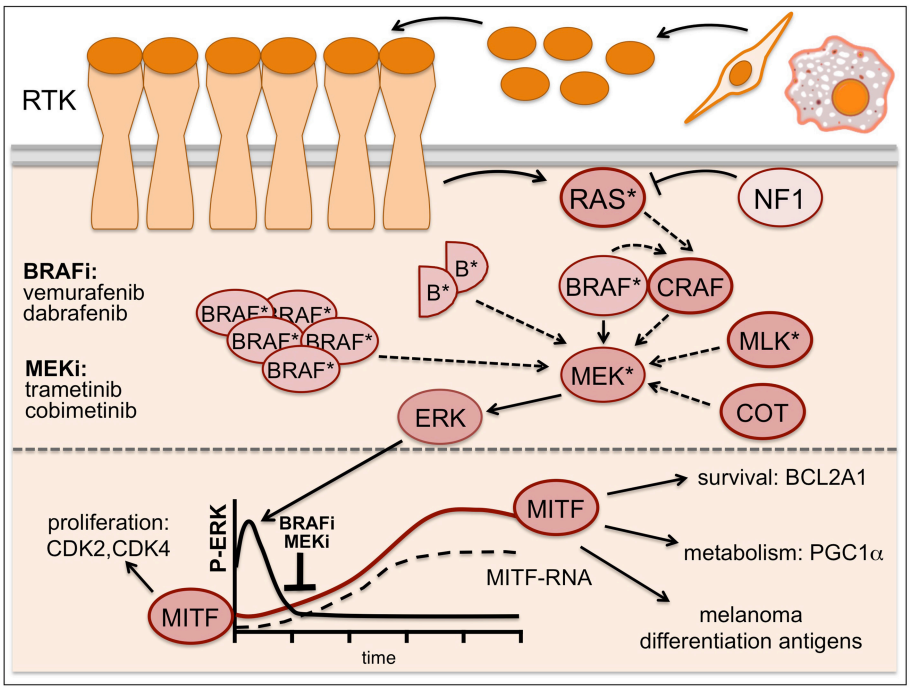

FIGURE 2 | Resistance mechanisms in MAPK-inhibitor treated melanoma. Resistance to BRAF inhibitors can occur through activating *NRAS* mutations (*), loss of the RAS suppressor *NF1*, *BRAF* amplification or alternative splicing leading to BRAF truncations (semicircles B*), overexpression or mutation of the MEK activators CRAF, COT/TPL2/MAP3K8 or MLKs as well as MEK mutations (*). Addition of a MEK inhibitor can buffer some of these resistance mechanisms, but eventually enhanced resistance signaling will overcome its effects. While ERK is inhibited -particularly in the initial phases of treatment- MITF expression is up-regulated and contributes to drug-tolerance. Solid arrows indicate signaling induced by mutant BRAF, dashed arrows indicate signaling brought about by the various resistance mechanisms.

has been linked to an intrinsically BRAF inhibitor resistant phenotype (Konieczkowski et al., 2014; Muller et al., 2014), which was unexpected, as RTK signaling was not perceived as being a major driver of human melanoma. Indeed, it appears that in heterogeneous tumors RTK-signaling melanoma cells are present with lower frequency. However, this balance changes in the presence of a BRAF inhibitor, when RTK-signaling becomes advantageous (Sun et al., 2014). That RTKs in fact can be very potent drivers of melanoma development is seen in Xiphophorus hybrids, where overexpression of an EGFR homolog stimulates proliferation (Wellbrock et al., 1998) and is sufficient to initiate and progress melanoma development (see **Figure 1B**).

Another BRAF/MEK inhibitor resistance mechanism is based on overexpression of pro-survival factors that allow melanoma cells to evade apoptosis even under complete/efficient ERK inhibition. Around 30% of melanomas display amplifications in the *BLC2A1* gene (Haq et al., 2013). Over-expression of the anti-apoptotic BLC2A1 protein blocks BRAF and MEK inhibitor induced apoptosis, and intriguingly, BCL2A1 expression is regulated by MITF (Haq et al., 2013; **Figure 2**). This together with other target genes might underlie the fact that MITF itself can confer resistance to BRAF and MEK inhibitors even when ERK is not re-activated (Smith et al., 2013; Muller et al., 2014). This becomes relevant on the initial phase of treatment, where (in line with low ERK activity being correlated with increased MITF levels; see **Figure 1A**) the majority of patients show significant up-regulation of MITF as early response (**Figure 2**). Importantly, this increased MITF expression can contribute to drug-tolerance in the initial phases of treatment (Smith et al., 2016).

Apart from cell-autonomous resistance, the tumor-stroma can also confer resistance to BRAF inhibitors (**Figure 2**). HGF secreted by stromal fibroblasts can circumvent BRAF inhibition by re-activating ERK through cMET/RAS/CRAF-signaling (Straussman et al., 2012) and stromal fibroblasts can alter the ECM and produce resistance by engaging integrin/FAK signaling (Hirata et al., 2015). Furthermore, tumor associated macrophages can induce resistance via the secretion of VEGF or TNFα (Smith et al., 2014; Wang et al., 2015). Secreted factors can also support the outgrowth of innate resistant cells that are otherwise slow cycling (Obenauf et al., 2015).

The above-described examples reflect the complexity of inhibiting the MAPK-pathway as therapy strategy, because interfering with this central pathway *in vivo* will inevitably have also an effect on non-cancer cells. As a consequence, it can be expected that the entire tumor microenvironment will readjust to the condition of reduced MAPK signaling and establish a new balance that eventually can "buffer" the drug effect.

THE FUTURE OF MAPK-PATHWAY TARGETING DRUGS IN MELANOMA

Resistance through BRAF-inhibitor bypass and the development of RAS-driven secondary cancers in responses to BRAF inhibition have prompted the development of combination therapies with BRAF and MEK inhibitors. These combinations prolong responses and significantly reduce the appearance of RAS-driven secondary malignancies, but unfortunately patients still develop resistance (Larkin et al., 2014; Long et al., 2015). Nevertheless, BRAF/MEK inhibitor combinations are now accepted as the standard of care for BRAF-mutant advanced melanoma and the trametinib/dabrafenib and cobimetinib/vemurafenib combinations received FDA approval in 2014 and 2015, respectively. In addition, drugs targeting both BRAF and CRAF and interfering with dimerization have been described (Girotti et al., 2015; Yao et al., 2015), but whether the use of such inhibitors might increase systemic toxicity will have to be assessed.

Currently, great effort is put into developing novel combination strategies to conquer resistance and prolong responses, and one of the main combination targets for such an approach is the PI3-kinase pathway (PI3-kinase, mTOR, AKT). The reason for this is its central role in melanoma, which is reflected in its frequent deregulation through mutations (CancerGenomeAtlasNetwork 2015), and these are found even more frequently in BRAF/MEK inhibitor resistant tumors (Shi et al., 2014; Van Allen et al., 2014). Furthermore, PI3-kinase signaling is also activated downstream of mutant *NRAS* suggesting possible MEK/PI3-kinase inhibitor combinations. While pre-clinical studies provide strong evidence for the rationale of these combinations, the latest clinical trials show that these combinations are poorly tolerated and toxicity limits efficacy (Bedard et al., 2015; Tolcher et al., 2015). Nevertheless, as the PI3-kinase pathway is central to many cancers the aim is to identify the crucial -and possibly cancer-specific- nodes within the pathway and design more specific and potent inhibitors (Kwong and Davies, 2013).

Other combinations (e.g., with RTK-inhibitors) are currently trialed, and of course it is considered to combine MAPK-pathway inhibitors with immunotherapies. However, the toxicities observed in the first attempts demonstrate that we require a much better understanding of the role of MAPK-signaling in the context of immunity.

CONCLUSIONS

Over the last 5 years the use of MAPK inhibitors in melanoma patients and the development of resistance to these drugs has revealed the vast complexity of MAPK signaling that occurs in a multicellular organism. However, while the ability of the MAPK pathway to rewire has so far played against its inhibition, there might be an opportunity to take advantage of this and target the rewiring. As such, pre-clinical studies support the concept of a drug-holiday, where drugs are administered intermittently to break the rewiring (Das Thakur et al., 2013). Another possibility is to directly target the "rewired phase." In this phase, in which cells display an almost uniform rewiring response and >80% of tumors react with MITF up-regulation, targeting the rewiring-mechanism produces impressive responses in pre-clinical studies (Smith et al., 2016) Thus, with all the excitement about the latest immunotherapy successes, it should not be forgotten that BRAF and MEK inhibitors produce immediate and impressive results and long-lasting (>4 years) responses are also observed in a number of melanoma patients (Puzanov et al., 2015). This clearly demonstrates that there is room for further improvement that will allow building on the remarkable achievements of these targeted therapies.

AUTHOR CONTRIBUTIONS

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p38MAPK and Chemotherapy: We Always Need to Hear Both Sides of the Story

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The p38MAPK signaling pathway was initially described as a stress response mechanism. In fact, during previous decades, it was considered a pathway with little interest in oncology especially in comparison with other MAPKs such as ERK1/2, known to be target of oncogenes like Ras. However, its involvement in apoptotic cell death phenomena makes this signaling pathway more attractive for many cancer research laboratories. This apoptotic role allows to establish a link between p38MAPK and regular chemotherapeutic agents such as Cisplatin or base analogs (Cytarabine, Gemcitabine or 5-Fluorouracil) which are currently used in hospitals across the world. In fact, and more recently, p38MAPK has also been connected with targeted therapies like tyrosine kinase inhibitors (vg. Imatinib, Sorafenib) and, to a lesser extent, with monoclonal antibodies. In addition, the oncogenic or tumor suppressor potential of this signaling pathway has aroused the interest of the scientific community in evaluating p38MAPK as a novel target for cancer therapy. In this review, we will summarize the role of p38MAPK in chemotherapy as well as the potential that p38MAPK inhibition can bring to cancer therapy. All the evidences suggest that p38MAPK could be a double-edged sword and that the search for the most appropriate candidate patients, depending on their pathology and treatment, will lead to a more rational use of this new therapeutic tool.

Keywords: p38MAPK, cancer, chemotherapy, targeted therapy, resistance, sensitivity, p38MAPK inhibitors

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INTRODUCTION

The p38MAPK pathway belongs to the group of stress-activated kinases, composed by p38MAPK (with four isoforms) and JNK (with, at least, 10 isoforms grouped into JNK1, 2, and 3) (reviewed in Kyriakis and Avruch, 2012). This MAPK signaling pathway, initially discovered as a stress response mechanism and as an osmolality sensor (Han et al., 1994), resulted to be implicated in different human pathologies such as rheumatoid arthritis (Clark and Dean, 2012), or neurodegenerative diseases (Corrêa and Eales, 2012) among others. The involvement of p38MAPK in cancer has been widely described (for review see Wagner and Nebreda, 2009). However, its role as an oncogene or tumor suppressor is unclear. In some experimental models, it has been shown to have tumor suppressor properties, for example through the control of oxidative stress (Dolado et al., 2007) while in others, it is clearly associated to survival and oncogenesis (Alvarado-Kristensson et al., 2004; Comes et al., 2007). Indeed, this dual role has been recently demonstrated in colorectal cancer, where it is described as a tumor suppressor in early stages, but in later stages it is required

for cell survival with oncogenic traits (Gupta et al., 2014). In any case, it is clear that, regardless of p38MAPK dual characteristic as a possible oncogene or tumor suppressor, this signaling pathway is implicated in different types of tumors (for review see Koul et al., 2013).

Moreover, p38MAPK has been connected with apoptotic cell death. In fact, key molecules in the apoptotic onset—such as Bcl2 superfamily members or p53—have been shown to be substrate of this MAPK (Sanchez-Prieto et al., 2000; Cai et al., 2006). This observation prompted to consider p38MAPK as a key player in the response to chemotherapy, considering that apoptosis is the main mechanism of cell death associated to it. Therefore, the study of p38MAPK in the last decades was focused in its implication in the response to chemotherapy. In the following pages, we will show some examples of how p38MAPK can modulate the response to several chemotherapeutic agents that are currently used in the clinical practicum. This includes conventional chemotherapeutic agents and novel therapeutic approaches like tyrosine kinase inhibitors or monoclonal antibodies. Although all the members of the p38MAPK family have been related to cancer and its therapy in different experimental models (Pillaire et al., 2000; Cerezo-Guisado et al., 2011; Hou et al., 2012; Del Reino et al., 2014; O'Callaghan et al., 2015; Zur et al., 2015), in this paper we will focus onto p38MAPKα as the most studied and representative member of the family.

p38MAPK AND CISPLATIN

Cisplatin (CDDP) is one of the most widely used drugs in cancer therapy and probably the best example of the connection between p38MAPK and conventional chemotherapy (Brozovic and Osmak, 2007). p38MAPK was related to the cellular response to CDDP through the c-Abl signaling pathway (Pandey et al., 1996). Moreover, p38MAPK mediates activation of p53 in response to CDDP suggesting a role in resistance (Sanchez-Prieto et al., 2000). In fact, this molecule can be activated by other platinum-based compounds even without toxicity as in the case of Trans-platin (Hernández Losa et al., 2003). However, the relationship between CDDP and p38MAPK has two sides: one is related to resistance and the other, to sensitivity. Initially, the role as a determinant of resistance was proposed mainly based on the inhibition of p38MAPK in different experimental models (Mansouri et al., 2003; Brozovic et al., 2004; Baldwin et al., 2006). Indeed, several substrates of p38MAPK seem to be implicated in resistance to CDDP such as $p18^{Hamlet}$ (Cuadrado et al., 2007) or ATF3 (St Germain et al., 2010). Furthermore, hyperactivation of p38MAPK through MKK3, that renders a non-functional pathway, has been correlated with resistance (Galan-Moya et al., 2011). However, recent evidences demonstrated a sensitizing role for the inhibition of p38MAPK both *in vivo* and *in vitro* through the production of Reactive Oxygen Species, which promotes the activation of the JNK pathway and thus sensitizing human tumor cells to CDDP-associated apoptosis (Pereira et al., 2013). In this regard, it has been proposed that certain p38MAPK downstream molecules (Hsp27, ERCC1, or Fox3a) can mediate sensitivity associated to p38MAPK inhibition (Planchard et al., 2012; Germani et al., 2014; Liu et al., 2016).In addition, inhibition of p38MAPK could also facilitate sensitivity in specific contexts as in the case of the presence of the adenoviral protein E1A (Cimas et al., 2015). Nonetheless, new platinum-based compounds have been developed and, some of them, for instance, Satraplatin or Picoplatin are in clinical use, (Doshi et al., 2012; Hamilton and Olszewski, 2013), but there is no clue about the role of p38MAPK. Only in the case of Monoplatin, a non-DNA binding platinum-based compound only used in cell culture so far, cell-type specific activation of p38MAPK has been demonstrated, but with no effect in terms of resistance/sensitivity (García-Cano et al., 2015). In conclusion, the dual role of p38MAPK as a mechanism of resistance/sensitivity to CDDP could be related to specific features such as cell type, downstream molecules or other signaling pathways.

