Regulations and actions mediated by c-Jun N-terminal kinase pathway

Jordi Lanuza Masdeu

UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA

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Memòria presentada per Jordi Lanuza Masdeu per optar al títol de doctor per la Universitat de Barcelona

Dra. Carme Caelles Franch

Jordi Lanuza Masdeu

INDEX

RESUM

INTRODUCCIÓ

Les Mitogen-activated protein kinases (MAPKs) són unes quinases eucariotes molt conservades evolutivament. Han estat implicades en vies de senyalització que responen d'una manera específica a estímuls com factors de creixement, pèptids vasoactius, polipèptids relacionats amb el Transforming Growth Factor (TGF), citoquines pro-inflamatòries i l'estrès ambiental (Chang & Karin, 2001). Aquests estímuls són amplificats per les vies de senyalització de les MAPK per tal de controlar programes cel·lulars com la proliferació, la mort cel·lular programada, la diferenciació, l'embriogènesi, l'homeòstasi, les respostes hormonals agudes i la inflamació. D'entre aquestes quinases, la c-Jun N-Termila Kinase (JNK) és activada específicament per citoquines pro-inflamatòries, estrès ambiental i factors de creixement. L'activació de JNK induïda per aquests estímuls condueix a una amplia gama de respostes cel·lulars (Kyriakis & Avruch, 2012).

Per altra banda, els receptors nuclears (NRs) constitueixen una superfamília de factors de transcripció que regulen diversos processos com el desenvolupament, la reproducció i el metabolisme, així com la inflamació. D'entre ells els Peroxisome Proliferator Activated Receptors (PPARs) i els Liver X Receptors (LXRs) són d'especial interès degut a la seva implicació en la regulació del metabolisme glucídic i lipídic, a més de la inflamació. En el grup de recerca, s'ha demostrat prèviament que la via de senyalització de JNK és inhibida per les Thiazolinediones (TZDs), que són lligands sintètics de PPARγ i que són emprats com a agents sensibilitzadors a la insulina en el tractament de la diabetis tipus 2 (Díaz-Delfín *et al*, 2007).

S'ha descrit que l'activitat de JNK està anormalment incrementada en diversos teixits en condicions de resistència a insulina i diabetis tipus 2 (Solinas & Karin, 2010). Per exemple, tant la producció i l'alliberació de IL-1β induïda per la hiperglicèmia i l'excés d'àcids grassos saturats relacionats amb l'obesitat,

condueixen a l'activació de JNK i la conseqüent disfunció en les cèl·lules productores d'insulina, les cèl·lules β-pancreàtiques (Solinas *et al*, 2006; Ammendrup *et al*, 2000). A més a més, s'ha descrit que la inhibició de JNK mitjançant inhibidors químics evita l'apoptosi dels illots pancreàtics induïda per IL-1β (Bonny *et al*, 2001).

La rellevància de l'activació de JNK en una gran varietat de malalties amb un component inflamatori, afegit a l'interès del grup en l'estudi de la regulació negativa dels glucocorticoids i els lligands de PPAR i LXR sobre JNK, va portar al grup a la generació d'un model de ratolí transgènic amb expressió condicionada a l'espressió de la recombinassa Cre. En absència de l'expressió de la recombinassa, s'expresa la Green Fluorescent Protein (GFP), mentre que en presència de Cre es produeix la recombinació i el resultat és l'expressió de la forma constitutivament activada de la MAP2K de JNK, la MKK7. D'aquesta manera, creuant el ratolí transgènc amb un altre que expressi Cre de manera teixit específica, podem aconseguir una activació de JNK en un teixit en concret. En aquest cas s'han estudiat els efectes de l'activació de JNK en les cèl·lules β-pancreàtiques.

RESULTATS

L'activació de JNK a les cèl·lules β-pancreàtiques genera intolerància a la glucosa causada per una resistència a insulina central

Tot i que aquests ratolins no tenen cap afectació morfoestructural en els seus illots pancreàtics ni diferències en el contingut total d'insulina, tenen una homeòstasi de la glucosa defectiva. Presenten intolerància a la glucosa i no secreten insulina en resposta a hiperglucèmia. Aquesta reducció de la secreció d'insulina és autònoma de la cèl·lula β ja que s'ha reproduït en secrecions estàtiques amb illots aïllats, i depenent de JNK, ja que s'ha aconseguit revertir el fenotip amb l'inhibidor TAT-JIPi, un inhibidor específic de JNK que es deriva de la regió on s'uneix JNK a la proteïna d'andamiatge JNK-interacting Protein 1 (JIP-1), ajuntada amb la seqüència del pèptid permeable HIV TAT.

Al nivell molecular, les cèl·lules β pancreàtiques amb JNK activa, tenen un bloqueig a la via de senyalització d'insulina que fa que es redueixi la secreció d'insulina i l'expressió de gens diana de la insulina. El tractament amb rossiglitazona, un fàrmac del grup de les TZDs, aconsegueix revertir el fenotip i recuperar la secreció d'insulina en resposta a glucosa i insulina tan en illots aïllats com *in vivo*. Tots aquests resultats indiquen que l'activació de JNK és suficient per promoure la resistència a insulina central però no n'hi ha prou per induir hiperplàsia dels illots o induir l'apoptosi de les cèl·lules β. A més a més, aquests ratolins estan protegits de la hiperinsulinèmia, la hiperglucèmia i l'obesitat induïdes per la dieta grassa o l'envelliment.

Estabilització de βTrCP mitjançada per JNK

També ha estat objecte d'estudi la interacció entre les vies de senyalització de JNK i NF-κB. Les dues vies de senyalització són essencials per la regulació de les respostes immune i inflamatòria, així com altres processos fonamentals com la proliferació cel·lular i la supervivència. Per aquesta raó, no n'hi ha prou en estudiar les vies independentment sinó que és molt important estudiar les interaccions que puguin tenir.

Fins ara, el mecanisme més estudiat és la inhibició mútua però en canvi se'n sap molt poc de l'existència d'una interacció positiva. S'ha demostrat que la quinasa MEKK1, que és una MAP3K, pot activar tant JNK com el complex IKK, que està implicat en la degradació proteosomal de l'inhibidor de NF-κB (IκBα). Uns anys més tard, es va provar que JNK era essencial en l'activació d'IκBα en resposta als estímuls específics tioredoxina i trombina. Simultàniament, es va publicar que JNK podia induir l'activació de NF-κB mitjançant l'estabilització del mRNA de la βTrCP, responsable de la degradació d'IκBα.

D'acord a aquesta interacció, resultats previs del laboratori han demostrat que JNK és necessària per a la poliubiquitinació i degradació d'IκBα i, per tant, l'activació de NF-κB. Sembla que l'activació de JNK afavoreix l'ensamblatge del complex d'ubiquinització $SCF^{\beta TrcP}$ i la seva unió al seu substracte, IκBα. La hipòtesis seguida és que existeixen dos mecanismes que explicarien aquesta interacció. Per un costat hi hauria l'estabilització del mRNA de βTrCP per a efectes a llarg termini, mentre que per altra banda hi hauria efectes post-traduccionals per a permetre respostes ràpides. A més a més, la inhibició de JNK mitjançant dexametasona, afecta a l'activació de NF-κB i contribueix a la transrepresió mitjançada pel receptor de glucocorticoids.

Les dues línies investigades han estat si l'estabilització del mRNA de βTrCP podia estar mitjançada per la interferència de JNK amb la interacció d'aquest mRNA amb el microRNA-183 (miR-183) i l'estudi de la funció de JNK sobre l'ensamblatge del complex d'ubiquitinització.

En quant al primer punt, JNK va resultar fosforilar a CRD-BP, una proteïna que s'uneix al mRNA de βTrCP per protegir-li de l'atac per part del miR-183. A més a més, un mutant de βTrCP amb la seqüència nucleotídica alterada perquè no s'hi unís el miRNA però amb la seqüència aminoacídica inalterada, es seguia estabilitzant en resposta a l'activació de JNK a nivell de proteïna però no de mRNA. Això indica que l'activació de βTrCP efectivament està passant a ambdós nivells i que JNK afecta al sistema mRNA (βTrCP) - miR-183 - CRD-BP.

A part d'aquest efecte de JNK, sembla que estigui fent de proteïna d'ensamblatge per a la unitat bàsica del complex de $SCF^{\beta TrCP}$, SKP1- βTrCP. L'activació de JNK resulta en l'increment de les dues proteïnes però la interacció entre elles és també necessària per a aconseguir la màxima estabilització.

A més a més, aquest efecte podria ser extensiu a altres complexes $SCF^{\beta TrCP}$ ja que altres dianes de βTrCP com β-catenina també estan afectades i els nivells d'altres F-Box com la SKP2 també es veuen incrementats, i el dels seus substrats disminuïts d'una manera dependent de JNK.

INTRODUCTION

BACKGROUND

Cell signaling

All living cells sense and respond to the environment by a set of mechanisms known as cell signaling: part of a complex system of communication that governs basic cellular activities and coordinates cell actions. Cells must respond appropriately to the environment, whether they live freely or are part of an organism. This need of communication is even more required in organisms with tissues with specialized functions. The ability of cells to perceive and correctly respond to the microenvironment is the basis of very important processes such as development, tissue repair, and immunity as well as normal tissue homeostasis.

Cells receive information from the environment through receptors, located in the plasma membrane or in intracellular compartments. The information is then processed through signaling pathways and decoded in cellular compartments, including the nucleus. To understand cell signaling, we need to understand the spatial and temporal dynamics of the receptors, the components of signaling pathways and their crosstalks. We need to know which specific components are actually present in a given cell, where are located, and what their functions are.

The diverse stimuli that cells receive constantly play an important role and determine cell processes such as proliferation, differentiation and apoptosis, as well as the coordinated control of multiorganic responses like inflammation. In this last example, while pathogen recognition begins at the receptor level, are the signaling components downstream of each receptor and the way they interact with each other that ultimately determine the specific transcriptional response and immunological outcome.

In the present work the main pathways studied have been the ones involving the c-Jun N-Terminal Kinase (JNK), the Nuclear Factor-κB (NF-κB), insulin and Nuclear Receptors, specifically the Peroxisome Proliferator-Activated Receptor-γ (PPAR-γ). Actually, the main goal of the thesis was to identify and characterize the molecular mechanisms by

which JNK interact with these other signaling pathways and evaluate their relevance to determine the final response. In the case of NF-κB, the interactions were studied in the context of inflammation whereas glucose homeostasis was the area of the study of the interaction JNK-Insulin signaling. Finally, through the JNK regulation, NRs could also exert their effects on NF-κB and insulin signaling pathway.

Mitogen-activated protein kinases (MAPK)

One of the main signal transduction pathways are the MAPKs, which are ubiquitous and evolutionarily conserved in eukaryotic cells. The MAPK pathways are present in all eukaryotic cells and enable coordinated and integrated responses to most, if not all, stimuli, including hormones, growth factors, cytokines, agents acting through G protein-coupled receptors, and environmental stresses.

Once activated, MAPK pathways exert major effects on cell physiology by coordinating the recruitment of gene transcription factors, protein biosynthesis, cell cycle control, apoptosis, and differentiation (Kyriakis & Avruch, 2012).

The conventional MAPKs, which include the extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun amino-terminal kinases 1 to 3 (JNK1 to 3), p38MAPK (p38) (α, β, γ, and δ), and ERK5 families, are the best known. In addition, atypical MAPKs, including ERK3/4, ERK7/8, and Nemo-like kinase (NLK), have distinct regulation and functions (Smaele *et al*, 2001; Tang *et al*, 2001). In particular and related to the thesis, JNK and p38 can be activated by cellular stresses and are collectively known as Stress-Activated Protein Kinases (SAPKs).

MAPKs contain the signature sequence –TXY–, where T and Y are threonine and tyrosine, and X is glutamate, proline or glycine, in ERK, JNK or p38, respectively. Phosphorylation of both the threonine and tyrosine residues within this signature sequence is required for MAPK activation (Kyriakis & Avruch, 2012). MAPKs are serine/ threonine-specific, proline-directed, protein kinases; however, substrate selectivity is

often conferred by specific MAPK docking sites present on physiological substrates. Interestingly, these docking sites often reside at considerable distance in the primary sequence from the MAPK phosphoacceptor site (Kyriakis & Avruch, 2012).

Dual phosphorylation of MAPKs is achieved via a signaling cascade involving a MAPK kinase (MAPKK or MAP2K) that is responsible for phosphorylation of the appropriate MAPK, and a MAP2K kinase (MAPKKK or MAP3K) that phosphorylates and activates the MAP2K (Fig. 1) (Huang *et al*, 2009). There are a total of approximately 20 MAP3Ks in mammalian cells, with each of them receiving and integrating specific upstream signals (Symons *et al*, 2006; Winter-Vann & Johnson, 2007). Upon activation, MAPKs regulate key cellular events in the cytoplasm by phosphorylation of membrane-associated and cytoplasmic proteins including other kinases and cytoskeletal elements. Activated MAPKs also translocate to the nucleus to phosphorylate transcription factors such as c-Jun, c-Fos, Elk-1, c-Myc and other components of the transcriptional machinery to coordinate the expression of downstream target genes (Kyriakis & Avruch, 2012).

In contrast to the high degree of MAPK substrate selectivity, MAP2K and MAP3K frequently function promiscuously in several pathways and are often subject to regulation by multiple stimuli (Biondi & Nebreda, 2003; Kallunki *et al*, 1994; Tanoue & Nishida, 2003). In order to preserve the selectivity and efficiency of the MAPK signaling pathways, there are scaffold proteins that select the particular MAPK pathway elements and bring them together. In some cases, scaffold proteins are distinct polypeptides that bind specific MAPK pathway components. Alternatively, core MAPK signaling components themselves can also possess intrinsic scaffolding properties (Morrison & Davis, 2003).

MAPK phosphatases or Dual Specificity Phosphatases (MKPs or DUSP) are protein phosphatases that dephosphorylate both the phosphothreonine and phosphotyrosine residues on activated MAPKs. Removal of the phosphates renders MAPKs inactive, effectively ensuring a fast and efficient control of the signaling pathway (Kyriakis & Avruch, 2012; Barr & Bogoyevitch, 2001). Depending on the MKP, they can act over one

or several of the MAPKs, such as the ERK specific inactivation by the cytoplasmic MKP-3/DUSP6 or the nuclear MKP-1/DUSP1, which is able to dephosphorylate ERK, JNK and p38 (Owens & Keyse, 2007).

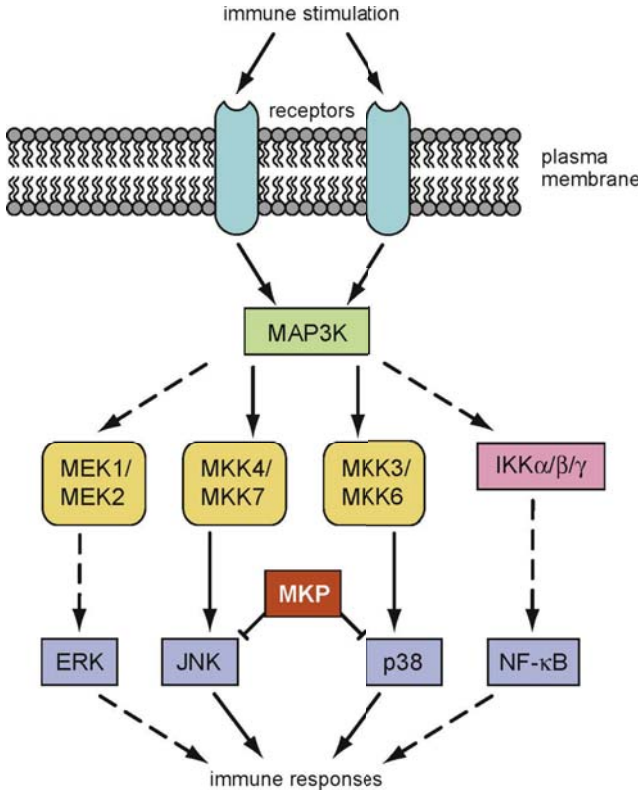

Figure 1. Organization of the MAPK cascade in the immune system. Stimulation of receptors in the immune system results in the activation of the three-tiered kinase module comprised of MAP3K, MAP2K and MAPK through sequential protein phosphorylation. Activated MAPKs are dephosphorylated by MKPs. Certain MAP3Ks such as TAK1 and MEKK3 also contribute to the activation of the IKK/NF-κB pathway. Although not depicted here, p38 can be also activated by MAP2K-independent mechanisms. Adapted from (Huang *et al*, 2009).

JNK pathway

JNK was initially identified and purified as a protein kinase activated in the liver of rodents exposed to cycloheximide and therefore named SAPK (Kyriakis & Avruch, 1990). Later studies identified JNK as a ultraviolet (UV) activated kinase that phosphorylated the c-Jun transcription factor in two residues (Ser63 and Ser73) of the transactivation domain ((Hibi *et al*, 1993; Adler *et al*, 1992; Pulverer *et al*, 1991). Subsequent studies led to the molecular cloning of JNK and the demonstration that it was a member of the MAPK group of signaling proteins (Derijard *et al*, 1994; Kyriakis *et al*, 1994).

The JNKs are encoded by three different genes, *jnk*1 to 3, which are also named *mapk*8 to 10 respectively (Kyriakis *et al*, 1994). All JNKs contain the characteristic Thr-X-Tyr MAPK motive, in particular the in the Thr183-Pro-Tyr185 sequence located in activation loop in the subdomain VIII. Whereas *jnk*1 and *jnk*2 are expressed ubiquitously, *jnk*3 expression is restricted to certain tissues (Gupta *et al*, 1996).

Each *jnk* gene is subjected to two alternative splicing sites, one site in a region between the corresponding subdomains IX and X (resulting the α and β isoforms) and a second one in the 3' terminus of the Coding DNA Sequence (CDS) (resulting the 1 and 2 isoforms). This last site is responsible for the differences in the molecular weight, from 46 to 55 KDa. In contrast to observations made for JNK1 and JNK2, JNK3 isoforms with alternative sequences within protein kinase subdomains IX and X have not been detected (Gupta *et al*, 1996). Accordingly, 10 polypeptides exist but the functional significance of each one remains unclear because of a high redundancy (Fig. 2). In the case of the α and β isoforms apparently differ modestly in their affinities for substrates (Derijard *et al*, 1994; Kallunki *et al*, 1994; Kyriakis *et al*, 1994). Some other isoforms have been predicted but not detected at the protein level yet.

The functional analysis of JNK by gene disruption in mice confirmed that there is extensive complementation between the JNK isoforms and although there are also tissue-specific defects in signal transduction that may reflect the JNK isoform profile of

individual tissues. This complicates the analysis of JNK knockout (KO) mice and indicates the need for studies of compound mutants that lack expression of all JNK isoforms in specific tissues, as double mice *jnk*1/2 KO are not viable (Tournier, 2000).