p38MAPK AND CYTARABINE

Cytarabine -also known as ara-C-, a deoxycytidine analog, is an antileukemic agent that incorporates into DNA promoting strand breaks (Fram and Kufe, 1982; Major et al., 1982). Cytarabine promotes both cell death and differentiation in leukemia cells (Grant et al., 1996). It has been demonstrated that Cytarabine induces apoptosis through p38MAPK and JNK in a c-Abl dependent fashion (Saleem et al., 1995; Pandey et al., 1996). In this sense, it has been suggested that Cytarabine-induced apoptosis can be blocked by the specific inhibition of p38MAPK in HL-60 cells, (Stadheim et al., 2000). Moreover, in chronic myeloid leukemia cells, the constitutive activation of p38MAPK by BCR/Abl renders a Cytarabine-insensitive phenotype (Sánchez-Arévalo Lobo et al., 2005), suggesting a role for p38MAPK in the resistance to Cytarabine. Interestingly, a study in acute myeloid patients treated with Cytarabine and Daunorubicin showed that active p38MAPK and JNK correlate with cell death in chemosensitive patients (Maha et al., 2009). Therefore, most of the evidences support that the lack of functionality in p38MAPK could mediate a resistant phenotype to Cytarabine.

p38MAPK AND GEMCITABINE

Gemcitabine is a deoxycytidine analog, widely used for treating different carcinomas such as pancreatic, bladder, breast and non-small cell lung cancer (Gesto et al., 2012). Cell death associated to Gemcitabine has been related to the p38MAPK pathway (Nakashima et al., 2011; Liu et al., 2014). Indeed, a study performed in human urothelial carcinoma sub-lines with acquired Gemcitabine resistance showed a marked repression in p38MAPK activity and an increase in gemcitabine sensitivity when expression of p38MAPK was forced (Kao et al., 2014). It has also been described that Gemcitabine induces phosphorylation of p38MAPK substrates like Hsp27 that could be mediating acquired resistance in pancreatic cancer cell lines (Kang et al., 2015). In addition, there are evidences showing how the p38MAPK/MK2 stress response pathway is required for the

cytotoxic effect of Gemcitabine in osteosarcoma and pancreatic cancer cells (Köpper et al., 2013, 2014). However, the use of p38MAPK as a putative biomarker for the response to Gemcitabine is still unexplored and, in the few studies performed so far, results are disappointing as in the case of platinum resistant recurrent ovarian cancer (Klotz et al., 2008). Therefore, all the evidences support a definitive role for p38MAPK and different p38MAPK substrates as key players in Gemcitabine response, in which blockage of p38MAPK seems to be a key mechanism of resistance that still needs to be more investigated for future clinical use.

p38MAPK AND 5-FLUOROURACIL

5-Fluorouracil (5FU) irreversibly inhibits thymidylate synthase. It is widely used in the treatment of solid tumors such as breast, colorectal, stomach, pancreatic, oesophageal and skin cancers (Longley et al., 2003). The implication of the p38MAPK signaling pathway in the response to 5FU has been studied since late 90's (Wu et al., 1998). 5FU-associated cell death works through the induction of p53-dependent apoptosis (Mariadason et al., 2003). The role of p38MAPK in terms of resistance or sensitivity is still not clear. On the one hand, a report demonstrated that p38MAPK inhibition renders a blockage of p53-dependent apoptosis allowing an autophagic response that mediates resistance (de la Cruz-Morcillo et al., 2012). On the other hand, other reports support that the inhibition of p38MAPK and the subsequent effect onto Hsp27 can promote sensitivity to this drug (Yang et al., 2011; Matsunaga et al., 2014). In this regard, the high dose of the inhibitor used, i.e., 50 μM SB203580, and the lack of a genetic approach suggest that the inhibition of other molecules, in addition to p38MAPK, could be implicated. Finally, the antiangiogenic properties through the induction of Thrombospondin-1, the mucositis and the release of pro-inflammatory cytokines associated to 5FU are also mediated by p38MAPK (Elsea et al., 2008; Zhao et al., 2008; Gao et al., 2014). In summary, most of the evidences support a role for p38MAPK in 5FU-based therapy in terms of therapeutic response, as well as in other aspects, that need to be fully elucidated for its clinical applications.

p38MAPK AND TARGETED THERAPY

The idea of a targeted therapy has become a gold standard in cancer therapy, being Imatinib (Gleevec, STI571), a specific inhibitor of BCR/Abl (Druker et al., 1996), its first example. Later, it was shown how the activation of p38MAPK was directly implicated in the survival of KT-1 cells in response to Imatinib (Parmar et al., 2004). Almost at the same time, it was also demonstrated how p38MAPK activation was a key event in the differentiation effect of Imatinib in K562 cells, but with no effect onto cell viability (Kohmura et al., 2004) probably due to the lack of effect of p38MAPK onto caspase activation (Jacquel et al., 2007). Indeed, in a resistant model by continuous co-culturing with this drug, p38MAPK showed a lack of implication in the acquired resistance phenotype (Aceves-Luquero et al., 2009). In fact, it has been proposed that the connection between Abl and p38MAPK is not related to the tyrosine kinase activity of Abl (Galan-Moya et al., 2008), indicating that the activation of p38MAPK in the presence of Imatinib could be a secondary event, rather than a direct activation by this compound. Nonetheless, it has been shown how p38MAPK could be implicated in the response to second generation of BCR/Abl inhibitors as in the case of Dasatinib (Dumka et al., 2009) with a direct implication in some of the side effects like hepatotoxicity (Yang et al., 2015). However, the role of p38MAPK in the response to other BCR/Abl inhibitor like Nilotinib remains unclear and has merely been investigated as side studies in other experimental models as sarcoma or colorectal cancer derived cell lines (Villar et al., 2012; Rey et al., 2015) or in combination with other drugs (Bucur et al., 2013).

But the role of p38MAPK is not restricted to the response to BCR/Abl inhibitors. For example, the multikinase inhibitor Sorafenib (BAY 43-9006), originally described as a Raf inhibitor, used in the treatment of several pathologies such as hepatocarcinoma or renal cell carcinoma among others (Wilhelm et al., 2008), has been also related to p38MAPK. In this sense, it has been shown how the activation of p38MAPK can be a mechanism of resistance and a novel biomarker for Sorafenib-based therapy in hepatocellular carcinoma (Rudalska et al., 2014). Indeed, it has been recently proposed how the combination with mTOR inhibitors can potentiate the effect of Sorafenib in malignant pleural mesothelioma through p38MAPK-dependent apoptosis (Pignochino et al., 2015). Finally, in the case of an EGFR inhibitor, known as Iressa (Genfitinib), early evidences demonstrate a lack of effect on the p38MAPK signaling pathway in different experimental models (Höpfner et al., 2003; Kokubo et al., 2005). However, in certain leukemic and intestinal epithelial cells, p38MAPK activation was observed (Moon et al., 2007; Sheng et al., 2007), but no definitive effect in terms of resistance/sensitivity has been reported. Nonetheless, the role of p38MAPK in Iressa-based therapy could be related to the combination with other compounds. For example, in the case of curcumin, p38MAPK attenuates the adverse gastrointestinal effects (Lee et al., 2011), or, in combination with metformin, p38MAPK inhibition can potentiate the effect of Iressa (Ko et al., 2013). Therefore, the full role of p38MAPK in Iressa-based therapy still needs to be elucidated.

Finally, another targeted therapy modality is the use of monoclonal antibodies (Dienstmann et al., 2012; Henricks et al., 2015). In this sense, the literature regarding p38MAPK is much less abundant. For example, for Trastuzumab, an antibody against Her2/neu, the few studies where p38MAPK is considered support a direct role for this MAPK in resistance to this antibody (Yong et al., 2013; Donnelly et al., 2014). In the case of EGFR-directed antibody Cetuximab, the main connection with p38MAPK has been established in combination with Oxaliplatin (Santoro et al., 2015), in which Cetuximab blocks Oxaliplatin-triggered p38-dependent apoptosis, explaining the lack of success for this combination in some patients of colorectal cancer. Finally, in the case of Bevacizumab, a monoclonal antibody against VEGF, there is no evidence linking directly MAPK to this

TABLE 1 | p38MAPK inhibitors used in ongoing clinical trials in which p38MAPK is used as a target in cancer therapy.

Inhibitor	Pathology	Combination (if Available)	Trial ID	Date	Phase
Ralimetinib (LY2228820 dimesylate)	Metastatic breast cancer (MBC)	Tamoxifen	NCT02322853	2014	II
LY2228820	Recurrent Ovarian Cancer	–	NCT01663857	2012	I/II
LY3007113	Advanced Cancer (Either Solid Tumors or Lymphomas)	–	NCT01463631	2011	I
LY2228820	Advanced Cancer (Either Solid Tumors or Lymphomas)	–	NCT01393990	2011	I
LY2228820	Glioblastoma	TMZ and Radiotherapy	NCT02364206	2015	I/II
ARRY-614	Myelodysplastic Syndrome	–	NCT00916227	2009	I
ARRY-614	Myelodysplastic Syndrome	–	NCT01496495	2011	I

Source: https://clinicaltrials.gov/ct2/results?term=p38+inhibitor+cancer&Search=Search (as accessed in April 2016).

antibody and only as a side study in mesenchymal stem cells, melanoma and pancreatic carcinoma cells s (De Luca et al., 2012; Jiang et al., 2012).

FUTURE DIRECTIONS: p38MAPK INHIBITORS

The development of new and more specific inhibitors is a critical step in a future therapy based on p38MAPK inhibition. Several pathologies are considered susceptible for this strategy. In the case of chronic obstructive pulmonary disease (COPD), the use of p38MAPK inhibitor PH-797804 (Selness et al., 2011) turned out to have preliminary positive results in healthy volunteers and in patients (MacNee et al., 2013; Singh et al., 2015). In addition, the p38MAPK inhibitor GW856553 (Losmapimod) succeeded to ameliorate exacerbations in patients suffering COPD with low eosinophil levels (Marks-Konczalik et al., 2015). Not only does Losmapimod bear a therapeutic potential for COPD, but also it has been tested against coronary artery diseases such as acute myocardial infarction through its anti-inflammatory activity (O'Donoghue et al., 2015). This specific p38MAPK inhibitor has become an alternative treatment to several disorders derived from acute coronary syndrome (thoroughly reviewed in Kragholm et al., 2015). Many other diseases—atherosclerosis, Alzheimer's disease, depression, immunological diseases and so on—are sought to be treated by the use of p38MAPK inhibitors (Supplementary Material 1). However, although in some pathologies, namely rheumatoid arthritis, the inhibition was considered as a promising therapeutic approach (McLay et al., 2001) clinical results are disappointing (Genovese et al., 2011), thus suggesting the complexity of the biological function for p38MAPK.

Regarding cancer therapy, p38MAPK inhibition by itself is also considered a promising target (Igea and Nebreda, 2015). In fact, in some cases like in colorectal cancer, experimental evidences support this novel therapeutic approach (Gupta et al., 2015), and it has also been reported recently that p38MAPK inhibition overcomes the resistance to compounds like Birinapant in primary acute myeloid leukemia (Lalaoui et al., 2016). Ongoing clinical trials are showing the safety of the p38MAPK inhibitors as in the case of Ralimetinib (Patnaik et al., 2016) or ARRY-614 (Garcia-Manero et al., 2015). Currently, there are 59 clinical trials in different stages, including healthy volunteers in phase I studies, in which p38MAPK is evaluated as a potential biomarker or target for different diseases such as arthritis or heart and pulmonary disease among others[1]. From this wide range, 14 of them are related to cancer and only in 7 of these ongoing clinical trials, p38MAPK is regarded as a target in cancer treatment, by using specific inhibitors alone or in combination with other therapeutic agents (**Table 1**). Therefore, it seems logical to look for adequate candidates that might be benefited with the use of p38MAPK inhibitors. The mutation ratio in p38MAPK or MAP2K3/6 in human cancer is extremely low[2] (i.e., Pritchard and Hayward, 2013), suggesting that the implication of an active p38MAPK in cancer is due to the pathologic context of the tumor rather than a genetic alteration in the MAPK or MAP2K able to render a constitutive pathway. One possibility could be the evaluation of the expression / activity levels of the different components of this signaling pathway by immunohistochemistry, as it has been shown for different pathologies, allowing us to choose the best candidate patient for a p38MAPK inhibition-based therapy. It is important to be as careful as possible due to the dual role of this signaling pathway in cancer. For example, MAP2K3 (MKK3) has been proposed as a tumor suppressor in breast cancer (MacNeil et al., 2014) thus suggesting that the inhibition of p38MAPK signaling pathway could be counterproductive in this type of tumors. However, recent evidences show how inhibition of this same molecule is a novel therapeutic approach in different experimental models (Baldari et al., 2015). Therefore, the antitumoural activity associated to p38MAPK could also be used in combination with current therapies in order to potentiate its effects, increasing the number of putative patients that can be benefited from the use of p38MAPK inhibitors. Nonetheless, we should not discard the potential side effects of p38MAPK inhibition as a mechanism of drug resistance as well as the loss of its tumor suppressor potential.

To sum up, the use of p38MAPK as a potential target for cancer therapy should be carefully considered based on the pathology and the therapy used in order to avoid adverse effects, as could be the generation of resistances or more aggressive phenotypes. The deep understanding of the role of p38MAPK in cancer therapy can lead to a new era of better prognosis and

[1]https://clinicaltrials.gov/
[2]http://cbioportal.com

personalized therapy in which p38MAPK inhibitors could be a cornerstone.

AUTHOR CONTRIBUTIONS

JG contributed in the Introduction and in the "Future Directions" section. He intervened in editing tasks and in gathering information for Supplementary Material 1 and **Table 1** and thoroughly proofread the manuscript for its submission. OR collaborated in the composition of "p38MAPK and Cytarabine" section, provided proofreading comments and helped in the edition. FC collaborated in the composition of "p38MAPK and Cisplatin" section, provided proofreading comments and helped in the edition. RP collaborated in the composition of "p38MAPK and Gemcitabine and 5-Fluorouracil" sections. MO collaborated in the composition of "Targeted Therapies" section. DF collaborated in the composition of Supplementary Material 1. RS designed and coordinated the whole review, collaborated composing all the sections, and thoroughly proofread the manuscript for its submission.

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SUPPLEMENTARY MATERIAL

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Gain-of-Function Mutations in the Toll-Like Receptor Pathway: TPL2-Mediated ERK1/ERK2 MAPK Activation, a Path to Tumorigenesis in Lymphoid Neoplasms?

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Lymphoid neoplasms form a family of cancers affecting B-cells, T-cells, and NK cells. The Toll-Like Receptor (TLR) signaling adapter molecule MYD88 is the most frequently mutated gene in these neoplasms. This signaling adaptor relays signals from TLRs to downstream effector pathways such as the Nuclear Factor kappa B (NFκB) and Mitogen Activated Protein Kinase (MAPK) pathways to regulate innate immune responses. Gain-of-function mutations such as MYD88[L265P] activate downstream signaling pathways in absence of cognate ligands for TLRs, resulting in increased cellular proliferation and survival. This article reports an analysis of non-synonymous somatic mutations found in the TLR signaling network in lymphoid neoplasms. In accordance with previous reports, mutations map to MYD88 pro-inflammatory signaling and not TRIF-mediated Type I IFN production. Interestingly, the analysis of somatic mutations found downstream of the core TLR-signaling network uncovered a strong association with the ERK1/2 MAPK cascade. In support of this analysis, heterologous expression of MYD88[L265P] in HEK293 cells led to ERK1/2 MAPK phosphorylation in addition to NFκB activation. Moreover, this activation is dependent on the protein kinase Tumor Promoting Locus 2 (TPL2), activated downstream of the IKK complex. Activation of ERK1/2 would then lead to activation, amongst others, of MYC and hnRNPA1, two proteins previously shown to contribute to tumor formation in lymphoid neoplasms. Taken together, this analysis suggests that TLR-mediated ERK1/2 activation via TPL2 may be a novel path to tumorigenesis. Therefore, the hypothesis proposed is that inhibition of ERK1/2 MAPK activation would prevent tumor growth downstream of MYD88[L265]. It will be interesting to test whether pharmacological inhibitors of this pathway show efficacy in primary tumor cells derived from hematologic malignancies such as Waldenstrom's Macroglobulinemia, where the majority of the cells carry the MYD88[L265P] mutation.

Keywords: MYD88, blood cancer, hematologic malignancies, NFκB, lymphocytes, B cells

OVERVIEW: THE TOLL-LIKE RECEPTOR (TLR) SIGNALING ADAPTER MYD88 IS THE MOST FREQUENTLY MUTATED GENE IN LYMPHOID NEOPLASMS

The adven Next Generation Sequencing has made available rich data sources to better understand biological processes. In the field of cancer, it is now possible to obtain a better idea of the mutations landscape of specific types of tumors. Analysis of this information can yield a greater insight into the pathogenesis of specific disorders. In this Hypothesis and Theory article, we have mined the Catalog Of Somatic Mutations In Cancer (COSMIC) to better understand the process of cellular transformation of lymphocytes (lymphoid neoplasms). We focused on the Toll-Like Receptor (TLR) signaling pathway as previous evidences had highlighted an important role in B cell transformation for MYD88, one of its main downstream signaling adaptor.

MYD88 is mutated in 22% of lymphoid tumor samples according to the COSMIC database (Sanger institute, UK; Forbes et al., 2014) (**Table 1**). This critical signaling adaptor normally relays signals from TLRs to downstream effector pathways such as the Nuclear Factor kappa B (NFκB) and Mitogen Activated Protein Kinase (MAPK) pathways to regulate innate immune responses (Kawai and Akira, 2010). Gain-of-function mutations such as MYD88[L265P] activate downstream signaling pathways in absence of cognate ligands for TLRs, resulting in increased cellular proliferation, and survival (Yang et al., 2013; Ansell et al., 2014; Avbelj et al., 2014). The medical and scientific literature investigating MYD88 role in cancer focuses on NFκB activation (Treon et al., 2012; Yang et al., 2013). However, TLR-mediated activation of MYD88 also leads to the activation of other signaling pathways such as ERK1/2, p38 MAPK, and JNK (**Figure 1**). The contribution of these other effector signaling pathways to tumor formation in the context of TLR-activation has been largely overlooked and deserves closer attention. In order to formulate a testable hypothesis on the identity of the TLR effector pathway(s) driving the tumorigenic process, we first investigated the frequency of mutations of TLR signaling components in lymphoid neoplasms.

ANALYSIS OF SOMATIC MUTATIONS FOUND IN THE TLR SIGNALING NETWORK IN LYMPHOID NEOPLASM

Overview of the TLR Signaling Network

The TLR family has 10 members in humans (Carpenter and O'Neill, 2009). Following dimerization, TLRs bind different adaptor molecules through their Toll/IL-1 receptor domain (TIR). The family can be sub-divided between receptors signaling through the adaptors MYD88 (TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8, and TLR9) or TRIF (TLR3 and TLR4). MYD88 mediates the sequential recruitment of IL-1R-associated protein kinases (IRAK) (Muzio et al., 1997; Wesche et al., 1997), TNF-receptor-associated factor 6 (TRAF6) (Cao et al., 1996) and TGF-β Activated Kinase (TAK1) (Ninomiya-Tsuji et al., 1999; Lee et al., 2000). This signaling cascade leads to the activation of four major signaling pathways: the NFκB pathway and the three MAPK pathways, ERK1/2, JNK, and p38 MAPK (**Figure 1**). The TRIF adaptor, which can be recruited directly to TLR3, or indirectly via TRAM to TLR4, lead to the production of type I interferons via the activation of the IKK family members IKKε and TBK1 and the phosphorylation of interferon response factors (IRFs) (Yamamoto et al., 2002). TRIF can also mediate NFκB activation via Pellino 1 and RIP1 (Meylan et al., 2004; Chang et al., 2009).

Non-silent Somatic Mutations in the TLR Pathway

Based on the current literature a TLR signaling network containing 79 distinct molecules involved in MYD88-signaling was assembled. A proposed network organization of these molecules in TLR signaling is illustrated in **Supplementary Figure 1** (Oda and Kitano, 2006; Padwal et al., 2014). Each of these molecules was investigated for the presence of non-synonymous somatic mutations in lymphoid neoplasms using data extracted from the COSMIC database (**Table 1**) (Forbes et al., 2014). The search parameters used were: Tissue selection [Haematopoietic and Lymphoid tissue]; Subtissue selection [Include All]; Histology Selection [Lymphoid neoplasm]; subhistology selection [Include All]. The search results were updated to the most recent values at the end January 2016. To determine the more likely pathway members contributing to tumorigenesis, a mutation frequency threshold was set based on a study looking for oncogenic driver mutations in Chronic Lymphocytic Leukemia (CLL) (Wang et al., 2011). In that study, 88 Tumor samples were studied by exome and whole genome sequencing. Oncogenic drivers were defined as genes having mutation rates significantly higher than the background taking into account sequence composition. The gene identified with the lowest mutation frequency deemed significant was ERK2 (MAPK1). In the gene set investigated in the current analysis, ERK2 was found mutated 4 times out of 1508 samples (**Table 1**). Therefore, as mutations for ERK2 were deemed significant oncogenic drivers in CLL and that the role of the ERK1/2 MAPK pathway in tumor growth of B cells is well established (Platanias, 2003; Rickert, 2013), the threshold of significance in this analysis was set at 0.25%.

Distribution of Mutations in the TLR Signaling Network

The TLR network architecture takes the shape of an hourglass, with multiple TLRs feeding into a core signaling module (MYD88-TRAF6-TAK1), before re-expansion downstream of the activation of TAK1 (**Figure 1**). When examining mutations identified in the TLR-network above the 0.25% threshold (**Table 1**), 2 are found in the upper part of the hourglass network (TLR2, and TLR5; 0.9% overall mutation frequency), 6 in the core network (MYD88, A20, ciap2, CYLD, TRAF3, and Pellino 2; 33.4% overall mutation frequency) and 7 in the downstream-activated signaling pathways (CBP, MYC, MKK1, IKKβ, hnRNPA1, MKK2, and ERK2; 13.2% mutation rate). Mutations that lead to activation of the upper part of the TLR network (TLR + core signaling components) account

TABLE 1 | Mutation frequency of TLR-signaling network components in lymphoid neoplasms.

Id.	Alt Id	No of mutations	Samples tested	% of mutations	Most common non-silent mutation(s)
MYD88	MYD88	1754	7854	22.3	1584[L265P]
A20	TNFAIP3	214	3073	7.0	25[whole gene del]
CBP	CREBBP	174	2605	6.7	12[R1446H]
cMyc	MYC	40	1500	2.7	6[F138S]
ciap2	BIRC3	110	4173	2.6	12 [whole gene del]
MKK1	MAP2K1	40	1694	2.4	4[C121S]
TLR2	TLR2	8	1415	0.6	4[D327V]
CYLD	CYLD	10	1832	0.5	6[whole gene del]
TRAF3	TRAF3	8	1673	0.5	2[whole gene del]
IKKβ	IKBKB	8	1727	0.5	8[K171E]
TLR5	TLR5	5	1415	0.4	2[N96K]
PELI2	PELI2	5	1415	0.4	2[R154W]
hnRNPA1	HNRNPA1	4	1496	0.3	[S22T]; [E9K]; [T138S]
ERK2	MAPK1	4	1508	0.3	[D162N];[D291G];[R124H];[Y316F]
MKK2	MAP2K2	4	1589	0.3	2[Q60P]
ciap1	BIRC2	3	1415	0.2	
TLR4	TLR4	3	1415	0.2	
TLR8	TLR8	3	1415	0.2	
MKK4	MAP2K4	3	1460	0.2	
TLR6	TLR6	2	1415	0.1	
pellino3	PELI3	2	1415	0.1	
TAB3	TAB3	2	1415	0.1	
ABIN1	TNIP1	2	1415	0.1	
HOIP	RNF31	2	1415	0.1	
SMAD6	SMAD6	2	1415	0.1	
TTP	ZFP36	2	1415	0.1	
TAB2	TAB2	2	1415	0.1	
TLR1	TLR1	2	1415	0.1	
ERK1	MAPK3	2	1423	0.1	
IRAK4	IRAK4	2	1423	0.1	
TPL2	MAP3K8	2	1423	0.1	
JNK2	MAPK9	2	1423	0.1	
MNK2	MKNK2	2	1423	0.1	
IRAK1	IRAK1	2	1508	0.1	

Genes with a mutation frequency >0.25 are in Yellow (upper and core TLR network) or Green (downstream effector pathways).
List of genes analyzed with: 1 mutation: TOLLIP, ATF2, CREB, SHARPIN, ECSIT, ELK1, MSK1, TBK1, TLR7, TAX1BP1, TAK1, MKK7, JNK1, p38α, IKKα, MSK2, IKKε, MAPKAPK2, PELI1.
No mutation: ABIN2, TIRAP, TRAF6, TAB1, p105, MKK6, JNK3, p38β, ERK5, NEMO, IκBα, IκBβ, NFκB(p65), cJun, cFOS, eiF4E, HSP27, UBC13, Uev1a, TANK, IRAKM, TIFA, SCF-βTRCP, HOIL1, hnRNPA0, MKK3, OTUL.

for more than a third of mutations found in lymphoid neoplasms. This assumes that all these mutations only target TLR-signaling, which is unlikely to be the case as regulators of multiple pathways such as CYLD and A20 would impact other pathways like the TNFR-activated pathway (Kovalenko et al., 2003; Trompouki et al., 2003; Shembade et al., 2010) or BAFF-R signaling in the case of ciap2 (Gardam et al., 2011). But even when removing these components from the equation more than a quarter of the mutations would be hypothesize to favor enhanced signaling of the global TLR signaling network. Looking at molecules assigned to effectors of TLR signaling (bottom part of the hourglass) a striking observation is that all of these molecules can be linked to ERK1/2 MAPK signaling (**Figure 1**). As was the case for ciap2, CYLD, and A20, these downstream effectors are not specific to TLR signaling and are targets of many other pathways. Nevertheless, they provide clues as the likely arm of TLR-signaling contributing to tumorigenesis.

TLRs-Linked to MYD88 Are More Frequently Mutated than Those Linked to TRIF

In the context of cancer biology, TLR-mediated activation of MYD88 pro-inflammatory signaling is associated with tumor formation and growth, whereas activation of the Type I IFN via

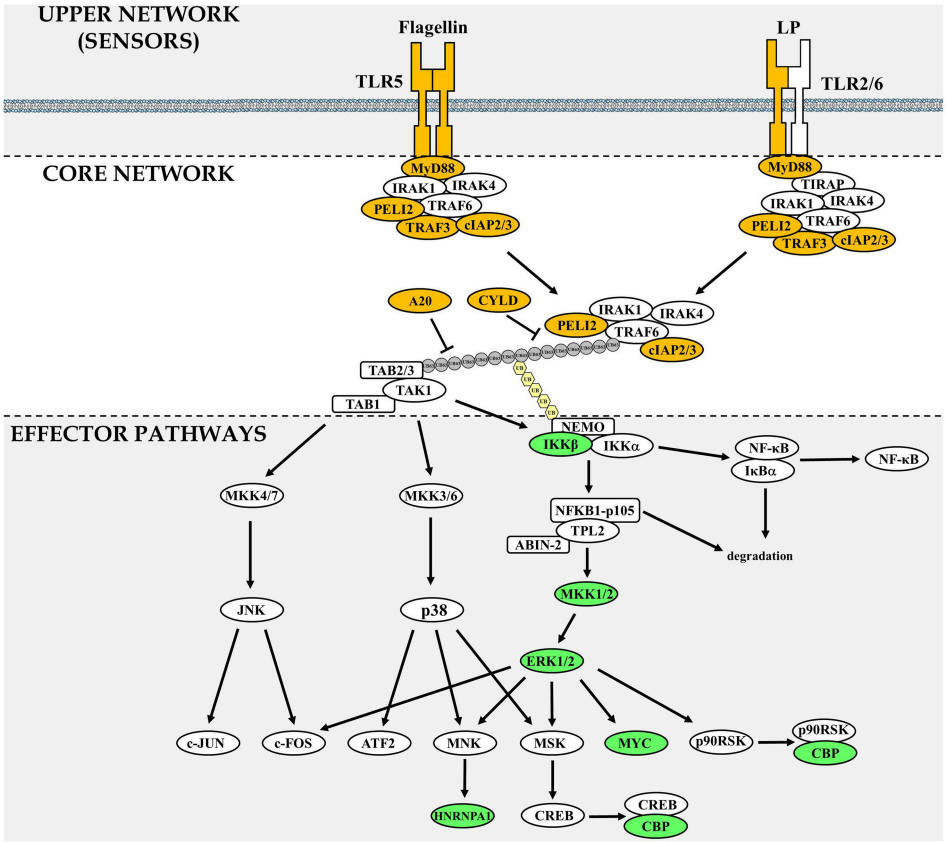

FIGURE 1 | Components of the TLR signaling network frequently mutated in lymphoid neoplasms. The TLR signaling network is divided in three sections: upper network, which comprises sensors, core network that is common to MYD88-dependent signaling and downstream effector pathways. Gene products that are found frequently mutated in lymphoid neoplasms are highlighted in yellow (upper and core network) or green (downstream signaling effector pathways). In the core network, the gray circles represent ubiquitin chains linked via Lysine 63 and the pale yellow hexagons represent linear ubiquitin chains. A pathway comprising all 77 network components investigated can be found in **Supplementary Figure 1**.

the TRIF adaptor has been associated with anti-tumor immunity (Lin and Karin, 2007). In accordance with those data, not only is MYD88 the most frequently mutated gene in lymphoid neoplasms, but the TLRs harboring non-silent mutations above the arbitrary threshold are those linked with MYD88 activation (TLR2 and TLR5). Interestingly, these mutations map to the extracellular domains of the TLRs and can be proposed to favor dimerization. In contrast, no non-silent mutations are reported in either TRIF, TRAM or TLR3, involved in type I IFN signaling. TLR4 leads to activation of both MYD88 and TRIF-dependent pathways. It would be interesting to check whether any of the three mutations in TLR4 favors MYD88 signaling to the detriment of TRIF signaling. These mutations could promote association with MYD88 or signaling at the cell surface instead of the endosome via decreased interaction with TRAF3 (Tseng et al., 2010). Two of the reported TRAF3 mutations are whole gene deletions and another two are premature stop codon that can be hypothesize to favor MYD88-dependent signaling as shown with RNA interference against TRAF3 (Tseng et al., 2010).