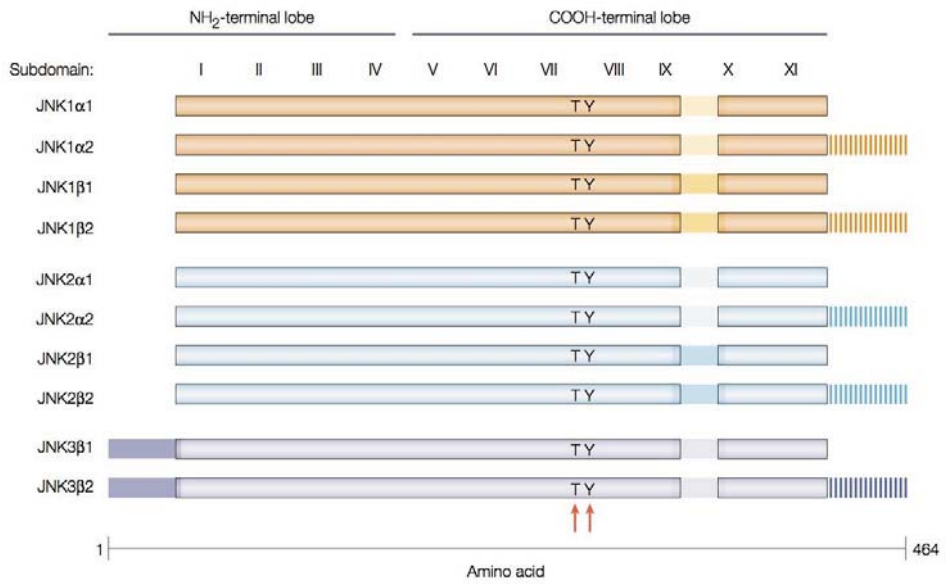

Figure 2. Structural features of the JNK proteins. The JNKs are composed by 11 protein kinase subdomains (indicated as I–XI). The protein kinase activation loop is located between subdomains VII and VIII, and contains the threonine (T) and tyrosine (Y) residues that are phosphorylated for full kinase activation. The members of the JNK family are generated by alternative splicing of three jnk genes to produce ten different isoforms. The shaded regions indicate the differences. There are two key alternative splicing sites: the first is between subdomain IX and X of the COOH-terminal lobe of the corresponding protein, which results in splice forms that demonstrate altered substrate specificity; the second alternative splicing site occurs at the 3' end of the CDS, and results in proteins that differ in length by either 42 or 43 amino acids (depicted as hatched regions) (Adapted from Manning & Davis, 2003).

JNKs, while activated by mitogens, are also vigorously activated by a variety of environmental stresses (heat shock, ionizing radiation, oxidants), genotoxins (topoisomerase inhibitors and alkylating agents), ischemic reperfusion injury, mechanical shear stress, vasoactive peptides, proinflammatory cytokines, PAMPs/DAMPs and by translational inhibitors like cycloheximide (Weston & Davis, 2007; Kallunki *et al*, 1994). For some of the stimuli, like TNF-α or IL-1β, the signaling cascade that leads to the phosphorylation and activation of JNK is well described, while

for other stimuli, such as osmotic stress and UV radiation, there is still some controversy.

In any case, it is known that JNKs are activated by the MAP2Ks, MKK4/SEK1 and MKK7/MEK7 (Rana *et al*, 1996; Yao *et al*, 1997). Comparison of the biochemical properties between them indicates that these protein kinases have different substrate specificity. Thus, MKK4 can activate both JNK and p38 MAPK (Dérijard *et al*, 1995; Lin *et al*, 1995). In contrast, MKK7 selectively activates JNK (Holland *et al*, 1997; Moriguchi *et al*, 1997; Tournier *et al*, 1997). Although both MKK4 and MKK7 are dual-specificity protein kinases that can phosphorylate JNK on Tyr and Thr, these residues are phosphorylated preferentialy by MKK4 and MKK7, respectively (Lawler *et al*, 1998). Because dual phosphorylation of these sites is required for JNK activation (Derijard *et al*, 1994), these in vitro experiments suggest that MKK4 and MKK7 may cooperate to activate JNK. However, JNK activation in response to TNF-α strictly needs MMK7 and MKK4 can not compensate it (Tournier *et al*, 2001).

At the same time, MKK4 and MKK7 are also activated by a double phosphorylation by a MAP3K. In this case there are several kinases capable to do it: MEKK1, ASK1, MLK, TAK1 and TPL2. Even so, there is a large functional redundancy as these MAP3K can activate other signaling pathways. Together, these protein kinases are able to form signaling cascades that can function as defined signaling modules that mediate JNK activation in response to specific stimuli.

As mentioned, scaffold proteins create a functional signaling module and to control the specificity of signal transduction. In the case of JNK there several proteins in charge like the JNK interacting proteins (JIP), Axin or POSH (Plenty of SH_3). These proteins collect specific MAP3K-MAP2K-MAPK core modules into organized signaling oligomers. Gene disruption of these scaffold proteins result in JNK deficient activation whereas overexpression has the opposite effect (Yasuda *et al*, 1999; Whitmarsh *et al*, 2001)

Activated JNK is inactivated through dephosphorylation of threonine and/or tyrosine residues. The dephosphorylation could be achieved by dual phosphatases and, in the case of JNK, the ones in charge are MKP-1/2/X/5/7 and M36 (Kondoh & Nishida, 2007). This way the magnitude and duration of the activation can be regulated.

Downstream JNK

The first described target of JNK was c-Jun, a member of the Activator Protein 1 (AP-1) transcription complex. c-Jun phosphorylation on Ser63 and Ser73 by JNK increases its activity regulating genes implied in cell processes such as proliferation, differentiation and apoptosis. AP-1 complex is a dimmer formed by members of the families JUN, FOS, ATF (Activating Transcription Factor) and MAF (v-maf musculoaponeurotic fibrosarcoma oncogene homolog). These proteins bind to form homo- and heterodimers with distinct binding affinities to different response elements in the DNA like TREs (TPA Response Elements) and CREs (cAMP Response Elements) (Eferl & Wagner, 2003). The activity of these AP-1 complexes can be regulated then for both, the dimmer composition and their post-translational modifications.

Phosphorylation of c-Jun by JNK increases its activity by reducing its ubiquitination (Musti *et al*, 1997) and also by favoring the dissociation of a transcriptional repressor complex that includes the histone deacetylase HDAC3 (Weiss *et al*, 2003). Moreover, activated c-Jun promotes its own gene transcription (Karin *et al*, 1997). In addition, other members of the AP-1 complex like ATF-2 can also be phosphorylated/activated by JNK (Livingstone *et al*, 1995). In the other hand, c-Jun phosphorylation can promote its recognition by the E3 ubiquitin ligase Fbw7 (Nateri *et al*, 2004), and JNK can also phosphorylate and activate Itch, another E3 ubiquitin ligase that targets c-Jun (Gao *et al*, 2004).

Finally, related to the nuclear activity of JNK, it has been described that it binds to a large set of active promoters during the differentiation process of stem cells into neurons (Tiwari *et al*, 2012). Additionally, it binds and phosphorylates the histone H3

which results in an increase in the transcription of the JNK-bound promoters containing phosphorylated H3 (Tiwari *et al*, 2012). Other transcriptional regulators that can be phosphorylated by JNK, a part from the already described c-Jun and ATF-2, are Elk-1, c-Myc and p53 (Lin, 2003; Fuchs *et al*, 1998). Regarding the cytoplasmatic actions of JNK through phosphorylation of different substrates, it has been implicated in a wide range of processes, like inflammation, apoptosis and insulin resistance, which will be described in further sections of this introduction.

Nowadays is also accepted a major role of JNK in the oncogenic transformation and tumor development. For example, it has been stated that JNK phosphorylation of c-Jun is essential for the transformation induced by Ras (Adjei, 2001) or β-catenin/TCF-4 (Nateri *et al*, 2005). Its activity has also been observed to be increased in tumoral cell lines (Davis, 2000) and its inhibition has been associated with a decrease of the tumorigenicity and a sensibilization against anti-cancer drugs (Potapova *et al*, 1997, 2000).

NUCLEAR RECEPTOR SUPERFAMILY

Nuclear Receptors (NRs)

Nuclear hormone receptors are ligand-regulated sequence-specific transcription factors that may activate or repress gene expression. Ligand-activated gene transcription is generally mediated by binding of NR to their cognate DNA elements (Beato *et al*, 1995). Though negative binding elements have been described, repression is mainly conducted by interference with other transcription factors, of which AP-1 and NF-κB are one of the most representatives.

Glucocorticoid Receptor (GR)

Glucocorticoids play a key physiological role in development, cellular proliferation and differentiation. In addition, the prominent pharmacological actions of these hormones have prompted their widespread medical use to treat diverse pathological conditions such as asthma, allergic rhinitis, rheumatoid arthritis and leukemia (Barnes, 1998). Glucocorticoids exert most of their actions by binding to an intracellular regulator that belongs to the NR superfamily.

Hormone-activated GR regulates gene transcription, either positively or negatively, by two major modes of action. Essentially, it can bind to promoters of target genes or interfering with other transcriptional regulators. This mechanism is known as transrepression and are DNA-Independent protein to protein interactions. Important sets of genes transrepressed by GR are those that are under positive control of AP-1 and/or NF-κB. Actually, this is thought to be the mechanism by which glucocorticoids exert their anti-inflammatory and immune-suppressive actions (Herrlich, 2001).