MYD88[L265P]: One Mutation to Rule Them All

MYD88 is the most frequently mutated gene in lymphoid neoplasm, all genes taken into account (**Table 1**). Moreover, one mutation dominates: the nucleotide substitution L265P. This mutant is present in 86–98% of patients with Waldenstrom's Macroglobulinemia (WM) (Treon et al., 2012; Jiménez et al., 2013; Poulain et al., 2013). However, it is not restricted to WM, with 29% of activated B-cell-like diffuse large B-cell lymphoma (ABC-DLBCL) harboring the L265P mutation, a subtype particularly difficult to cure. Overexpression of MYD88[L265P] is linked with increase cell survival and NFκB signaling (Yang et al., 2013). The mutant MYD88 activates downstream signaling via allosteric TIR-domain oligomerization (Avbelj et al., 2014) that activates TRAF6, IRAK1, and TAK1 (Ansell et al., 2014). Myddosome formation (oligomerization of MYD88 and subsequent recruitment of accessories signaling protein) is a key event linking TLR-dimerization upon ligand binding to activation of effector pathways. Therefore, if TLR-signaling contributes to lymphocyte tumorigenesis, it makes

sense that one of the first step in the activation the signaling cascade is the most frequently mutated event in lymphoid neoplasms.

CYLD and A20: Negative Regulator of MYD88-Dependent Signaling Have Frequent Gene Deletions

CYLD and A20 act as negative regulators of TLR and TNFR signaling. CYLD and TNFAIP3 are found frequently deleted in lymphoid neoplasms, which contrasts with the presence of single nucleotide substitutions more frequently found in positive regulators of MYD88 signaling (**Table 1**). Loss of these negative regulators would prolonged TLR-signaling and contribute to tumor growth and survival. It is important to note that it is difficult to determine whether the contribution of CYLD and A20 to tumor growth acts through TLR, TNFR, both TLR and TNFR or other pathways.

Mutation Analysis of the TLR Downstream Effector Pathways Points to Activation of ERK1/2 as a Target of TLR-Driven Lymphocyte Transformation

TLR-signaling leads to the activation of four major effector pathways: the NFκB pathway and the three MAPK pathways, ERK1/2, JNK, and p38 MAPK. The process of TLR-mediated tumorigenesis may implicate all or some of these pathways. Individually, each of these pathways have been linked to cancer, and the discussion of these roles is beyond the scope of this article. In the context of TLR-signaling, emphasis has been put on NFκB activation, for both historical and technical reasons. Nevertheless, the MAPK pathways may be important contributor to tumorigenesis.

Interestingly, all of the seven genes identified carrying non-silent mutations above the 0.25% threshold that are part of the TLR effector pathways can be linked to the ERK1/2 MAPK pathway (**Figure 1**). This suggests that the ERK1/2 pathway is a path to tumorigenesis in TLR-driven lymphoid neoplasms. This does not rule out a role for the JNK, p38 MAPK, or NFκB, but simply mutations within these pathways are not selected during clonal selection of tumor cells. Therefore, the analysis of mutations found in the TLR network leads to the formulation of the following hypothesis: "Inhibition of ERK1/2 MAPK activation would impair lymphocytes transformation dependent on MYD88-activation."

ACTIVATION OF ERK1/2 A CRITICAL COMPONENT OF THE TRANSFORMATION PROCESS IN LYMPHOID NEOPLASMS?

Constitutive ERK activity is a hallmark of many B-cell malignancies (Platanias, 2003), consistent with the findings of the mutational analysis presented in Section 'Analysis of Somatic Mutations Found in the TLR Signaling Network in Lymphoid Neoplasm' and a whole genome sequencing study in CLL (Wang et al., 2011). Surprisingly, the upstream signals regulating ERK activation in B-cells are poorly understood, as reported in a recent article (Rickert, 2013).

The Protein Kinase Tumor Promoting Locus 2 (TPL2) Is a Key Activator of ERK1/2 Downstream of the IKK Complex

Activation of ERK1/2 can occur downstream of the classical RAS-RAF-MKK1/2 pathway in response to growth factor activation (Macdonald et al., 1993). However, ERK1/2 can also be activated by another signaling pathway via the activation of the Tumor Promoting Locus 2 (TPL2, also known as MAP3K8 or COT) protein kinase (Dumitru et al., 2000). Activation of TPL2 requires phosphorylation and degradation of NFκB1 p105 by the IκB Kinase (IKK) complex (Beinke et al., 2004). Once activated TPL2 phosphorylates MKK1/2, direct upstream activators of ERK1/2. This pathway is well established as essential to ERK1/2 activation following TLR activation (Dumitru et al., 2000; Beinke et al., 2004; Banerjee et al., 2006; Rousseau et al., 2008; Martel et al., 2013). It is important to re-emphasize that following activation of TLR-signaling, ERK1/2 activation will occur in parallel to NFκB via a shared upstream activator. This means that experimental data obtained with IKKβ inhibitors where the results were solely assigned to NFκB activity may have overlooked an important contribution of the ERK1/2 MAPK cascade. Interestingly, the IKKβ [K171E] mutant identified in some lymphoid neoplasms has greater activity toward NFκB activation but has not been tested for its capacity to activate ERK1/2 (Kai et al., 2014). Based on the critical role of IKKβ in activating TPL2, it is interesting to speculate that this mutant will also lead to greater ERK1/2 activity.

MYD88[L265P] Leads to ERK1/2 Activation via TPL2 in a Heterologous Expression System

Heterologous expression of MYD88[L265P] in HEK293 cells, not only activates the NFκB pathway (**Figure 2A**), but also the ERK1/2 MAPK but only in the presence of TPL2 exogenous expression (**Figure 2B**). This activation can be blocked with an inhibitor of TPL2 (**Figure 2B**). Consistent with previously published data on NFκB activation, MYD88[L265P] leads to ERK1/2 phosphorylation in a TAK1 (MAP3K7) and MKK1/2-dependent fashion (**Figure 2B**).

MKK1/2 Hot Spot for Resistance to RAF and MEK (MKK) Inhibitors

MEK inhibitors have been of great interest as novel anti-cancer agents. MEK162, showed improvement in progression-free survival of patients with metastatic melanoma (Flaherty et al., 2012) and is currently in phase II clinical trials for the treatment of myeloid leukemia. The MKK1[C121S] mutation leads to greater kinase activity and confers resistance to RAF and MEK inhibitors (Wagle et al., 2011). Similarly, the MKK2[Q60P] was found in tumor cells with sustained MAPK activation and resistance to BRAF and MEK inhibitors (Villanueva et al., 2013). Therefore, these mutations are not only oncogenic drivers but of

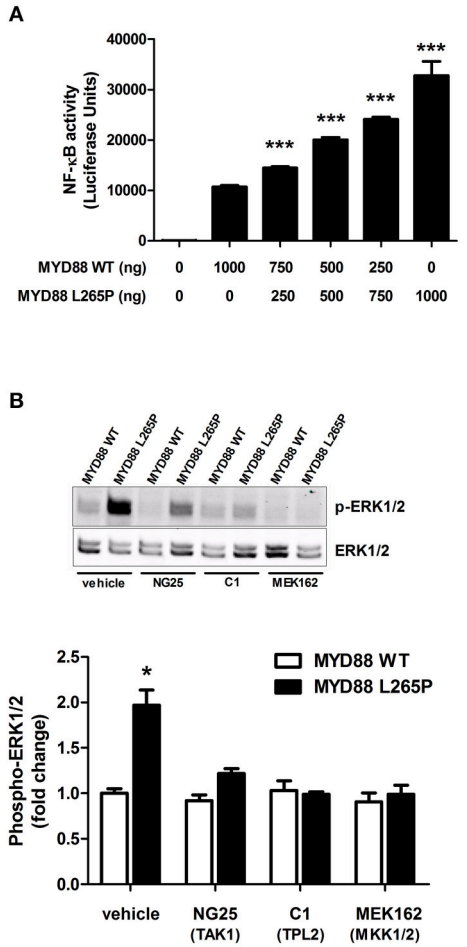

FIGURE 2 | TPL2-mediates the MYD88[L265P] ERK MAPK activation in HEK 293 cells. (A) Cells were grown to confluence, lysed with Promega's reporter buffer and subjected to luminescence analysis as previously described. ***$p < 0.005$ vs. Myd88WT. All values are expressed as fold ± S.E.M. from four different experiments. **(B)** Cells were serum starved overnight and left untreated or pre-treated for 1 h with vehicle, NG25 (TAK1 inhibitor, 10 μM), C1 (TPL2 inhibitor, 2 μM), or MEK162 (MKK1/2 inhibitor, 1 μM). ERK1/2 phosphorylation was determined by immunoblotting. *$p < 0.05$ vs. to Myd88WT and Myd88WT/L265P treated cells.

important concern in considering treatment options due to their role in resistance to therapy.

The Complex Role of ERK1/2 Signaling in Cellular Transformation

Oncogenic ERK1/2 activation leads to its translocation to the nucleus and induction of transcription factor linked with proliferation such as FOS, JUN, and MYC (**Figure 1**). However, the role of ERK in tumorigenesis is not as simple as MYC-driven cell proliferation. It is highly context dependent and reflected by the fact that ERK1/2 activation is also linked with growth arrest and differentiation in both normal and transformed cells. Sustained ERK1/2 activation but not its transient activity was linked with PC12 cell differentiation (Traverse et al., 1992). Moreover, PMA-stimulation of the K562 human leukemia cell line leads to growth arrest and differentiation in a ERK1/2-dependent manner (Herrera et al., 1998). Interestingly, acute ERK1/2 hyper-activation in tumors by the oncogenic BRAF[V600E] mutant leads to tumor cell senescence (Serrano et al., 1997; Michaloglou et al., 2005). This phenomenon is not the result of ERK1/2 hyperactivity but the induction of negative signaling feedback mechanisms acting as tumor suppressors (Courtois-Cox et al., 2006). Therefore, the capacity of cells to tolerate high level of ERK1/2 activity without inducing senescence requires other transformation events, such as the loss of negative feedback regulators. Supporting this notion, acute activation of oncogenic signals in pre-B cells leads to the majority of cells dying with only a fraction progressing to malignant transformation (Shojaee et al., 2015).

In addition to its role in promoting cell proliferation when escaping senescence, ERK1/2 MAPK activation is also linked with increased cell survival and resistance to treatment like their upstream activators MKK1/2. In hairy-cell leukemia, sustained ERK1/2 activation promotes cell survival (Kamiguti et al., 2003). Sustained BCR-signaling that prolong ERK1/2 and AKT(PKB) signaling, increases the expression of the antiapoptotic protein myeloid cell leukemia-1 (Mcl-1), promoting cell survival in CLL (Petlickovski et al., 2005). Similarly, CXCR4 somatic mutations frequently found in WM lead to sustained ERK1/2 and AKT activation linked with resistance to the BTK inhibitor Ibrutinib (Cao et al., 2015).

Although no experimental data is currently available for the four ERK2 mutations identified in this analysis, it is interesting to note that the tumor samples in which they have been identified did not have mutations in MYD88 or other TLR-related signaling network component. Coexistence of MYD88 and ERK2 mutations would have undermined the theory that one of the main outcome of MYD88 activation in tumor formation is increase ERK1/2 activity.

MYC, CBP and hnRNPA1, Downstream Targets of the ERK1/2 Pathway with Potential Link with Tumorigenesis

The ERK1/2 MAPKs are Key Regulators of MYC Expression and Function

Activation of MYC is associated with increased proliferation, more aggressive disease and poorer outcomes in Richter's Transformation, a transformation of CLL into a clonally related aggressive Diffuse Large B Cell Lymphoma (DLBCL) (Scandurra et al., 2010). B-cell Receptor (BCR)-mediated expression of MYC is regulated by ERK1/2-mediated phosphorylation of the transcription factor ELK-1 (Yasuda et al., 2008). Moreover, it has long been established that phosphorylation of MYC at Ser62 by ERK1/2 promotes cellular transformation (Pulverer et al., 1994). Therefore, the ERK1/2 MAPKs are involved at two levels of MYC regulation contributing in both cases to its transformation potential. Mutations in MYC, which also include chromosomal rearrangements, lead to increase activity that may render it independent of upstream signaling regulation. Therefore, pharmacological strategies targeting upstream MYC signaling in cells harboring MYC activation are hypothesized to

be less effective than treatments inducing increase cell death via antagonizing for example BCL-2 that cooperates with MYC in transformation of pre-B cells (Vaux et al., 1988).

Loss of CBP Function Increases ERK1/2 Tumorigenic Potential

CBP act as a transcriptional co-activator for numerous transcription factors, including many oncogenes. In addition, it can also act as an histone acetylase with p300 (another gene frequently mutated in cancer) to dynamically regulate gene expression. It is tempting to hypothesize that based on its interaction with TFs known to be oncogenes, CBP promotes tumor formation. However, most evidences point to CBP as a tumor suppressor. This has been clearly demonstrated in mice, where CBP heterozygotes developed hematologic malignancies that were associated with the loss of the second CBP allele (Rebel et al., 2002). Moreover, patients suffering from Rubinstein-Taybi Syndrome, associated with mutations and deletions in CBP, have an increased risk for leukemia (Jonas et al., 1978).

Interestingly, during persistent ERK1/2 activation, CBP associates with MAPKAP-K1 (p90RSK) to promote growth arrest and cell differentiation (Wang et al., 2003). Accordingly, in the analysis presented in this paper, CBP is found frequently deleted, which could be a permissive event in presence of persistent ERK1/2 signaling to promote cellular growth instead of arrest. This idea is supported by a study investigating relapsed acute lymphoblastic leukemia (ALL). Non-synonymous somatic mutations in CBP were found in conjunction with RAS signaling pathway mutations within the same tumor samples (Mullighan et al., 2011). The CBP mutations identified impaired histone acetylation and transcriptional activity of CBP. These results not only support the idea that loss of CBP activity enhances ERK1/2-mediated transformation, but lend further strength to the idea presented above about the importance of secondary events impairing high or persistent ERK1/2 activation-induced senescence.

hnRNPA1 Expression and Phosphorylation Occurs Downstream of ERK1/2 Activation

In T lymphocytes, hnRNPA1, a TNFα AU-rich element binding protein (Rousseau et al., 2002) is phosphorylated by MNK1, a protein kinase activated by ERK1/2 and p38 MAPK (Buxadé et al., 2005). Phosphorylation of hnRNPA1 contributes to post-transcriptional regulation of TNFα (Buxadé et al., 2005). Interestingly, recent evidences have highlighted roles for genes involved in post-transcriptional regulation, such as mRNA splicing, as oncogenic driver in CLL (Wang et al., 2011). Moreover, MYC has been shown to increase hnRNPA1 expression leading in turn to higher expressing of pyruvate kinase, contributing to aerobic glycolysis frequently observed in tumor cells (David et al., 2010). In support for a role of hnRNPA1 in lymphoid neoplasms, aberrant expression of hnRNPA1 was described in acute leukemia (Choi et al., 2014). Therefore, increased expression of hnRNPA1 driven by MYC and its phosphorylation by MNK1 (two ERK1/2-dependent events), or its aberrant expression resulting from gain-of-function mutations, would increase pro-inflammatory gene synthesis and aerobic glycolysis to contribute to tumorigenesis.