In the case of AP-1, it exerts the inhibition by binding directly to c-Jun (Schüle et al, 1990) and attracting transcriptional correpressors like TIF2/GRIP1 (Rogatsky et al, 2001, 2002). In addition, GR can inhibit JNK activation, and therefore AP-1 activation, by a direct interaction (Caelles et al, 1997; Bruna et al, 2003). Moreover, JNK inhibition by

A successful acute inflammatory response results in the elimination of the infectious agent followed by a resolution and repair phase, which is mediated mainly by tissue-resident and recruited macrophages (Serhan & Savill, 2005). In case it fails to eliminate the pathogen, chronic inflammatory state starts. In addition to persistent pathogens, chronic inflammation can occur as a result of autoimmune responses or undegradable foreign bodies (Medzhitov, 2008).

Inflammation is essential for life and preservation of function, as reflected by the increase of risk of suffering diseases and reduced life span in patients carrying genetic mutations in the principal genes involved in the inflammatory process (Biesma *et al*, 2001; Bunting *et al*, 2002). However, tuning the magnitude of the response is crucial: insufficient responses result in immunodeficiency, which can lead to infection and cancer, and over reaction causes morbidity in diseases such as Crohn's disease, atherosclerosis or rheumatoid arthritis.

Thus, the medical focus is centered in both, inhibiting inflammation and learning how to induce inflammation more effectively. Deeper understanding of the molecular basis involved in the control of the inflammatory processes is fundamental for the design and refinement of new, powerful and specific drugs. Given the fine line in between the healing inflammatory process from the malignant one, a tight, efficient and precise mechanism is needed. The regulation of the inflammation response as been widely studied, resulting the most relevant signaling pathways implicated JNK/AP-1 and IκBα Kinase/Nuclear Factor-κB (IKK/NF-κB).

Moreover, in the last decade has become evident that metabolic and immune response pathways or nutrient- and pathogen-sensing systems have been evolutionarily conserved throughout species. As a result, immune response and metabolic regulation are highly integrated and the proper function of each is dependent on the other. This interface can be viewed as a central homeostatic mechanism, dysfunction of which can lead to a cluster of chronic metabolic disorders, particularly obesity, type 2 diabetes and cardiovascular disease (Hotamisligil, 2006).

Introduction to the NF-κB family

NF-κB was described for first time more than 30 years ago as a nuclear factor required for the transcription of the light chain κ of the immunoglobulins produced by the B-lymphocytes (Sen & Baltimore, 1986). Besides its role in the transcriptional regulation of the immune system, NF-κB is also involved in many other processes such as apoptosis (Yang *et al*, 1993) and oncogenesis (Mayo & Baldwin, 2000).

The NF-κB family is composed by two subfamilies: the Rel and the NF-κB proteins. All of them share at the N-terminus a RHD (Rel Homology Domain) of about 300 amino acids, which is responsible for the dimerization and binding to the DNA. Another important domain is the NLS (Nuclear Localization Sequence). Normally, the Rel members contain a transactivation domain located in their C-terminus, while the NF-κB members do not. As a result of this, the members of the NF-κB subfamily require dimerization with a Rel protein to become transcriptional activators (Gilmore, 2006; Hayden & Ghosh, 2004, 2012) (Fig. 4).

NF-κB activation

In a non-stimulated situation, NF-κB would stay bound to IκB (Inhibitor of NF-κB) in the cytoplasm and therefore, it will remain in an inactive state (Baeuerle & Baltimore, 1988). When the pathway is activated by a stimulus, such as the cytokines TNF-α, IL-1β or LPS (lipopolysaccharide), IκB is degraded so NF-κB is free to go into the nucleus and exert its action.

The extracellular signals detected by specific receptors and, with the collaboration of some adaptor proteins, activate the IκB Kinases (IKKs). The IKK complex is composed of two catalytic subunits (IKKα of IKK1 and IKKβ or IKK2) and two regulatory subunits (IKKγ or NEMO, the NF-κB Essential Modifier) (Fig. 4). Once active, IKKs phosphorylate the IκBs in the residues Ser 32 and Ser 36 (for IκBα). This phosphorylation is detected by the

E3 ubiquitin ligase $SCF^{\beta TrCP}$ and results in the poly-ubiquitination of the inhibitor in the residues Lys 21 and Lys 22 and its fast degradation through the proteasome pathway (Winston *et al*, 1999) (Fig. 5).

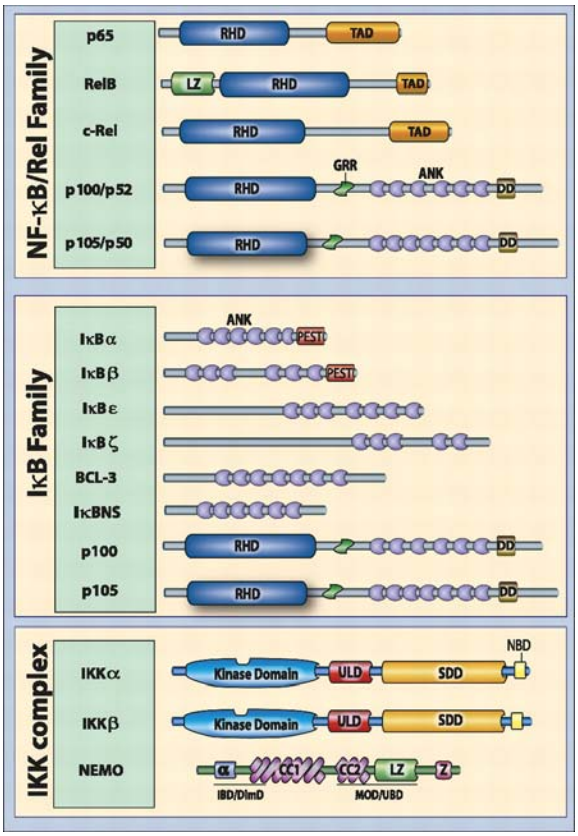

Figure 4. Components of the NF-κB pathway. The mammalian Rel (NF-κB) protein family consists of five members: p65 (RelA), RelB, c-Rel (Rel), and the precursor proteins p100 (NF-κB2) and p105 (NF-κB1), the latter giving rise to p52 and p50, respectively. The IkB family consists of eight bona fide members, IκBα, IκBβ, IκBε, IkBζ, BCL-3, IκBNS, p100, and p105, which are typified by the presence of multiple ankyrin repeat domains. The IKK complex consists of IKKα , IKKβ, and NEMO. Relevant domains typifying each protein family are indicated. (ANK) Ankyrin repeat domain; (DD) death domain; (RHD) REL homology domain; (TAD) transactivation domain; (LZ) leucine zipper domain; (GRR) glycine-rich region; (SDD) scaffolding and dimerization domain; (ULD) ubiquitin-like domain; (Z) zinc finger domain; (CC) coiled-coil domain; (NBD) NEMO-binding domain; (α) α-helical domain; (IBD/DimD) IKK-binding domain/dimerization domain; (MOD/UBD) minimal oligomerization domain/ubiquitin-binding domain; (PEST) proline-rich, glutamic acid-rich, serine-rich, and threonine-rich (Adapted from Hayden & Ghosh, 2012).

There are several IκB (IκBα, IκBβ, IκBγ, IκBε, …), every one with different affinity to the diverse NF-κB dimers and different patterns of phosphorylation and degradation. Structurally, they are characterized by a series of ankyrin-repeat sequences, from five to seven, that interact with the RHD domain from the NF-κB. This way, IκBs not only inhibit the nuclear translocation but also they inhibit their ability to bind to the DNA (Chen & Ghosh, 1999) (Fig. 4).

IκBα is largely the most abundant and studied while the standard NF-κB dimer is the p65/p50. In the binding of IκBα with p65/p50, only the NLS of p65 is masked, causing a balance in between the import-export of the NF-κB complexes form the cytoplasm and nucleus. When the pathway is activated this equilibrium is broken and the degradation of IκBα favors the nuclear translocation of NF-κB (Ghosh *et al*, 2002). In the case of IκBβ and IκBε, they have a more slow degradation so they are especially important in the persistent stimulations of the NF-κB pathway (Hoffmann *et al*, 2002).

For the NF-κB dimers containing the proteins p100 and p105, there is no IκB. The protein itself is masking the NLS and, once the pathway is stimulated and p100 or p105 phosphorylated, the same βTrCP is in charge to remove the IκB-like C terminus allowing the active p52 N-terminal half to function in transcriptional regulation (Xiao *et al*, 2001; Pomerantz & Baltimore, 2002). This route is called the non-canonical pathway (Fig. 5).

Other atypical mechanisms to activate NF-κB have been described, most of them characterized by being IKK-independent. For example, the IκBα phosphorylation by CK2 in response to UV radiation (Kato *et al*, 2003), or the activation by chemotherapeutic drug doxorubicin, that can happen independently of IKK and where the classical Ser 32 and Ser 36 IκBα phosphorylations are not required (Tergaonkar *et al*, 2003).

Once in the nucleus, NF-κB is capable to bind some specific sequences called κB sites and activate transcription of many genes (Hayden & Ghosh, 2004). Actually, some of the genes whose transcription is activated are *IκB*α, constituting a negative feedback

regulatory mechanism to self-limit the activation of the pathway (Brown *et al*, 1993; Ito *et al*, 1994).

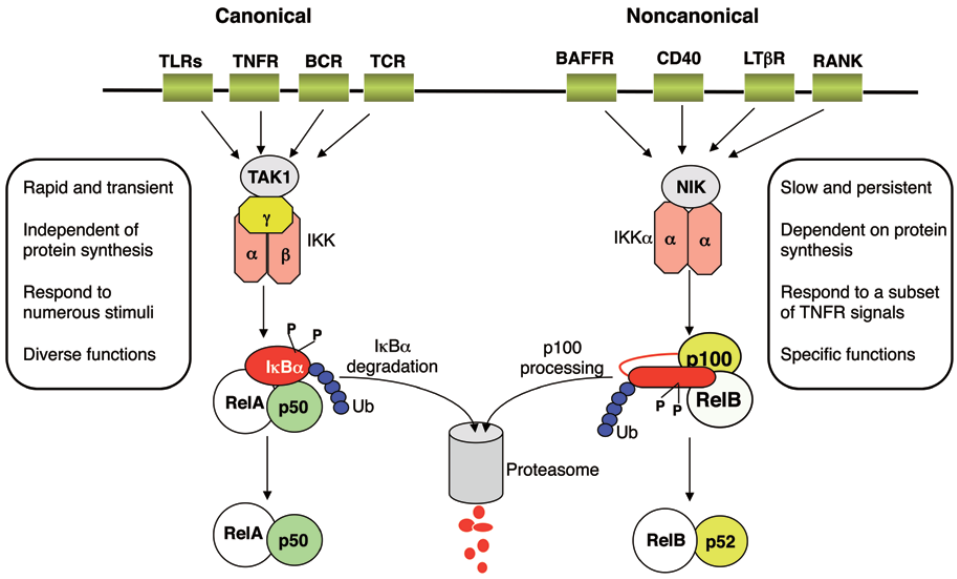

Figure 5. Canonical and non-canonical NF-κB signaling pathways. Canonical pathway is triggered by numerous signals, including those mediated by innate and adaptive immune receptors. It involves activation of IKK complex by Tak1, IKK-mediated IκBα phosphorylation, and subsequent degradation, resulting in rapid and transient nuclear translocation of the prototypical NF-κB heterodimer RelA/p50. Non-canonical NF-κB pathway relies on phosphorylation-induced p100 processing, which is triggered by signaling from a subset of TNFR members. This pathway is dependent on NIK and IKKα, but not on the trimeric IKK complex, and mediates the persistent activation of RelB/p52 complex (Modified from Sun, 2011).

The regulation of the NF-κB may involve other levels of regulation like different protein abundance or post-translational modifications that can alter its activity, such as ubiquitination, sumoylation and acetylation (Perkins, 2006; Natoli & Chiocca, 2008).

Introduction to the $SCF^{\beta TrCP}$ Ubiquitination complex

Ubiquitination of proteins requires three sequential steps that are regulated by three different enzymes. First of all, an E1 ubiquitin-activating enzyme activates ubiquitin by a two-step reaction using ATP as an energy source and resulting in a thioester linkage between the C-terminal carboxyl group of ubiquitin and E1 cysteine sulfydryl group.

Secondly, ubiquitin is transferred from the E1 to a cysteine group in the active site of a ubiquitin-conjugating enzyme E2 via a trans(thio)esterification reaction.

The final step of the ubiquitination cascade creates an isopeptide bond between the side chain of a lysine residue of the target protein and the COOH-terminal glycine residue of ubiquitin. This step requires the activity of an E3 ubiquitin-protein ligase (often termed simply ubiquitin ligase). Repeated rounds of ubiquitination result in the formation of long chains of ubiquitin molecules (poly-ubiquitination), which labels the target protein for 26S proteasomal degradation (Hershko & Ciechanover, 1998).

In most eukaryotes, there is only one E1, a considerable but limited number of E2s (about 60 in mammals), and a much larger number of E3s. In this scheme, the E3s serve the critical role of selecting specific substrates for ubiquitin conjugation (Gao & Karin, 2005). E3 enzymes function as substrate recognition modules of the system and are capable of interaction with both E2 and substrate. Each E3 recognizes a set of substrates and cooperates with one or a few E2s to catalyze substrate ubiquitination. A single E2 can interact with several E3s and thereby affect a large number of targets. Thus, regulation of E3 catalytic activity or the E3-substrate interaction allows for a greater degree of selectivity than regulation of E2 catalytic activity.

In the case of NF-κB activation, IκBα degradation by poly-ubiquitination is a key step and $SCF^{\beta TrCP}$ is the E3 responsible of it. $SCF^{\beta TrCP}$ multiprotein complex forms part of the RING (Really Interesting New Gene Domain) E3 ligase family (Joazeiro & Weissman, 2000), that differs from the other main family, HECT (Homologous to E-AP C-terminus domain), because they enable they direct transfer of the ubiquitin from the E2 to the substrate.

SCF complexes are composed of the scaffold protein Cul1, the RING-domain protein Rbx1/Roc1 (RING box protein/Regulator of cullins 1), the adaptor protein Skp1 (S-phase kinase associated protein 1), and an F box protein that binds the substrate. Rbx1

associates with Cul1 and the E2, while Skp1 interacts simultaneously with Cul1 and with the F box protein, in this case βTrCP (Skowyra *et al*, 1997; Wu *et al*, 2003) (Fig. 6).

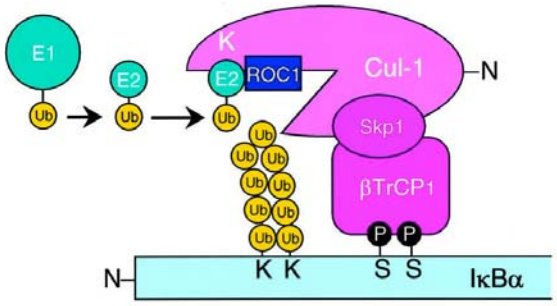

Figure 6. Schematic representation of IκBα ubiquitination. (Adapted from Kawakami *et al*, 2001). See the main text for further explanation.

As an F-Box protein, βTrCP has a characteristic F-Box domain that is used to bind to the adaptor protein. The C-terminal domain of the F-box, the part in charge of the substrate recognition, is variable and determines the three subfamilies: the Fbws, that contain several repeats of WD-40; the Fbls, that have leucine rich repeats; and the Fbxs, that include all the rest (Cenciarelli *et al*, 1999). βTrCP belongs to the first of the subfamilies, the Fbw. Two isoforms have been described: βTrCP1 (or Fbw1) and βTrCP2 (or Fbw2) that have a 75% similarity. Concerning the WD-40 domain, they have seven and four WD-40 repeats each that bind to their substrate at the same motif: DS*GXXS* (where * indicates the residues that have to be phosphorylated) (Winston *et al*, 1999).

A part from IκBα and other members of the NF-κB pathway (IκBβ, IκBε, p100 and p105) (Shirane *et al*, 1999; Fong & Sun, 2002; Lang *et al*, 2003), other substrates for βTrCP have been described: β-catenin, the activator of the transcription factor Tcf; Emi1, the APC (Anaphase-Promoting Complex) inhibitor; and ATF4 (Latres *et al*, 1999; Margottin-goguet *et al*, 2003; Lassot *et al*, 2001). This wide variety of substrates that it is able to recognize and target to degradation gives βTrCP a very important role in the regulation of biological processes like cell cycle, inflammatory response or oncogenesis.

Regulation of the activity of the $SCF^{\beta TrCP}$ complex

As long as the levels of βTrCP in the cell are generally low, it was suggested that the processes that would affect the abundance and localization of βTrCP would be of great importance in the regulation of the activity of the $SCF^{\beta TrCP}$. Indeed, the function of the complex can be downregulated by expressing βTrCP negative dominant forms (Yaron *et al*, 1998) or knocking out βTrCP (Nakayama *et al*, 2003; Guardavaccaro *et al*, 2003b). Oppositely, the overexpression of βTrCP leads to a greater and faster degradation of its substrates (Fuchs *et al*, 1999; Kroll *et al*, 1999).

The two βTrCP paralogs have similar biochemical properties. Regarding its cellular localization, βTrCP1 is mainly in the nucleus, even though its may have cytoplasmic substrates as IκBα. A nuclear protein, hnRNP-u, works as a pseudosubstrate sequestering active βTrCP1 in the nucleus until a higher affinity substrate (phospho-IκBα) appears (Davis *et al*, 2002). The other isoform, βTrCP2, is mainly cytoplasmic, most probably because of its inability to bind hnRNP-u. Even so, its abundance and affinity to IκBα is lower and βTrCP1 seems to be the main responsible of its degradation (Davis *et al*, 2002; Suzuki *et al*, 2000; Putters *et al*, 2011).

Actually, many studies show that the βTrCP isoforms are partially redundant and they are able to compensate each other's function in some situations (Nakayama *et al*, 2003). This has been reported for the degradation of Wee1, IκB, Per1 and the RE1-silencing transcription factor (Rest) (Suzuki *et al*, 2000; Watanabe *et al*, 2004; Shirogane *et al*, 2005; Westbrook *et al*, 2008). Conversely, the early mitotic inhibitor 1 (Emi1) explicitly requires both βTrCP1 and βTrCP2 for its degradation (Guardavaccaro *et al*, 2003b). In addition, genetic evidence from gene knock-out experiments in mice shows that βTrCP2 is essential in early development while deletion of βTrCP1 does not affect viability but impairs spermatogenesis and reduces fertility (Guardavaccaro *et al*, 2003b). βTrCP1 hereafter will be referred as βTrCP.

In relation to protein abundance, it has been described that βTrCP levels can be increased by the wnt and JNK pathways (Spiegelman *et al*, 2000, 2001). Regarding

βTrCP mRNA, it is very unstable, but unlike the majority of mRNAs where 3'UTR determines the rate of mRNA turnover, βTrCP mRNA contains cis-acting destabilizing elements within its coding region. It has been shown that degradation of mRNA of βTrCP is dependant on miR-183, a microRNA that interacts with the coding region of βTrCP mRNA. This binding targets it to degradation through binding to argonaute (Elcheva et al, 2009). The degradation, however, is prevented by the RNA-binding protein CRD-BP (also known as IMP-1 or IGF2BP1) that binds to the coding region of βTrCP mRNA and stabilizes it (Noubissi et al, 2006).