CONCLUSIONS

TLR-MYD88-IKKβ-TPL2-MKK1/2-ERK1/2, a Key Path Cell to Transformation?

Previously published experimental data have established the important contribution of MYD88-mediated signaling in a number of B-cell malignancies (Ngo et al., 2011; Treon et al., 2012; Wang et al., 2014). This is further supported by the mutational analysis presented in this hypothesis and theory article. Moreover, by looking at the frequency of gene mutations downstream of MYD88 activation, the ERK1/2 MAPK pathway is highlighted as a potential key effector pathway of tumorigenesis via activation of downstream targets such MYC and hnRNPA1. The overall outcome of this TLR-mediated signaling would be at least two fold: (1) increased in cell proliferation via MYC activation and anaerobic glycolysis (2) enhanced pro-inflammatory signaling, in particular the expression of TNFα, which would contribute to promote changes in the tumor microenvironment favoring tumor growth.

Limitations of the Analysis

There are a number of limitations to the current analysis. First, for a number of mutations identified, no experimental data is available to support their role in modifying TLR signaling. Moreover, these mutations were mostly considered in isolation from each other. But as discussed in the ERK1/2 and CBP sections, tumor samples have multiple mutations that may act together in order to promote tumorigenesis. Furthermore, lymphoid neoplasms are numerous, affecting T and B lymphocytes at different stages of their differentiation. Some factors play more important roles in one disease over the other, such as MYD88[L265P] in WM, or in pre-B cells vs. mature B cells (Rickert, 2013). Finally, mutations may have different roles in the tumor microenvironment vs. cells grown in tissue culture. The tumor microenvironment is likely playing an important role in understanding the breadth of impact that inflammation has on tumorigenesis, particularly related to TLR-mediated pro-inflammatory signaling.

Testing the Hypothesis in Waldenstrom's Macroglobulinemia

A number of hypothesis have been formulated along the way, but the overarching one would be that inhibition of ERK1/2 MAPK activation would prevent tumor growth downstream of MYD88[L265]. In the context of MYD88 driven tumorigenesis, a particularly attractive target would be the protein kinase TPL2, which should abrogate excess ERK signaling. Pharmacological inhibitors have been developed that target TPL2 as shown in **Figure 2B**, and it would be highly interesting to test these in a more physiologically relevant setting than an heterologous expression system. Since WM tumor cells have an overwhelming presence of MYD88[L265P] mutations, they represent an excellent model to test the hypothesis put forth in this article.

EXPERIMENTAL PROCEDURES

Materials

The TAK1 inhibitor NG25 and the TPL2 inhibitor Compound 1 were kindly provided by Professor Sir Philip Cohen (MRC PPU, University of Dundee, UK). The MKK1/2 inhibitor MEK162 (Binimetinib) was purchased from Selleck Chemicals (Houston, TX, USA).

ERK1/2 Immunoblotting

100,000 HEK293TLR5 cells (Invivogen, San Diego, USA, #HKB-HTLR5) were seeded in a 24-wells plate and transfected with 200 ng of pCDNA3.1-TPL2 and 800 ng of pCDNA3.1-Myd88 or the empty vector for 48 h using polyethylenimine (PEI). Cells were grown to confluence, lysed and lysates were subjected to SDS-PAGE. Quantitative analysis graph was obtained with the signal intensity of an antibody recognizing the phosphorylated forms of ERK1/2 at Thr202/Tyr204 normalized to the signal obtained with antibody that recognizes all forms of ERK1/2.

NFκB Luciferase Assay

Cells were transfected with 200 ng of pGL4.28-NF-κB and a combination of varying amounts of pCDNA3.1-Myd88 WT and/or pCDNA3.1-Myd88 L265P for a total of 1000 ng of DNA per transfection. Cells were grown to confluence, lysed with Promega's reporter buffer and subjected to luminescence analysis.

AUTHOR CONTRIBUTIONS

SR and GM have made substantial contributions to the conception, design, acquisition, analysis and interpretation of data for the work. SR has drafted the work and revised it critically for intellectual content. SR and GM have approved the final version to be published and agreed to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcell.2016.00050

Supplementary Figure 1 | TLR signaling network assembled for mutation analysis. Schematic representation of TLR5/TLR5 and TLR2/TLR6 MYD88-dependent intracellular signaling network built using CellDesigner. The network comprises 77 components, that were assembled based on the current literature (refer to text for references). LP stands for di-acylated lipopeptides; circled "P" denotes phosphorylation; circled "Ub" denotes ubiquitination; Ub(K63) denotes Lysine 63 poly-ubiquitin chains; Ub(Met-1) denotes linear ubiquitin chains; black lines, denote positive signal flow; red lines, denote negative regulatory events; the color purple highlights some commonly used inhibitors.

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ERK5 and Cell Proliferation: Nuclear Localization Is What Matters

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ERK5, the last MAP kinase family member discovered, is activated by the upstream kinase MEK5 in response to growth factors and stress stimulation. MEK5-ERK5 pathway has been associated to different cellular processes, playing a crucial role in cell proliferation in normal and cancer cells by mechanisms that are both dependent and independent of its kinase activity. Thus, nuclear ERK5 activates transcription factors by either direct phosphorylation or acting as co-activator thanks to a unique transcriptional activation TAD domain located at its C-terminal tail. Consequently, ERK5 has been proposed as an interesting target to tackle different cancers, and either inhibitors of ERK5 activity or silencing the protein have shown antiproliferative activity in cancer cells and to block tumor growth in animal models. Here, we review the different mechanisms involved in ERK5 nuclear translocation and their consequences. Inactive ERK5 resides in the cytosol, forming a complex with Hsp90-Cdc37 superchaperone. In a canonical mechanism, MEK5-dependent activation results in ERK5 C-terminal autophosphorylation, Hsp90 dissociation, and nuclear translocation. This mechanism integrates signals such as growth factors and stresses that activate the MEK5-ERK5 pathway. Importantly, two other mechanisms, MEK5-independent, have been recently described. These mechanisms allow nuclear shuttling of kinase-inactive forms of ERK5. Although lacking kinase activity, these forms activate transcription by interacting with transcription factors through the TAD domain. Both mechanisms also require Hsp90 dissociation previous to nuclear translocation. One mechanism involves phosphorylation of the C-terminal tail of ERK5 by kinases that are activated during mitosis, such as Cyclin-dependent kinase-1. The second mechanism involves overexpression of chaperone Cdc37, an oncogene that is overexpressed in cancers such as prostate adenocarcinoma, where it collaborates with ERK5 to promote cell proliferation. Although some ERK5 kinase inhibitors have shown antiproliferative activity it is likely that those tumors expressing kinase-inactive nuclear ERK5 will not respond to these inhibitors.

Keywords: MAP kinase, ERK5, nuclear translocation, transcriptional co-activator, cell proliferation, cancer, Hsp90, Cdc37

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INTRODUCTION

Extracellular signal-re gulated kinase 5 (ERK5, also named big MAP kinase 1, Bmk1) is a member of the Mitogen-activated protein kinases (MAPKs). ERK5 is ubiquitously expressed throughout all mammalian tissues and cell lines (Lee et al., 1995; Zhou et al., 1995; Buschbeck and Ullrich, 2005), where it is activated in response to several growth factors and oxidative and hyperosmotic

stress (Kato et al., 2000). ERK5 is twice the size of the classical MAPKs (816 aa for human ERK5), containing an N-terminal kinase domain (aa 78–406) and a unique C-terminal tail (aa 410–816) with no homology to any other protein, which has an autoinhibitory function (Buschbeck and Ullrich, 2005). This C-terminal tail contains a myocyte enhancer factor 2 MEF2-interacting region (aa 440–501, Kasler et al., 2000), a bipartite nuclear localization signal NLS (aa 505–539), and a transcriptional activation domain (TAD, aa 664–789, Kasler et al., 2000), which associates with and activates several transcription factors (Morimoto et al., 2007; Woo et al., 2010). Activation of ERK5 requires dual phosphorylation of a Threonine and Tyrosine residues within a TEY motif in the activation loop of the kinase domain (Mody et al., 2003). MEK5 is the only kinase that activates ERK5, and it has a unique specificity for ERK5, not phosphorylating any other MAPKs: MEK5 knockout mice dye at early stages of embryo development, showing similar defects in cardiac development and angiogenesis as those reported for ERK5 knockout mice (Wang et al., 2005).

ROLE OF ERK5 PATHWAY IN NORMAL AND CANCER CELL PROLIFERATION

ERK5 plays a crucial role in cell proliferation. First evidences reported that ERK5 activation is required for EGF-dependent proliferation in HeLa cells (Kato et al., 1998), by inducing transcription of *c-jun* (an essential component in cell proliferation) through the transcriptional activation of MEF2C (Kato et al., 1997). Since then, several authors have shown activation of ERK5 in response to other mitogenic factors, such as Nerve growth factor (NGF, Shao et al., 2002), Granulocyte colony-stimulating factor (G-CSF, Dong et al., 2001), Fibroblast growth factor (FGF, Kesavan et al., 2004), or Platelet-derived growth factor (PDGF, Rovida et al., 2008).

ERK5 regulates cell cycle progression, being necessary for G1/S transition. In this regard, ERK5 inhibition prevents cells from entering the S phase of the cell cycle (Kato et al., 1998) by stabilizing the cyclin-dependent protein kinase (CDK) inhibitors p21 and p27 (Perez-Madrigal et al., 2012). In human breast cancer MDA-MB-231 cells, activation of ERK5 promotes c-Myc-dependent transcriptional activation of miR-17-5p and miR-20a, resulting in blockade of p21 mRNA translation (Perez-Madrigal et al., 2012). ERK5 also mediates in G1/S transition by regulating expression of cyclin D1. Activation of MEK5/ERK5 pathway induces transcription of Cyclin D1, resulting in cell cycle progression in G1. Conversely, ERK5 inhibition diminishes serum-induced Cyclin D1 protein levels (Mulloy et al., 2003). Additionally, ERK5 is also implicated in G2/M transition. ERK5 is activated at G2/M, it is required for timely mitotic entry, and constitutively active ERK5 increases the mitotic index (Cude et al., 2007; Girio et al., 2007). The mitotic entry induced by ERK5 depends on the activation of the transcription factor NF-kB, which upregulates mitosis-promoting genes such as cyclins B1 and B2, and cdc25B (Cude et al., 2007). During mitosis, active ERK5 prevents caspase activation by binding and inactivating the pro-apoptotic protein Bim, suggesting that active ERK5 contributes to cell survival in mitosis (Girio et al., 2007). The role of ERK5 in controlling cell survival and differentiation, as well as angiogenesis, has been already covered in excellent reviews (Wang and Tournier, 2006; Drew et al., 2012; Lochhead et al., 2012; Nithianandarajah-Jones et al., 2012).

During the last years, different laboratories have shown that the MEK5-ERK5 pathway plays a key role in cancer cell proliferation. For instance, overexpression of either MEK5 or ERK5 in prostate adenocarcinoma PC-3 cells results in increased proliferation index (McCracken et al., 2008; Erazo et al., 2013). Consequently, ERK5 kinase inhibitors (such as the XMD8-92 compound) or ERK5 silencing show antiproliferative activity in different cancer cell lines and block tumor growth in animal models (human tumor xenografts). **Table 1** summarizes the different human cancers where it has been reported an effect of ERK5 silencing/inhibition on cell proliferation and/or tumor growth. Importantly, there are increasing evidences pointing to an important role of nuclear ERK5 in cancer, both *in vitro* (cell lines) and *in vivo* (mouse models). For instance, there is a strong correlation between nuclear ERK5 and poor prognosis in prostate cancer patients. Expression of nuclear ERK5 is upregulated in prostate cancers showing high-grade Gleason and bone metastasis (McCracken et al., 2008; Clape et al., 2009; Ramsay et al., 2011; Ahmad et al., 2013). However, and as explained below, ERK5 can promote cell proliferation independently of its kinase activity, acting as a moonlighting protein. For instance, hepatocellular carcinoma (HCC) tumors show increased nuclear ERK5, which does not correlate with an increase on ERK5 kinase activity (Rovida et al., 2015). Also, ERK5 localizes at the nucleus of CLB-BAR and CLB-GE human neuroblastoma cell lines, even in the presence of the ERK5 inhibitor XMD8-92 (Umapathy et al., 2014). These findings suggest that nuclear ERK5 expression, instead of ERK5 phosphorylation, might be used as prognostic biomarker of some cancers.

ERK5 NUCLEAR SUBSTRATES

The best characterized ERK5 substrates are nuclear transcriptional factors, whereas very few ERK5 cytosolic substrates have been characterized so far. Although ERK5 silencing affects the phosphorylation state of cytosolic proteins such as Akt and p90RSK kinases or the pro-apoptotic protein BAD, this is controversial since a direct ERK5 phosphorylation of these proteins has not been shown. For instance, Ranganathan et al. described that ERK5 phosphorylates p90RSK *in vitro* (Ranganathan et al., 2006), but other authors have shown that the MEK1/2 inhibitor PD184352 blocks p90RSK activation in response to EGF, at concentrations that do not block ERK5 activity (Mody et al., 2001). The use of the new synthesized specific ERK5 inhibitors—such as the XMD8-92 compound—will help to address these controversies.

ERK5 phosphorylates the transcription factor Sap1, a member of ternary complex factors (TCFs). ERK5-mediated Sap1 phosphorylation activates transcription through the Serum Response Element (SRE), which induces the expression of c-Fos (Kamakura et al., 1999). Although a direct phosphorylation

TABLE 1 | Effect of ERK5 silencing or inhibition on cancer cell proliferation and tumor growth.