In addition to the mechanisms regulating the abundance and localization of βTrCP, a potential role of post-translational modifications of βTrCP and other members of the complex is likely to happen in the assembly and activity of the $SCF^{\beta TrCP}$ E3 ubiquitin ligases (Fuchs *et al*, 2004). On the other hand, the low availability of βTrCP in the cell, can affect the ubiquitination of some specific target due to the competition with the other targets plus other proteins that can interact with βTrCP, such as the HIV viral protein Vpu (Bour *et al*, 2001; Besnard-Guerin *et al*, 2004).

Furthermore, there exists an extra level of control of the $SCF^{\beta TrCP}$ activity, which is the assembly of the complex. The abundance of the rest of the proteins of the complex in a normal situation is not a limiting factor, but their post-translation modifications have a key role in the assembly and the functionality of the complex. Importantly, CSN (COP9 signalosome) was shown to control Nedd8 modification of the Cul1 subunit (Lyapina *et al*, 2001). Nedd8 conjugation to Cul1 stimulates the activity of SCF, and the CSN inhibits the complex by deneddylating it (Cope *et al*, 2002). In contrast, there are several studies indicating that CSN is required to sustain CRL activity in cells (Bosu & Kipreos, 2008). This apparent paradox is resolved by the observation that CSN-mediated inactivation of $SCF^{\beta TrCP}$ counteracts autocatalytic breakdown of the F-Box and adaptor proteins (Cope & Deshaies, 2006; Wee *et al*, 2005).

JNK and NF-κB

As mentioned before, JNK and NF-κB are key signaling cascades in many biological functions. Several of these processes are shared, like inflammatory response and apoptosis, so, as it would be expected, their activity is coordinated. In this regard, they have both activators and targets in common. Both pathways can by activated by the MAP3K MEKK1 (Hirano *et al*, 1996; Lee *et al*, 1997), by IRAK-1 (Li *et al*, 2001), by TRAF6 (Song *et al*, 1997; Cao *et al*, 1996) and by PKCζ (Castrillo *et al*, 2003; Leitges *et al*, 2001). Concerning the downstream targets, they are able to activate a wide number of genes implicated in inflammation, apoptosis and cancer (Read *et al*, 1997; Kanakaraj *et al*, 1998).

The possibility of the existence of a crosstalk in between the two pathways has been studied in the last years by many groups in the field, resulting in the discovery of a complex interacting network at different levels and with distinct nature depending on the stimuli and cell type.

JNK/NF-κB activation in response to TNF-α

TNF-α is proinflammatory cytokine mainly produced by the activated macrophages and plays an important role in the septic shock, induction of other cytokines, proliferation, differentiation and apoptosis. Moreover it has been related to diseases such as malaria, SIDA or cancer. Many of the TNF-induced cellular responses are mediated by either one of the two TNF receptors, TNFR1 and TNFR2, both of which belong to the TNF receptor super-family (Loetscher *et al*, 1990; Smith *et al*, 1990). In response to TNF-α treatment, the transcription factor NF-κB and MAP kinases, including JNK, are activated in most types of cells and, in some cases, apoptosis or necrosis could also be induced (Smith *et al*, 1994).

The pathogenic stimuli induce the TNF-α production by macrophages and T-cells. TNF-α is primarily produced as a 212-amino acid-long type II transmembrane protein arranged

in stable homotrimers. From this membrane-integrated form, a soluble form is released via proteolytic cleavage by the metalloprotease TNF-α converting enzyme (TACE) (Black *et al*, 1977). Then TNF-α triggers the local expression of chemokines and cytokines, promoting the adhesion, extravasation, attraction, and activation of leukocytes at the site of infection. Later, TNF-α facilitates transition from innate to acquired immunity by enhancing antigen presentation and T cell costimulation (Varfolomeev & Ashkenazi, 2004)

When TNFR1 binds to TNF-α, the conformation of the intracellular domain is changed such it can interact with TNFR-associated factor containing death domains (TRADD), which in turn recruits TNFR-associated factors (TRAFs) including TRAF2 and TRAF5, as well as the cellular inhibitor of apoptosis proteins 1 and 2 (c-IAP1/2) to form the TNF receptor signaling complex (TNF-RSC), which is important for both the activation of IKK and the MAPK pathways, including JNK and p38 (Varfolomeev *et al*, 2012). In the case of TNFR2, it does not contain a binding domain for TRADD, but it is able to form a complex with TRAF2 and TRAF5.

A linear ubiquitin assembly complex (LUBAC) containing HOIL-1, HOIP and Sharpin has recently been identified (Ikeda *et al*, 2011). LUBAC is recruited to the TNF-RSC by TRADD, TRAF2/5 and c-IAP1/2 (Fig. 7). LUBAC stabilizes TNF-RSC, but it also ubiquitinates the receptor-interacting protein 1 (RIP1) and the regulatory subunit of IKK (also called NEMO) (Niu *et al*, 2011) (Fig. 7). This results in the formation of the IKK complex and the TAK1 (TGFβ-activated kinase 1)/TAB1/2 (TAK1 binding protein 1 and 2) complex.

Polyubiquitinated RIP1 triggers TAK1 activation, which in turn activates IKKα/β, JNK and p38 leading to NF-κB and AP-1 activation, respectively. AP-1 and NF-κB bind to DNA binding sites located in the promoter regions of their target genes and initiate gene expression.

In the case of NF-κB, a LUBAC/TAK-1 independent activation can happen in the non-canonical pathway. Upon TRAF binding to a TNFR, the NF-κB-inducing kinase (NIK) is displaced from the TRAF complex. As a result of displacement of NIK, NIK binds and phosphorylates IKKα, leading to activation of IKKα kinase activity (Sanjo *et al*, 2010).

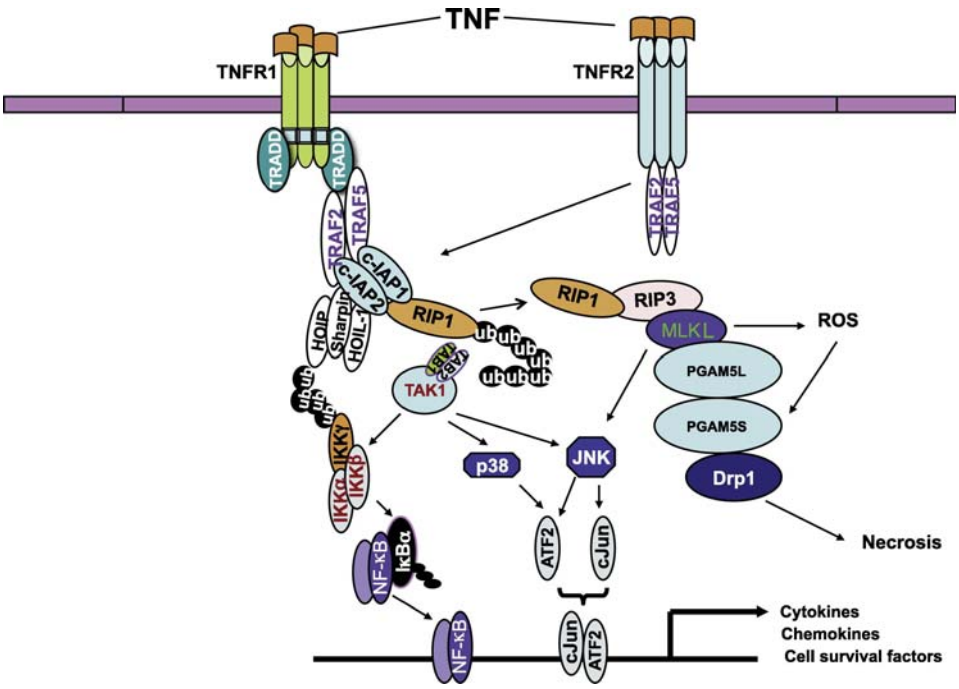

Figure 7. A model of TNF-α signaling in inflammation, apoptosis and necrosis. TNF binds to TNFR1 and TNRF2. TNFR1 interacts with TRADD, in turn recruiting TRAF2, TRAF5 and c-IAP1/2. RIP1 and LUBAC are then recruited, leading to ubiquitination of RIP1 and IKKγ and the formation of the IKK and TAK1 complexes. Polyubiquitinated RIP1 triggers TAK1 activation, which in turn activates IKKα/β, JNK and p38 leading to NF-κB and AP-1 activation, respectively (Adapted from Chu, 2012).

JNK/NF-κB interaction in response to TNF-α

The interaction between JNK and NF-κB is essential in regulation of TNF-α induced apoptosis. It has been demonstrated that NF-κB controls the expression of various anti-apoptotic proteins including XIAP, IAP and GADD45α, which inhibit caspase-dependent apoptosis, as well as genes that inhibit JNK activation (Wang *et al*, 1998; Barkett & Gilmore, 1999; Smaele *et al*, 2001). In this regard, TNF-α induces TRAF2 phosphorylation at serines 11 and 55 and that leads to a prolonged IKK activity and NF-

κB–dependent gene expression (Blackwell *et al*, 2009; Thomas *et al*, 2009). This activation exerts an inhibitory action over of JNK, protecting the cells against oxidative stress–induced cell death (Zhang *et al*, 2011a).