Type of cancer	Target strategy	Effect	References
Leukemia	Silencing	shERK5 blocks tumor formation	Garaude et al., 2006; Charni et al., 2009
Lung carcinoma	XMD8-92 inhibition	XMD8-92 blocks tumor proliferation and angiogenesis in LL/2 and A59 xenograft models	Yang et al., 2010
Prostate cancer	Silencing	ERK5 silencing inhibits PC-3 cell proliferation and invasion. ERK5 overexpression induces more metastatic lesions in an orthotopic prostate model	Ramsay et al., 2011
Osteosarcoma	Silencing	ERK5 silencing reduces the number of invading cells	Kim et al., 2012
Malignant mesothelioma	Silencing	Injection of shERK5 malignant mesothelioma cell lines into SCID mice shows reduction in tumor growth	Shukla et al., 2013
Clear cell renal carcinoma	Silencing	ERK5 knockdown reduces proliferation and migration of 769-P and 786-O cells	Arias-Gonzalez et al., 2013
Hepatocellular carcinoma (HCC)	XMD8-92 inhibition Silencing	ERK5 inhibition or silencing inhibits EGF-induced cell migration. XMD8-92 reduces size of HCC xenograft tumors	Rovida et al., 2015
Triple negative breast cancer	XMD8-92 inhibition	XMD8-92 synergizes with chemotherapy (docetaxel + doxorubicin) or Hsp90 inhibitors to reduce growth of TNBC xenograft tumors	Al-Ejeh et al., 2014
Triple negative breast cancer	Silencing	ERK5 knockdown blocks TNBC cell proliferation	Ortiz-Ruiz et al., 2014
Pancreatic ductal adenocarcinoma	XMD8-92 inhibition	XMD8-92 inhibits growth of AsPC-1 tumor xenografts	Sureban et al., 2014
Neuroblastoma	XMD8-92 inhibition	XMD8-92 reduces growth of CLB-BAR and CLB-GE tumor xenografts. Also, synergizes with crizotinib to reduce growth of these tumors	Umapathy et al., 2014
Skin cancer	XMD8-92. ERK5 conditional KO in epidermis	XMD8-92 blocks skin tumor development and potentiates doxorubicin action. ERK5-KO keratinocyte show impair inflammation-driven tumorigenesis	Finegan et al., 2015

by ERK5 has not been shown, co-expression of ERK5 and catalytically active MEK5 in COS-7 cells induces phosphorylation and stabilization of c-Fos and Fra-1 transcription factors (Terasawa et al., 2003). Activation of the ERK5 pathway results in phosphorylation of few Ser/Thr residues in c-Fos and Fra-1, generating more stable proteins and enhanced transactivation activity of these factors (Terasawa et al., 2003).

The three members of the MEF2 (myocyte enhancer factor-2) family of transcription factors MEF2A, MEF2C, and MEF2D are the best characterized ERK5 substrates. MEF2 proteins regulate cell differentiation in myocytes and neurons (Potthoff and Olson, 2007), and act as a nodal point for *stress-response* in adult tissues (Kim et al., 2008). An interaction of MEF2C with ERK5 has been shown in two hybrid and co-immunoprecipitation assays. To our knowledge, MEF2C is the only nuclear protein whose interaction with ERK5 has been mapped. MEF2C interacts with a region of the C-terminal tail of ERK5 (aa 440–501) through its N-terminal end (Yang et al., 1998; Kasler et al., 2000). More importantly, activation of ERK5 pathway by either serum or EGF stimulates the transactivation activity of the MEF2A, MEF2C, and MEF2D transcription factors. Furthermore, ERK5 seems to be determinant for EGF-stimulated activation of MEF2A and MEF2D, since expression of ERK5 dominant negative mutant abolishes this activation (Kato et al., 2000). ERK5 phosphorylates different sites in MEF2A, MEF2C, and MEF2D proteins, however many of these residues are conserved in all three members, suggesting the existence of additional structural determinants to achieve recognition of the phosphorylated sites. Thus, ERK5 phosphorylates Thr312, Thr319, and S355 in MEF2A, Ser386/7 in MEF2C, and Ser179 and in MEF2D, and mutation to alanine of these residues abrogates MEF2 transcriptional activity (Kato et al., 1997, 2000).

The promyelocytic leukemia protein (PML) is a transcription factor that acts as a tumor suppressor, inhibiting proliferation and inducing cellular senescence and apoptosis through activation of the CDK inhibitor p21 (Bernardi and Pandolfi, 2007). Yang et al. have shown that ERK5 interacts with PML at the nuclear bodies in cancer cells, and inhibits its tumor suppressor activity by phosphorylating PML protein at Ser403 and Thr409 (Yang et al., 2010). ERK5-mediated phosphorylation impairs PML-dependent activation of p21, through disrupting PML-MDM2 interaction, and downregulating expression of the p53 tumor suppressor (Yang et al., 2013).

MECHANISMS INVOLVED IN ERK5 NUCLEAR TRANSLOCATION

ERK5 acts as a transcriptional co-activator, regulating MEF2C, AP-1, and c-Fos transcriptional activities in the nucleus. Therefore, translocation of ERK5 to nucleus is essential to regulate ERK5-mediated gene transcription. ERK5 is a big protein (110 kDa) so it cannot enter the nucleus by passive diffusion through the nuclear pores, as described for small proteins.

Recently, we have shown that inactive ERK5 binds the cytoplasmatic chaperone Hsp90 and the co-chaperone cell division-cycle 37 (Cdc37), which helps Hsp90 in the stabilization of ERK5 (Erazo et al., 2013). Cdc37 is the co-chaperone that

specifically promotes association of Hsp90 with many protein kinases (Smith and Workman, 2009). This trimeric complex, ERK5-Hsp90-Cdc37, not only stabilizes inactive ERK5, but also keeps ERK5 in a suitable conformation for MEK5 recognition and activation (Erazo et al., 2013).

In basal conditions, ERK5 binds the cytoplasmatic chaperone Hsp90, which serves as a cytosolic anchor for ERK5 (Erazo et al., 2013). This inactive ERK5 adopts a closed conformation where the C-terminal tail interacts with the kinase domain and the NLS motif is hidden and not available for the nuclear transport (reviewed in Kondoh et al., 2006). Nuclear shuttling of ERK5 requires both a conformational change to allow exposure of the NLS motif and the release of Hsp90. This mechanism is analogous to the one described for the progesterone and androgen receptors, which also requires Hsp90 dissociation for their nuclear translocation (Picard, 2006).

So far, two different mechanisms have been proposed for the ERK5 shuttling to the nucleus: one of them requires C-terminal phosphorylation, while the other does not. Once in the nucleus, ERK5 enhances gene transcription by either phosphorylating transcription factors, or by interacting with these factors through the transactivation TAD domain located at the C-terminal. Strikingly, ERK5 does not require kinase activity to interact with and activate transcription factors and therefore, forms of nuclear ERK5 devoid of kinase activity are able to activate transcription (Diaz-Rodriguez and Pandiella, 2010; Inesta-Vaquera et al., 2010; Erazo et al., 2013). Kinase-independent nuclear functions have been also proposed for other MAP kinases that lack a TAD domain. For instance, ERK2 promotes cell cycle entry by disrupting retinoblastoma-lamin A complexes in a kinase-independent fashion (Rodriguez et al., 2010), and also binds DNA acting as a transcriptional repressor for interferon gamma-induced genes (Hu et al., 2009). It would be interesting to study if ERK5 can also bind DNA.

Nuclear Translocation Dependent of ERK5 C-Terminal Phosphorylation

In response to EGF stimulation or different stresses MEK5 becomes activated, which in turn activates ERK5 by dual phosphorylation of the TEY motif. Then, active ERK5 phosphorylates its C-terminal tail resulting in a sequence of events that include: (1) dissociation of the cytosolic anchor Hsp90 from ERK5-Cdc37 complex; (2) adoption of a conformation in which the NLS motif is exposed; and (3) nuclear translocation (**Figure 1**).

ERK5 autophosphorylates several Thr/Ser residues within its C-terminal region. Mody et al., using purified recombinant active ERK5 and mass-spectrometry analysis, first identified five autophosphorylation sites at the C-terminal region: Ser421, Ser433, Ser496, Ser731, and Thr733 (Mody et al., 2003). Another laboratory also reported autophosphorylation in residues Ser760, Ser764, and Ser766, in addition to Thr733 (Morimoto et al., 2007). Inhibition of ERK5 autophosphorylation prevents the release of Hsp90 and nuclear entry (Erazo et al., 2013), reflecting that C-terminal tail autophosphorylation plays a critical role in ERK5 nuclear shuttling in response to MEK5 stimulation. Consequently, mutant forms of ERK5 in which the autophosphorylated residues at the C-terminal were mutated to alanine show cytoplasmatic localization and constitutive association to Hsp90 (Erazo et al., 2013), whereas the mutant in which these residues were mutated to glutamic acid does not bind Hsp90 (Erazo et al., 2013) and shows nuclear localization (Morimoto et al., 2007; Diaz-Rodriguez and Pandiella, 2010). Therefore, autophosphorylation of the C-terminal tail induces the release of Hsp90 from the ERK5-Cdc37 complex, a step essential previous nuclear translocation.

ERK5 C-terminal tail can also be phosphorylated by other kinases. During mitosis, ERK5 is phosphorylated at residues Ser567, Ser720, Ser731, Thr733, Ser753, and Ser830 (Diaz-Rodriguez and Pandiella, 2010; Inesta-Vaquera et al., 2010). These phosphorylations are not MEK5-dependent but induce the nuclear translocation of a kinase-inactive form of ERK5 which retains its transcriptional activity. These events might represent a second pathway controlling ERK5 C-terminal phosphorylation, which is activated in mitotic cells and involves kinase activities distinct from MEK5. Cyclin-dependent kinase-1 (CDK1) might well phosphorylate these sites, since either roscovitine or the RO3306 inhibitor reverse mitotic phosphorylation of ERK5 (Diaz-Rodriguez and Pandiella, 2010; Inesta-Vaquera et al., 2010).

All in all, C-terminal phosphorylation integrates different signals that converge in ERK5 nuclear shuttling and activation of transcription. ERK5 C-term autophosphorylation would represent a MEK5-dependent mechanism that integrate signals such as growth factors (EGF) and oxidative and osmotic stresses that activate MEK5-ERK5 pathway. On the other hand, C-terminal phosphorylation by other kinases (such as CDK1 during mitosis) represents a mechanism of nuclear translocation that does not require ERK5 kinase activity, but results in ERK5-mediated activation of transcription.

Nuclear Translocation Independent of ERK5 C-Terminal Phosphorylation

We have described a new mechanism for ERK5 nuclear translocation which is independent of C-terminal phosphorylation. Expression of high levels of Cdc37 induces the release of Hsp90 and the nuclear shuttling of a kinase-inactive form of ERK5 that retains its transcriptional activity (Erazo et al., 2013). This mechanism does not involve ERK5 activation or C-terminal phosphorylation by other kinases; overexpression of Cdc37 induced nuclear translocation and ERK5-mediated gene transcription in the presence of the specific inhibitor XMD8-92 or in MEK5 KO cells (Erazo et al., 2013).

The relevance of this new mechanism relays on the fact that Cdc37 acts as an oncogene, stabilizing other oncogenes that are mutated or overexpressed in cancer cells such as Akt, Her-2, or BRAF (Smith and Workman, 2009). Overexpression of Cdc37 has been observed in prostate adenocarcinoma, where it collaborates with c-Myc and cyclin D1 in the transformation of this tumor (Stepanova et al., 2000; Gray et al., 2007). Remarkably, *in vitro* evidences suggest that Cdc37 collaborates with ERK5 to promote proliferation of PC3

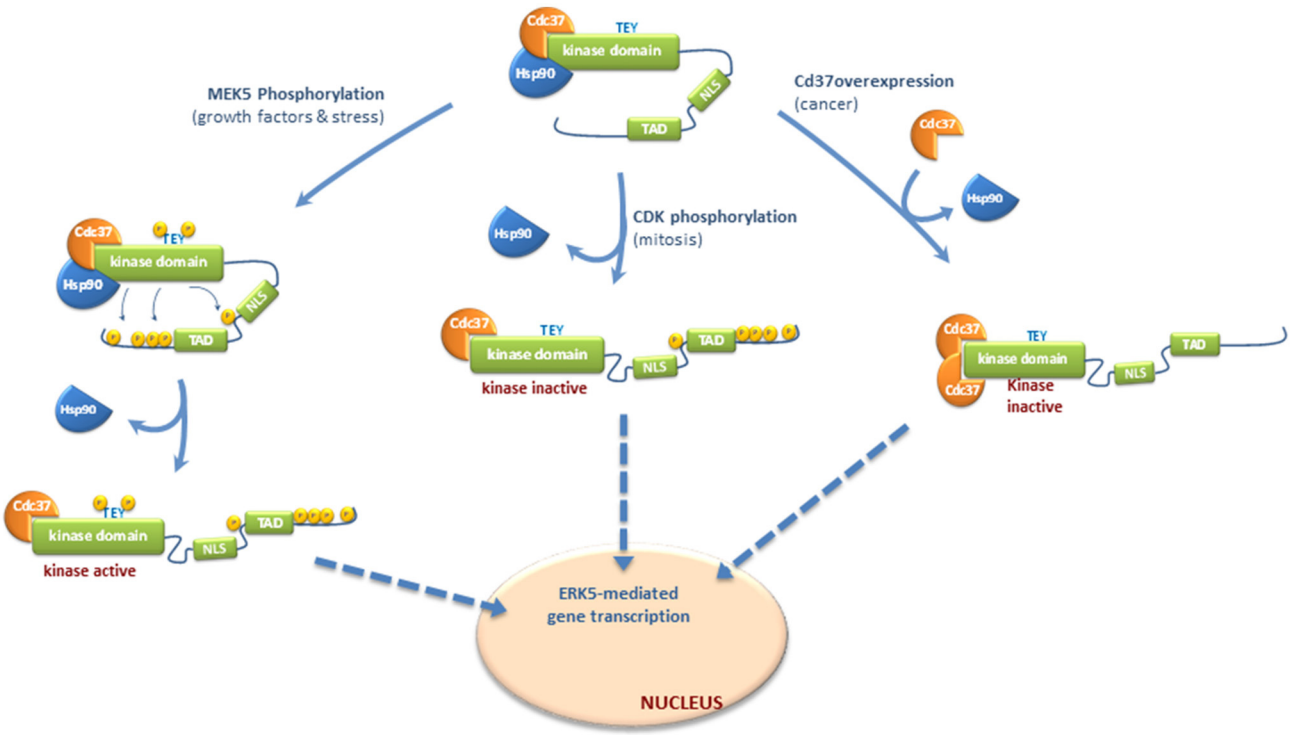

FIGURE 1 | Molecular mechanisms controlling nucleocytoplasmatic transport of ERK5. Different mechanisms have been proposed for ERK5 shuttling to the nucleus, depending or not of C-terminal phosphorylation. Once in the nucleus, ERK5 enhances gene transcription by either phosphorylating transcription factors, or by interacting with these factors through the transactivation TAD domain located at the C-terminal. ERK5 does not require kinase activity to interact with and activate transcription factors and therefore, forms of nuclear ERK5 devoid of kinase activity are able to activate transcription. Inactive ERK5 binds the cytoplasmatic chaperone Hsp90 and co-chaperone Cdc37, which helps Hsp90 in the stabilization of ERK5 (Erazo et al., 2013). In basal conditions, Hsp90 serves as a cytosolic anchor for ERK5, and inactive ERK5 adopts a closed conformation where the NLS motif is hidden and not available for the nuclear transport. Nuclear shuttling of ERK5 requires both a conformational change to allow exposure of the NLS motif and the release of Hsp90 (Erazo et al., 2013). In response to growth factors stimulation or different stresses, activated MEK5 phosphorylates and activates ERK5. Then, active ERK5 phosphorylates its C-terminal tail resulting in dissociation of the cytosolic anchor Hsp90 from ERK5-Cdc37 complex, adoption of an open conformation in which the NLS motif is exposed, and nuclear translocation. ERK5 C-terminal tail can also be phosphorylated by other kinases, such as Cyclin-dependent kinase-1 (CDK1) during mitosis. This phosphorylation is not MEK5-dependent but induce the release of Hsp90 and nuclear translocation of a kinase-inactive form of ERK5 which retains its transcriptional activity (Diaz-Rodriguez and Pandiella, 2010; Inesta-Vaquera et al., 2010). Finally, overexpression of Cdc37 (reported to happen in some cancers) induces the release of Hsp90 and nuclear shuttling of a kinase-inactive but transcriptionally active form of ERK5 (Erazo et al., 2013).

prostatic adenocarcinoma cells (Erazo et al., 2013). Cdc37 is also overexpressed in acute myelocytic leukemia and multiple myeloma (Casas et al., 2003; Katayama et al., 2004). It would be interesting to explore in these cancer cells if ERK5 shows constitutive nuclear localization and also collaborates with Cdc37 to promote cell proliferation. If so, and given the fact that Cdc37 induces nuclear shuttling of a kinase-inactive form of ERK5, we predict that these cancers will not respond to ERK5 inhibitors.