In the other hand, JNK is not only involved in several forms of cell-specific and stress-induced apoptosis but also JNK activation has also been described as an antiapoptotic agent by phosphorylating the apoptosis regulator protein BAD (Yu *et al*, 2004). Therefore, a balance in between JNK and NF-κB is decisive for the cell survival/apoptosis and the inhibition of NF-κB activation would be required for prolonged JNK activation, which would results in apoptosis (Smaele *et al*, 2001; Zhang *et al*, 2004).

Moreover, oxidative stress can also partly activate JNK pathway. This occurs when the levels of reactive oxygen species (ROS) in the cells are higher than the capacity of antioxidant factors they have. In response to persistent TNFα stimulation, ROS formation and accumulation induces cell damage by inhibiting NF-κB activity and activating JNK (Bubici *et al*, 2006).

In the case of NF-κB, it has been shown that various antioxidant enzyme genes including manganese-dependent SOD (MnSOD), metallothionein, glutathione S-transferase, and ferritin heavy chain (fhc), are induced by TNF-α in an NF-κB-dependent fashion (Pham *et al*, 2004), which would prevent ROS accumulation and JNK activation. At some point, however, ROS are accumulated either by impaired induction of antioxidant enzymes or because NF-κB fails to suppress some latent signaling cascades and therefore induce ROS (Nakano *et al*, 2006).

This accumulation of ROS inactivates the MKPs (Kamata *et al*, 2005) and also induces ASK1 (Tobiume *et al*, 2001; Matsuzawa *et al*, 2005). Hence, ROS activates the JNK pathway downstream of TNFRs both by inducing MAP3Ks and inhibiting MKPs (Zhang *et al*, 2011a). Being JNK activated but not NF-κB signaling, apoptosis will occur.

Several studies demonstrate that the proapoptotic JNK cascade ultimately induces apoptosis via the mitochondria-dependent pathway. JNK phosphorylates members of the Bcl-2 family of proteins, such as Bcl-2 and Bcl-xL, and inactivates their antiapoptotic function (Yamamoto *et al*, 1999; Fan *et al*, 2000). Furthermore, JNK also activates proapoptotic members of the Bcl-2 family, Bim and Bmf, and has a role in the induction of the caspase 8-independent cleavage of Bid (Deng *et al*, 2003). Finally, it has been reported that JNK phosphorylates the 14-3-3 protein, a cytoplasmic anchor of Bax, and that phosphorylated 14-3-3 fails to sequester Bax into the cytoplasm, therefore inhibiting its translocation to the mitochondria (Tsuruta *et al*, 2004).

In conclusion, the biological response of cells to TNF-α is determined by both the physiological context and the time course of JNK/NF-κB signaling. Essentially, JNK and NF-κB would be transiently activated for 10 to 30 min, which would favor survival. After NF-κB activation, JNK would be turn down to inhibit its pro-apoptotic effects. However, if the stimuli persists for more than 6 hours, then ROS will accumulate causing NF-κB inhibition and JNK activation, in this case in a relatively smaller scale but more persistent, what would lead to apoptosis (Ventura *et al*, 2006).

JNK effect on βTrCP

In addition to collaborate and antagonize in response to TNF-α, a positive interaction of JNK over NF-κB pathway has been described. Activation of JNK leads to accumulation of βTrCP mRNA in a transcriptional-independent manner (Spiegelman *et al*, 2001). Therefore, IKK and JNK collaborate in response to stress to increase IκBα degradation and NF-κB activation. However, JNK is not sufficient to activate NF-κB but is able to multiply the activation started by IKK. This is explained because despite the increase in βTrCP, IκBα still needs to be phosphorylated by the IKK so it is targeted to degradation (Spiegelman *et al*, 2001). Nonetheless, JNK is necessary as NF-κB is not activated in MEFs JNK1/2 KO. At the same time, JNK favors the assembly of the $SCF^{\beta TrCP}$ complex and the activated form can be found bound to it (Arevalo, 2008).

It is also of particular interest that some JNK actions besides NF-κB activity are also mediated through $SCF^{\beta TrCP}$, like the ubiquitination of the cell cycle phosphatase Cdc25B (Uchida *et al*, 2011). In this case, JNK may be targeting the Cdc25B for ubiquitination by phosphorylation but also increasing the amount of ubiquitination complex.

These crosstalk events have been related to be present in some inflammatory pathologies, like rheumatoid arthritis, and other biological processes, such as chemotherapeutic resistance. Clarifying the exact mechanisms might help the development of new pharmacological strategies.

GLUCOSE HOMEOSTASIS AND INSULIN SIGNALING

Glucose homeostasis

Glucose is a monosaccharide that represents an essential biological energy source, enabling the generation of ATP following glycolysis. Although many tissues can also use fats and protein as an energy source, red blood cells can only use glucose and the brain does it preferentially. Glucose is stored in the body, importantly in the liver and the skeletal muscle, as glycogen. Circulating levels of glucose are controlled in an opposite manner by two hormones: insulin and glucagon.

In response to high blood glucose levels, insulin is released to the blood stream from β-cells in the islets of Langerhans in the pancreas. Insulin stimulates the glucose uptake (through the membrane transporters Glut) and storage in the tissues as glycogen (glycogenesis). The individual Glut subtypes differ in their tissue distribution, substrate specificity, kinetic properties, and intracellular localization, allowing members of the Glut family to finely regulate whole-body glucose homeostasis. The more important glucose transporters are Glut1, specially important in fetal tissues and adult erythrocytes; Glu2, which is a bidirectional transporter present in liver, renal and pancreatic β-cells; Glut3, expressed in neurons; and Glut4, found in adipose tissues and the skeletal muscle.

On the other hand, low glucose levels cause secretion of glucagon from pancreatic α-cells. Glucagon promotes the conversion of liver glycogen to glucose (glycogenolysis) and release of glucose back into the blood thanks to the bidirectionality of its glucose transporter Glut2. In contrast, in pancreatic β-cells, free flowing glucose is required so that the intracellular environment of these cells can accurately gauge the serum glucose levels

During starvation and intense exercise, glucose can also be generated from non-carbohydrate precursors (i.e. pyruvate, amino acids and glycerol), in a process called gluconeogenesis that takes place in liver and kidney.

Insulin signaling

Insulin circulates through the blood stream until it finds an Insulin Receptor (IR) embedded in the cell membrane. The main endocrine insulin target tissues are skeletal muscle, liver and adipose tissue. Insulin also has an autocrine function by stimulating the release of new insulin and inducing its own gene expression (Muller *et al*, 2006), together with other glucose homeostasis related genes such as Glut2.

The binding of ligand to extracellular domain of the IR induces structural changes within the receptor leading to autophosphorylation of various tyrosine residues in its intracellular domain. These changes enable the recruitment of specific adapter proteins such as the IR substrate proteins (IRS). Multiple tyrosine residues of IRS itself are then phosphorylated by hormone bound-IR. This enables IRS engage and activate several signaling pathways.

Among them, PI3K has a major role in insulin signal transduction, mainly via the activation of the Akt/PKB cascade (Cross *et al*, 1995; Alessi *et al*, 1996). Activated Akt induces glycogen synthesis, through inhibition of GSK-3 (Weeren *et al*, 1998); protein synthesis via mTOR and downstream elements (Pullen *et al*, 1998); cell survival, through inhibition of several pro-apoptotic agents (Datta *et al*, 1997) and glucose uptake through Glut translocation from vesicles to the cytoplasmic membrane (Tanti *et al*, 1997) (Fig. 8). At the same time it produces a JNK-mediated negative feedback results in serine phosphorylation and inactivation of IRS signaling (Lee *et al*, 2003) (Fig.9).

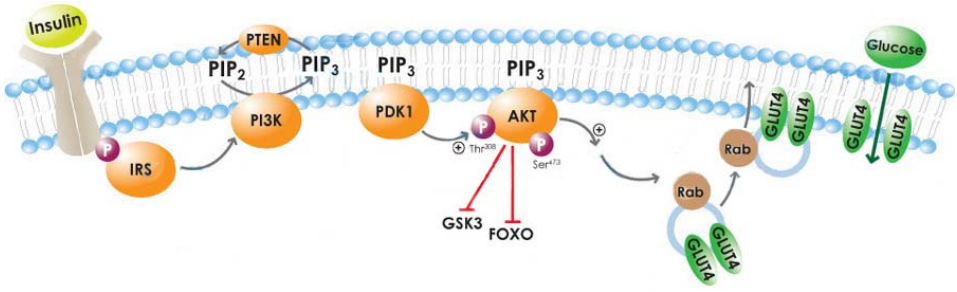

Figure 8. Schematic representation of Insulin signaling pathway. (Adapted from www.caymanchem.com). See the main text for further explanation.

Diabetes Mellitus

Type 1 and type 2 diabetes are conditions in which glucose cannot be properly moved into the cells, either due to a lack of insulin or due to cellular resistance to insulin. This creates two problems: high blood glucose levels (hyperglicemia) and a depletion of stored glucose. When the blood glucose concentration is higher 9-10 mmol/L, reabsorption of glucose in the proximal renal tubules is incomplete, and part of the glucose is excreted through the urine (glycosuria). This increases the osmotic pressure of the urine and inhibits reabsorption of water by the kidney, resulting in increased urine production (polyuria). Lost blood volume causes dehydration and increased thirst.

Type 1 diabetes results from the body's failure to produce insulin due to the autoimmune destruction of the pancreatic β-cells. In contrast, type 2 diabetes results from insulin resistance, a condition in which cells fail to respond to insulin properly.