PERSPECTIVES

During the last years, efforts of many laboratories have led to delineate the importance of ERK5 in controlling cell proliferation in normal and cancer cells, by mechanisms that are both dependent and independent of its kinase activity: nuclear ERK5 activates transcription factors by either direct phosphorylation or acting as co-activator thanks to a unique transcriptional activation domain located at its C-terminal tail. Consequently, ERK5 has been proposed as an interesting target to tackle different cancers, and either inhibitors of ERK5 activity or silencing the protein have shown antiproliferative activity in cancer cells and to block tumor growth in animal models. However, and as we have seen above, ERK5 kinase inhibitors such as XMD8-92 might not be useful in cancers showing kinase-inactive nuclear ERK5. On the other hand, the anticancer activity of ERK5 inhibitors should be carefully interpreted. It has been recently reported that several classes of kinase inhibitors can strongly inhibit the bromodomain-containing protein-4 Brd4, a general transcription co-activator (Ciceri et al., 2014). This is the case for XMD8-92, which inhibits Brd4 and ERK5 with similar potency (Lin et al., 2016), and therefore some of the antitumor effects of this compound could be mediated by the Brd4-inhibiting activity. A new generation of specific ERK5

inhibitors are required to clarify the exact role of ERK5 in cancer cell growth.

There are still many open questions that remain to be addressed in order to describe the precise mechanism involved in ERK5 nuclear shuttling. For instance, is CDK1 the only kinase that phosphorylates ERK5 C-terminal tail? Does Cdc37 overexpression mimic a physiological mechanism that allows nuclear shuttling of inactive ERK5? Are other post-translational modifications, such as SUMOylation, required for ERK5 nuclear translocation? What is the role of ERK5 phosphatases in this process? The very near future seems an exciting time to deal with these questions, and hopefully will provide clues to design new compounds with antiproliferative activity.

AUTHOR CONTRIBUTIONS

All three authors wrote and corrected the manuscript. TE desgined **Table 1**. JL designed **Figure 1**.

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p38γ and p38δ Mitogen Activated Protein Kinases (MAPKs), New Stars in the MAPK Galaxy

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The protein kinases p38γ and p38δ belong to the p38 mitogen-activated protein kinase (MAPK) family. p38MAPK signaling controls many cellular processes and is one of the most conserved mechanisms in eukaryotes for the cellular response to environmental stress and inflammation. Although p38γ and p38δ are widely expressed, it is likely that they perform specific functions in different tissues. Their involvement in human pathologies such as inflammation-related diseases or cancer is starting to be uncovered. In this article we give a general overview and highlight recent advances made in defining the functions of p38γ and p38δ, focusing in innate immunity and inflammation. We consider the potential of the pharmacological targeting of MAPK pathways to treat autoimmune and inflammatory diseases and cancer.

Keywords: p38γ, p38δ, inflammation, innate response

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INTRODUCTION

The activation of p38 Mitogen Activated Protein Kinases (p38MAPKs) is one of the main signal transduction mechanisms by which the cell adapts to changes in the environment. There are four p38MAPK isoforms in mammalian cells encoded by different genes: p38α (*MAPK14*), p38β (*MAPK 11*), p38γ (*MAPK 12*), and p38δ (*MAPK 13*) (Cuenda and Rousseau, 2007). p38α was the first p38MAPK family member identified, therefore the most studied and best-characterized isoform; most of the literature on p38MAPK refers to p38α.

The four p38MAPK isoforms are widely expressed, but their expression pattern varies in tissues. p38α is ubiquitously expressed in all cell types and tissues, although expression levels are lower in the brain, liver, and pancreas than in other tissues. p38β is highly expressed in the brain, thymus, and spleen; its expression is lower in the adrenals, lung, kidney, liver, pancreas, and heart, and it is not expressed in skeletal muscle (Beardmore et al., 2005). p38γ is very abundant in skeletal muscle, although its expression in most other tissues is lower (Mertens et al., 1996; Beardmore et al., 2005). p38δ levels are high in pancreas, intestine, adrenal gland, kidney, and heart (Goedert et al., 1997; Jiang et al., 1997; Beardmore et al., 2005).

p38MAPKs are strongly activated by a wide variety of environmental and cellular stresses or by inflammatory cytokines, but are poorly activated by serum or growth factors (Cuenda and Rousseau, 2007). All p38MAPKs are Serine/Threonine kinases that catalyze the reversible phosphorylation of proteins. They are activated by dual phosphorylation of the TGY activation motif mediated by the MAPK kinases (MAP2K) MKK3, and MKK6, and in the case of p38α also by MKK4 (Remy et al., 2010). The activation of distinct p38MAPK isoforms is regulated by the selective and synchronized action of two kinases, MKK3 and MKK6. These two MAP2Ks

are implicated in p38α, p38γ, and p38β activation in response to general environmental stresses in mouse embryonic fibroblasts (Remy et al., 2010). However, MKK3 is the major kinase responsible for p38δ activation (Remy et al., 2010). MKK3 and MKK6 are in turn activated upon phosphorylation of Serine/Threonine residues by a MAPK kinase kinase (MAP3K). Several MAP3Ks, including MAPK/ERK kinase kinases (MEKK), TAO1 and 2, ASK1 (apoptosis signal-regulating kinase-1), MLKs (mixed-lineage kinases), and TAK1 (TGF β-activated kinase 1) activate p38MAPK cascade; the specific MAP3K that is required appears to be stimulus and cell type specific (Cuenda and Rousseau, 2007) (**Figure 1**).

p38MAPK family can be further divided into two subsets, p38α/p38β and p38γ/p38δ, based on sequence homology, substrate specificities, and sensitivity to chemical inhibitors. In this review, we provide an overview of p38γ and p38δ (also called alternative p38MAPKs), which play important roles in the innate immune response, in inflammation and inflammation-related diseases such as cancer or arthritis.

GENERAL FEATURES OF p38γ AND p38δ SIGNALING PATHWAYS

One of the hallmarks used for the division of p38MAPK into two subgroups is the protein sequence similarity. p38γ and p38δ sequences are highly similar to each other (∼70% identity), whereas p38α and p38β display higher similarity (75% identity). In contrast, p38γ and p38δ are more divergent in sequence to p38α (∼60% identical to p38α) (Risco and Cuenda, 2012). These two p38MAPK subgroups also differ on their sensitivity to kinase inhibitors. Pharmacological experiments demonstrated that only p38α and p38β are inhibited by certain compounds, which are ATP competitors, such as SB203580 and other pyridinyl imidazoles, whereas p38γ and p38δ are not affected by these drugs (Goedert et al., 1997; Kuma et al., 2005; Bain et al., 2007). However, the diaryl urea compound BIRB796, a molecule that functions allosterically and is a potent inhibitor of p38α and p38β, also inhibits p38γ and p38δ at higher concentrations in cell-based assays. This compound has provided a good tool for identifying physiological substrates and roles of the alternative p38MAPK isoforms (Kuma et al., 2005; Cuenda and Rousseau, 2007; Risco and Cuenda, 2012). Nonetheless, due to the lack of specific p38γ and p38δ inhibitors, the information about the physiological substrates and the biological function of these kinases is limited compared to the extensive knowledge of p38α functions.

The use of kinase inhibitors and the genetic deletion of specific p38MAPK isoforms has showed that they have some overlapping substrates and functional redundancy; however, there are particular proteins that are better substrates for p38α/p38β than for p38γ/p38δ and the other way around (Kuma et al., 2005; Cuenda and Rousseau, 2007; Risco and Cuenda, 2012). Even more, protein kinases such as MAPK-activated protein kinase 2 (MK2) or MK3 are good substrates for p38α and p38β, but cannot be phosphorylated by other p38MAPK isoforms (Cuenda et al., 1997; Goedert et al., 1997; Cuenda and Rousseau, 2007; Arthur and Ley, 2013) (**Figure 1**).

It has been reported that p38δ kinase phosphorylates the neuronal microtubule-associated protein Tau (Feijoo et al., 2005), the eukaryotic elongation factor 2 kinase (eEF2K) (Knebel et al., 2001), the protein kinase D1 (PKD1) (Sumara et al., 2009), which controls insulin exocytosis in pancreatic beta cells and chemotaxis in neutrophils, and the signal adaptor p62, which controls mTORC1 activation, autophagy, and tumor growth (Linares et al., 2015).

Several physiological substrates for p38γ have been described taking advantage of a feature that makes p38γ unique among other MAPKs. p38γ possesses a short C-terminal sequence (-KETXL), which binds to PDZ domains. p38γ associates with PDZ-domain containing proteins, such as α1-syntrophin, SAP (synapse-associated protein) 90/PSD (post-synapse density) 95, hDlg (human disc large also known as SAP97) and the protein tyrosine phosphatase PTPH1 and under stress conditions it is able to phosphorylate them and modulate their activity (Hasegawa et al., 1999; Sabio et al., 2004, 2005; Hou et al., 2010). For example, changes in the osmolarity of the environment trigger p38γ activation in the cytoplasm, which phosphorylates hDlg. Phosphorylation of hDlg leads to its dissociation from the cytoskeletal guanylate kinase-associated protein (GKAP) and therefore from the cytoskeleton (Hasegawa et al., 1999; Sabio et al., 2004, 2005; Hou et al., 2010). In addition, the interaction of p38γ with the single PDZ domain of PTPH1 enables this phosphatase to dephosphorylate p38γ, but not p38α, *in vitro* and in cells over-expressing both proteins (Hou et al., 2010; Chen et al., 2014). So far, the only physiological p38γ substrate that does not require PDZ domain binding interactions is the transcription factor MyoD, whose phosphorylation by p38γ results in a decrease in its transcriptional activity (Gillespie et al., 2009).

p38MAPKs act normally by direct phosphorylation of substrates on Serine or Threonine residues followed by Proline, however, there are some examples showing that p38α and also p38γ may also have kinase independent roles by associating to protein targets and modulating their function in the absence of phosphorylation (reviewed in Cuadrado and Nebreda, 2010; Risco and Cuenda, 2012). For example, it has been shown that p38γ regulates nuclear protein complexes independently of its kinase activity. Changes in the osmolarity cause the accumulation of p38γ in the nucleus where it interacts with nuclear hDlg. In the nucleus, hDlg forms a complex with the proteins polypyrimidine tract-binding (PTB) protein-associated splicing factor (PSF) and p54nrb, and with various RNAs. p38γ regulates hDlg-PSF complex dissociation independently of hDlg phosphorylation by displacing PSF from hDlg, since both proteins, p38γ and PSF, bind to PDZ1 domain of hDlg. This has been shown comparing cells from knockin mice expressing an endogenous kinase-inactive p38γ mutant with cells from mice lacking p38γ (Sabio et al., 2005, 2010; Remy et al., 2010; Risco and Cuenda, 2012). The studies on p38γ-hDlg-GKAP and p38γ-hDlg-PSF protein complexes indicate that, through its ability to shuttle between cytoplasm and nucleus, p38γ might provide

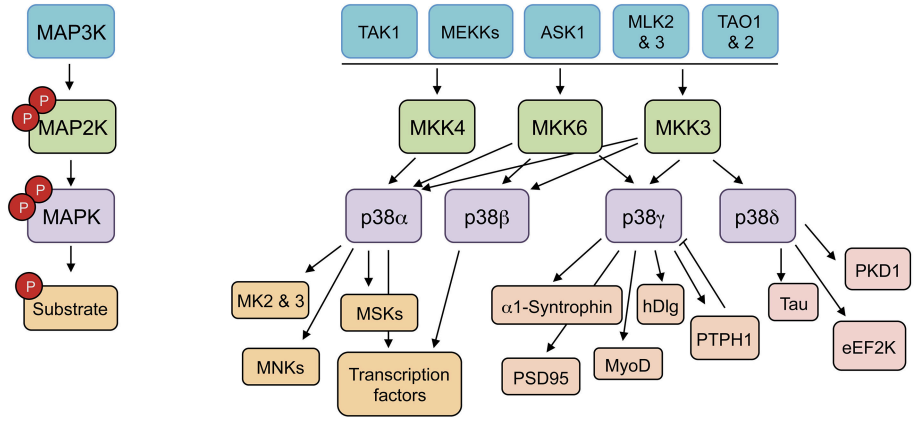

FIGURE 1 | p38MAPK pathways consist of several MAPK kinase kinases (MAP3K), three MAPK kinase (MAP2K), and four p38MAPK. These pathways can be activated by many stimuli, including growth factors, inflammatory cytokines, and a wide range of cellular stresses. The p38MAPKs phosphorylate different substrates, including protein kinases, cytosolic substrates, and transcription factors. ASK1 (apoptosis signal-regulating kinase-1), eEF2K (eukaryotic elongation factor 2 kinase), hDlg (human disc large), MEKK (MAPK/ERK kinase kinases), MK (MAPK-activated protein kinase), MLKs (mixed-lineage kinases), MNK (MAPK-interacting protein kinase), MSK (mitogen and stress-activated kinase), PKD1 (protein kinase D1), PSD95 (post-synapse density 95), PTPH1 (protein tyrosine phosphatase H1), TAK1 (TGF β-activated kinase 1), TAO (thousand-and-one amino acid).

a connection between two processes critical for adaptation to environmental changes: gene expression and cytoskeletal reorganization.

SOME PHYSIOLOGICAL ROLES OF p38γ AND p38δ MAPK PATHWAYS

Studies using knock-out mice have provided important information concerning p38γ and p38δ functions *in vivo* and in pathological conditions (**Figure 2**). p38γ and p38δ deficient mice are viable and have not apparent phenotypes (Sabio et al., 2005, 2010; Remy et al., 2010; Risco and Cuenda, 2012). Nonetheless, there are reports showing the implication of p38γ and p38δ in tissue regeneration, cancer, and metabolic diseases (Sabio et al., 2005, 2010; Remy et al., 2010; Risco and Cuenda, 2012). Thus, it has been described that p38δ regulates insulin secretion and pancreatic β cells death implying a central role in diabetes (Cuenda and Nebreda, 2009; Sumara et al., 2009). p38δ is also crucial in neutrophil chemotaxis pathway, contributing to acute respiratory distress syndrome (ARDS) (Ittner et al., 2012), and in mediating IL-13-driven mucus overproduction in human airway epithelial cells in chronic inflammatory lung diseases (Alevy et al., 2012).

Since p38γ expression is very high in skeletal muscle and its expression is induced during muscle differentiation (Cuenda and Cohen, 1999; Tortorella et al., 2003; Perdiguero et al., 2007), it is not surprising that it plays a fundamental role in this process. Thus, p38γ knockdown impairs cardiomyocyte formation (Ramachandra et al., 2016) and p38γ and p38δ promote cardiac hypertrophy by modulating the mTOR pathway (González-Terán et al., 2016). Moreover, studies in p38γ deficient mice reported that p38γ plays a pivotal role in blocking the premature differentiation of skeletal muscle stem cells, the satellite cells that participate in adult muscle regeneration (Gillespie et al., 2009). Also, p38γ is required for the upregulation of PGC-1α [peroxisome proliferator-activated receptor-γ (PPARγ) coactivator-1α] in mitochondrial biogenesis and angiogenesis in response to endurance exercise in mice, which is critical for skeletal muscle adaptation (Pogozelski et al., 2009).

In addition, p38γ and p38δ are involved in the modulation of some processes implicated in cellular malignant transformation, such as proliferation, cell cycle progression, apoptosis, or cell migration. Using mouse embryonic fibroblasts derived from mice lacking p38γ or p38δ, it has been shown that deletion of either p38γ or p38δ increases cell migration and metalloproteinase-2 secretion, whereas only p38δ deficiency impairs cell contact inhibition. Also, lack of p38γ in K-Ras-transformed fibroblasts leads to increased cell proliferation as well as tumorigenesis both *in vitro* and *in vivo* (Cerezo-Guisado et al., 2011). These pieces of evidence indicates that p38γ and p38δ have a role in tumor suppression; however, there are other studies reporting a pro-oncogenic function for these kinases, for example in the development of breast and skin cancer (reviewed in Risco and Cuenda, 2012).

p38γ AND p38δ IN THE INNATE RESPONSE AND IN INFLAMMATION

The use of genetically modified mice lacking one or more p38 isoform has provided strong evidence of the p38γ and p38δ importance in the innate immune response and in inflammation (**Figure 2**). The innate immune system is the front line of defense against invading pathogens, and uses evolutionarily conserved high-affinity receptors (pattern recognition receptors, PRRs) that recognize specific constituents of bacteria or virus, endogenous cytokines and host cell-derived components (Kawai and Akira, 2007). The activation of PRRs in the innate immune cells leads to

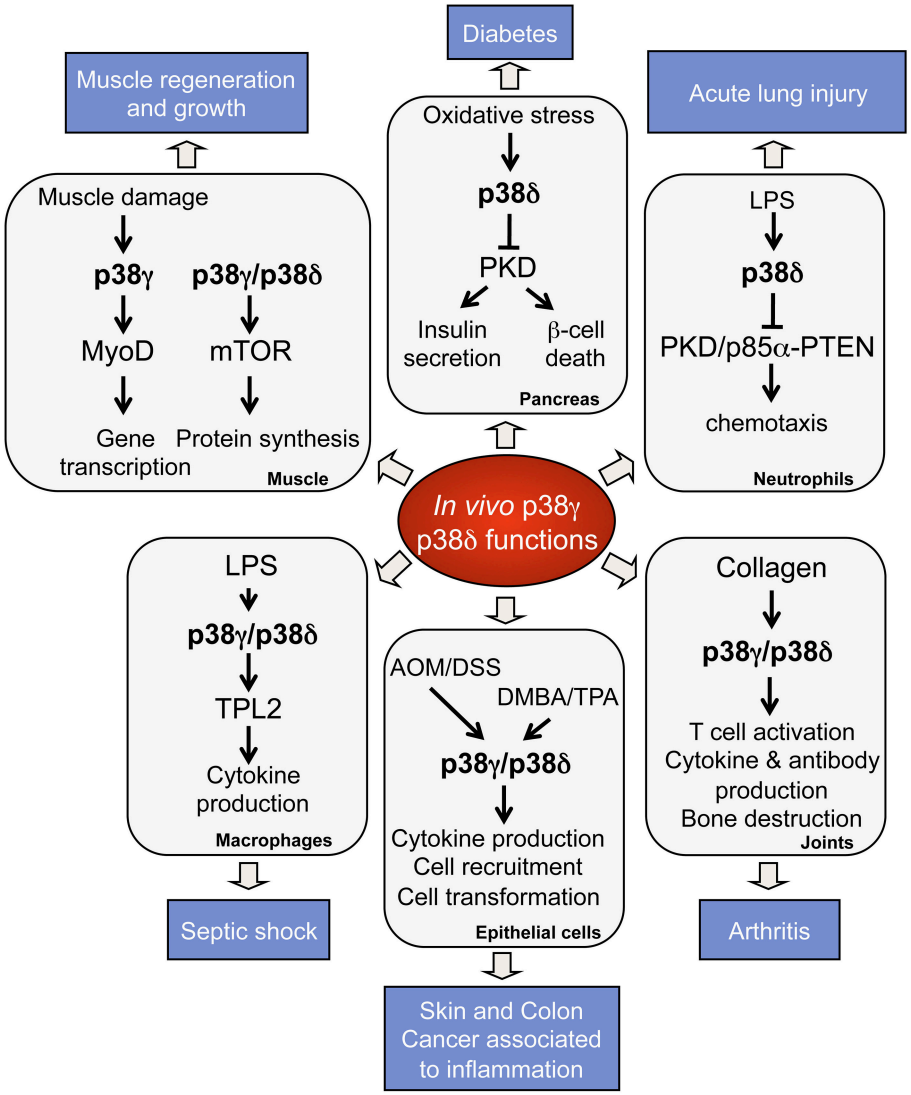

FIGURE 2 | Physiological roles and pathological implications of p38γ and p38δ. p38γ and p38δ are key players in the regulation of many biological functions, which contribute to physiological processes. Deregulation of p38γ and p38δ leads to the development of several pathological conditions.

secretion of inflammatory cytokines and other mediators, which induce an inflammatory response. This inflammatory response promotes the recruitment of additional immune cells, the elimination of infectious agents, and the induction of tissue repair (Kawai and Akira, 2007). The production of cytokines during the innate response is also important for the subsequent induction of the adaptive immune response (Iwasaki and Medzhitov, 2010). The stimulation of all PRRs by pathogen-associated molecules activates both MAPKs and NFκB pathways, which are crucial to generate immune responses (Cuenda and Rousseau, 2007; Gaestel et al., 2009; Arthur and Ley, 2013).

The important role of the MAPK p38α in the innate immune response and in inflammation has been uncovered mainly by studies using a range of p38α inhibitors or the constitutive deletion of its physiological substrates, or activators (Cuenda and Rousseau, 2007; Gaestel et al., 2009; Arthur and Ley, 2013). Much less is known about the importance of p38γ and p38δ in these processes. However, recent studies show that in macrophages and dendritic cells (DC), which are key mediators of the inflammatory response, the deletion of both p38γ and p38δ impaired the innate immune response to lipopolysaccharide (LPS), a Toll-like receptor 4 (TLR4) ligand (Risco et al., 2012). In these cells p38γ and p38δ are necessary to maintain steady-state levels of TPL-2, the MKK kinase that mediates ERK1/2 activation in response to TLR stimulation (Gantke et al., 2011). ERK1/2 are other MAPK family members that play a central role in cytokine production. p38γ and p38δ deficient macrophages ($p38\gamma/\delta^{-/-}$) showed substantially lower levels of TPL2 protein, and therefore lower MKK1-ERK1/2 activation and inflammatory cytokine production (Risco et al., 2012). Exogenous expression

of TPL-2 in p38γ/δ$^{-/-}$ macrophages not only increased ERK1/2 activation, but also rescued TPL-2-dependent TNFα production in response to LPS (Risco et al., 2012).

p38γ and p38δ signaling has complex pro- and anti-inflammatory effects on cytokine production in innate immune responses. Production of TNFα, IL-1β, and IL-10 is severely reduced in LPS-stimulated macrophages from p38γ/δ-deficient mice, whereas IL-12 and IFNβ production increases (Risco et al., 2012). p38γ and p38δ regulate IL-1β and IL-10 production at the transcriptional level, whereas regulation of TNFα is at the secretion level in bone marrow derived macrophages stimulated with LPS (Risco et al., 2012). In LPS-stimulated liver macrophages, p38γ and p38δ are required for the translation of *Tnf* mRNA through inhibitory phosphorylation of eEF2K that leads to activation of eEF2 (González-Teran et al., 2013). Furthermore, in TPA-stimulated keratinocytes, p38γ and p38δ are required for IL-6, IL-1β, and CXCL1 transcription (Zur et al., 2015). The exact mechanisms by which p38γ and p38δ regulate the production of cytokines and chemokines in different cells are still largely unknown and further studies are needed to determine them.

Using p38δ deficient mice it has been shown that this p38MAPK isoform is important in neutrophils migration and in their recruitment into inflammatory sites in lung (Ittner et al., 2012). The degree of inflammation and the associated organ damage is a consequence of complex pro- and anti-inflammatory responses, which involve the regulation of neutrophil recruitment and migration in a cell-autonomous manner. p38δ-deficient neutrophils show a defect in chemotaxis, which is caused by increased activity of the p38δ substrate, the kinase PKD1 (Sumara et al., 2009). PKD1 phosphorylates p85α to enhance its interaction with PTEN, leading to increased PTEN activity and lower cell migration (Ittner et al., 2012). Appropriate signaling in neutrophils is essential to resolve inflammation without causing inappropriate organ damage.

Overall, these data strongly suggest that p38γ and p38δ have a key role in the mechanisms leading to inflammation.

p38γ AND p38δ IN INFLAMMATORY DISEASES

The role of p38γ and p38δ in inflammation *in vivo* is further supported by experiments in other mouse animal models. The reaction to bacterial LPS is a well-characterized innate immune response that leads to endotoxic or septic shock, due primarily to TNFα overproduction. Thus, p38γ/δ-deficient mice are less sensitive to endotoxic shock than wild type mice following LPS challenge and this is associated with a decrease in serum levels of inflammatory cytokines such as TNFα, IL-1β, or IL-10 (Risco et al., 2012). The acute liver failure caused by LPS is also suppressed in mice that lack p38γ and p38δ in myeloid cells (González-Teran et al., 2013). In addition, p38δ deletion results in decreased alveolar neutrophil accumulation, reduces acute lung inflammation, and protect from acute lung injury (ALI) induced by LPS (Ittner et al., 2012). Also, there are evidence that p38δ mediates mucus production in chronic inflammatory lung disease, since either the knockdown or inhibition of p38δ, but not of p38α, can block inflammatory IL13-induced mucus production in human airway epithelial cells (Alevy et al., 2012).

The role of p38γ and p38δ isoforms in other inflammatory diseases such as arthritis has recently been shown in a collagen-induced arthritis (CIA) mouse model. Combined p38γ and p38δ deficiency markedly reduced arthritis severity by suppressing clinical disease and bone destruction, compared with that in wild type mice (Criado et al., 2014). p38γ/δ deficient mice have lower mRNA expression of IL-17 and IFNγ in joints, and lower levels of pathogenic anti-collagen antibodies, IL-1β, and TNF-α in the serum than wild type mice (Criado et al., 2014). p38γ and p38δ also seem to control T cell activation, for example lymph node T cells from p38γ/δ-deficient mice show reduced proliferation and interferon (IFN)γ and IL-17 production (Criado et al., 2014). Moreover, p38γ/δ deficient mice showed a lower Th17 cell frequency and a greater Treg/Th17 ratio, both of which are linked to successful therapy in rheumatoid arthritis. The crucial role of p38γ/δ in synovial inflammation, bone erosion, as well as cytokine production suggests that they could serve as targets of therapy in rheumatoid arthritis as an alternative to traditional p38α inhibitors, which have proven minimally effective in human disease (Gaestel et al., 2009; Arthur and Ley, 2013).

During the last few years the role of p38γ and p38δ in cancer associated with chronic inflammation has been studied. Chronic inflammation is linked with an increase in malignant disease. Almost 20% of human cancers are related to chronic inflammation caused by infections, exposure to irritants or autoimmune diseases (Hanahan and Weinberg, 2011; Crusz and Balkwill, 2015). Colitis-associated cancer (CAC) is a colon cancer subtype associated with inflammatory bowel disease, such as that occurring in ulcerative colitis or Crohn's disease. Using the azoxymethane (AOM)/dextran sodium sulfate (DSS) mouse model of CAC it has been shown that p38γ and p38δ have a pro-oncogenic role by regulating inflammatory signaling to promote colon tumorigenesis, thus linking inflammation and cancer in CAC (Del Reino et al., 2014). Mice deficient in p38γ and p38δ display a decrease in cytokines and chemokines production and in inflammatory cell infiltration in the colon of treated animal and produce fewer colon tumors than control mice (Del Reino et al., 2014). p38γ and p38δ in hematopoietic cells are important for CAC development. Lethally irradiated wild type mice reconstituted with bone marrow from p38γ/δ-null mice exhibited less tumor formation, cytokine production, and immune cell infiltration, whereas p38γ/δ-deficient mice reconstituted with wild type bone marrow showed more tumor formation, cytokine production, and immune cell infiltration than controls (Del Reino et al., 2014). The pro-oncogenic role of p38γ and p38δ was also confirmed in the two-step 7,12-dimethylbenz(*a*)anthracene (DMBA)/12-*O*-tetradecanoylphorbol-13-acetate (TPA) chemical skin carcinogenesis model (Schindler et al., 2009; Zur et al., 2015). p38γ/δ-deficient mice showed diminished cytokine production and are resistant to tumorigenesis (Zur et al., 2015). Overall, all of these results suggest potential therapeutic prospects by targeting p38γ and p38δ for treatment of cancer. Future studies examining the effects of cell type-selective p38γ and p38δ targeting at different stages of carcinogenesis will elucidate the functional roles of these two alternative p38MAPKs in

the tumorigenesis process, and will guide future therapeutic strategies.

CONCLUSION AND PERSPECTIVE

The role of p38γ and p38δ has been some times ignored since most of the studies to date have focused on p38α, which is the most abundant p38MAPK isoform. Nonetheless, in the last years significant progress in understanding the functions of p38γ and p38δ *in vivo* has been achieved. It is now clear that p38γ and p38δ are crucial in innate response, inflammation, and inflammatory diseases. Therefore, they deserve to be studied in greater depth as they represent pharmacological target for the development of drugs that might be useful for the treatment of inflammatory pathologies. In fact, development of more specific p38δ inhibitors has been shown to reduce mucus production in human airway epithelial cells (Alevy et al., 2012). However, the molecular mechanisms of how p38γ and p38δ regulate innate response, including the significance of the regulation of other signaling pathways components, cytokine production and the recruitment of immune cells, remain to be fully established. A better mechanistic understanding of p38γ/p38δ-regulated innate response will permit the design of p38γ and p38δ-based therapies, alternative to traditional p38α inhibitors, which have proven minimally effective in human inflammatory diseases (Gaestel et al., 2009; Arthur and Ley, 2013). The prospective that basic research on p38γ and p38δ could be translated to the treatment of human disease provides an exciting goal for future studies in the field.

AUTHOR CONTRIBUTIONS

AC wrote the manuscript. AE, AR, DA made substantial contributions to conception and design, and acquisition of information. All authors contributor to the revision of the manuscript and approved the final version.

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