The incidence of type 2 diabetes is increasing alarmingly especially as a result of the tremendous increase in human obesity, a major risk factor for the development of this disease. Type 2 diabetes is characterized by defects in both insulin sensitivity and insulin secretion – either of which may predominate – that result in glucose intolerance and hyperglycemia with severe consequences. The obesity epidemic is caused by changes in eating habits and a decrease in physical activity, which together lead to an increased energy intake and reduced energy expenditure. Consequently, there is a massive expansion of adipose tissue. Obesity is often associated with a low-grade, chronic inflammatory state.

Moreover impaired insulin signaling also contributes to elevated plasma FFA levels. Insulin resistance leads to an increased release of FFA from adipose tissue that reduces skeletal muscle FFA uptake. The net consequence of this may be an augmented influx of FFA to the liver, which leads to fatty liver and exacerbates IR (Pansuria *et al*, 2012).

JNK and insulin resistance

In fact, the increased levels of pro-inflammatory cytokines, such as TNF-α, interleukin IL-1β, IL-6, and others, have a major role in promoting peripheral insulin resistance (Gregor & Hotamisligil, 2011). Furthermore, other conditions found in obesity, namely the induction of endoplasmic reticulum stress caused by adipocyte hypertrophy and the increased plasma level of FFAs, also contribute to peripheral insulin resistance (Ozcan *et al*, 2004; Solinas *et al*, 2006). At the molecular level, several serine/threonine protein kinases are activated by these stimuli, including JNK and the IKK, which both target IRS-1 for serine phosphorylation (Fig.9). As a result, IRS-1 recruitment to the hormone-bound IR is inhibited, thereby preventing activation of the insulin-signaling cascade (Solinas & Karin, 2010). In contrast, in normal conditions, insulin-bound IR triggers IRS-1 tyrosine phosphorylation, thus allowing the recruitment and activation of downstream effectors, such as the phosphatidylinositol 3-kinase (PI3K)-Akt cascade (Taniguchi *et al*, 2006) (Fig.9).

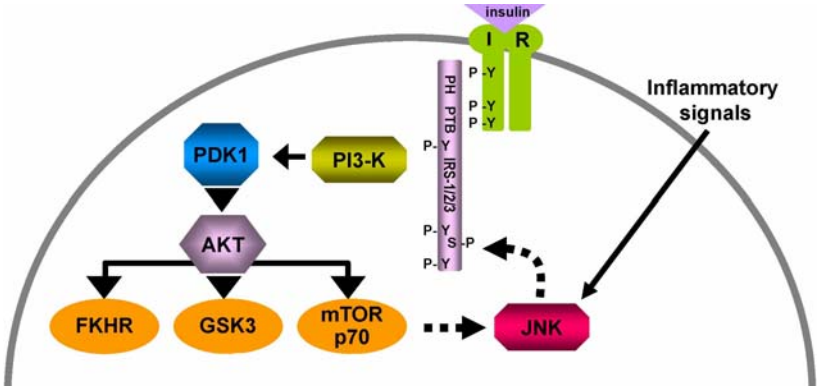

Figure 9. JNK pathway in the regulation of insulin signaling. Insulin acts on its receptor to increase tyrosine phosphorylation of the IR and IRS. Tyrosine phosphorylated IRS proteins lead to activation of the PI3 kinase pathway to increase AKT phosphorylation. Increased AKT phosphorylation regulates the activation of mTOR/p70 (S6K). Activation of mTOR signaling has been shown to trigger IRS-1 degradation, a feedback inhibition for insulin signaling, through kinases that increase serine phosphorylation of IRS-1, such as JNK. Inflammatory signals lead to insulin resistance through several inflammatory kinases, including JNK (Yang & Trevillyan, 2008).

In both dietary and genetic mouse models of obesity, there is a significant increase in JNK activity in peripheral insulin-target tissues, such as skeletal muscle, adipose tissue and liver, which promotes insulin resistance (Hirosumi *et al*, 2002). Consistently, the administration of the JNK inhibitory peptide JNKi-1 to these mice markedly improves insulin signaling in these tissues by reducing IRS-1 serine phosphorylation and in doing so increasing Akt phosphorylation (Kaneto *et al*, 2004).

In addition to the negative interaction of JNK with IR signaling in peripheral insulin-responsive tissues, this kinase has also been implicated in promoting insulin resistance in pancreatic β-cells. In particular, FFA treatment results in sustained JNK activation in these cells concomitantly with the inhibition of the autocrine insulin action as a result of JNK-mediated IRS-1 and IRS-2 phosphorylation at serine residues that interfere with their binding to activated IR (Solinas *et al*, 2006). Accordingly, treatment with JNKi-1 or jnk1 deficiency relieves the inhibition of glucose-induced insulin transcription by FFAs and enhances obesity-inhibited and glucose-induced insulin secretion in isolated pancreatic islets (Solinas *et al*, 2006; Kaneto *et al*, 2004; Bennett, 2003; Varona-Santos *et al*, 2008). Insulin signaling within the β-cells has also been shown to play a critical role in maintaining the essential function of the β-cells (Wang *et al*, 2010).

JNK and apoptosis in pancreatic β-cells

JNK is involved not only in the inhibition of insulin secretion but also in the loss of pancreatic β-cells induced by pro-inflammatory stimuli such as IL-1β (Ammendrup *et al*, 2000; Kim *et al*, 2005). In this regard, treatment of insulin-secreting cell lines or cultured pancreatic islets with JNK inhibitors, such as JNKi-1, SP600125 or the TZD rosiglitazone, prevents IL-1β-induced apoptosis (Díaz-Delfín *et al*, 2007; Bennett, 2003; Bonny *et al*, 2001). In addition, *jnk*1-deficient islets are more resistant to cytokine-induced cell death than wild type or *jnk*2-deficient islets (Varona-Santos *et al*, 2008).

Finally, activation of the JNK pathway may also be relevant for islet transplantation since it is induced during islet isolation (Abdelli *et al*, 2004); moreover, JNK inhibitors

have been shown to preserve whole-islet mass (Fornoni *et al*, 2008; Noguchi *et al*, 2005) and prevent islet apoptosis (Noguchi *et al*, 2005) and graft loss (Noguchi *et al*, 2007).

Nonetheless, in vivo data supporting these roles of JNK have been obtained using mainly genetic and/or chemical inhibition of the kinase, tools that allow the assessment of JNK requirement but not JNK sufficiency to achieve all these effects.

JNK and obesity

The rapid increase in the prevalence of obesity is a major global health problem. Overweight is a known risk factor for many diseases like type 2-diabetes, non-alcoholic fatty liver disease (NAFLC), hypertension, stroke as well as some forms of cancer. The production of proinflammatory cytokines is important early events in the development of obesity-associated complications. As explained in previous sections of this introduction, JNK has a central role in obesity and insulin resistance and it has been shown that its activity in the peripheral tissues is abnormally elevated in obesity.

So far studies using JNK1 knockout mice have demonstrated that they exhibit protection from diet-induced obesity, glucose intolerance, and insulin resistance (Hirosumi *et al*, 2002). In order to recapitulate the protection of the conventional knockout, JNK1 has been conditionally inactivated in classical insulin sensitive tissues including adipose tissue, muscle and liver (Sabio *et al*, 2009, 2008, 2010) but also in the hypothalamus and the pituitary gland (Velloso *et al*, 2008; De Souza *et al*, 2005; Bruning *et al*, 2000). Second, JNK inhibition prevents a state of β-cell dysfunction, which might contribute to the development of overt diabetes (Kaneto *et al*, 2004; Bennett, 2003).

On the other hand, some studies connect lack of insulin secretion and protection to high-fat diet-induced obesity. Some examples are the cholecystokinin gut-deficient mice (Lo *et al*, 2011, 2010); the β-pancreatic cells specific fatty acid receptor GPR40 knockout mice (Kebede *et al*, 2008; Steneberg *et al*, 2005); and the Cav1.2 calcium

channel β-cell depletion (Schulla *et al*, 2003; Uebele *et al*, 2009). All those animals are significantly leaner and they exhibit protection against HFD induced-insulin resistance.

Besides that, obesity is associated with increased risk of development of severe Acute Pancreatitis (AP) through unclear mechanisms that may include augmented necrosis of intra- and peripancreatic fat, fatty pancreas, alterations in the network of cytokines and adipokines, metabolic dysregulation, and reduced respiratory excursion. It has been demonstrated that Rosiglitazone prevents development of severe AP in HFD-fed mice through a mechanism including PPARγ (Pini *et al*, 2012). In the same line, JNK1 signaling plays an essential role in macrophage induced β-cell apoptosis and the development of hyperglycemia in streptozotocin-induced pancreatic injury (Fukuda *et al*, 2008).

JNK and insulin in ageing

Ageing is a complex process of accumulation of molecular, cellular and organ damage, which leads to a loss of function an increase in the vulnerability to disease and death. Despite the complexity of ageing, it has been shown that dietary and genetic alteration can substantially increase healthy life span of laboratory model organisms.

Essentially, when the food intake of organisms such as yeast and rodents is reduced to a certain extent (dietary restriction), they live longer than organisms fed a normal diet. A similar effect is seen when the activity of nutrient-sensing pathways, such as the Insulin-like Growth Factor-1 (IGF-1)/insulin and the TOR (target of rapamycin) pathways, is reduced by mutations or chemical inhibitors suggesting that they may induce a physiological state similar to that resulting from periods of food shortage (Fontana *et al*, 2010). Actually, caloric restriction, the most proven treatment known to increase mammalian lifespan, invariably reduces circulating IGF-I and insulin levels (Gems & Partridge, 2001).

In mice, as in yeast, worms, and flies, reduced activity of nutrient-sensing pathways can increase mouse life span. Mutations in growth hormone (gh) and *igf*-1 genes can

substantially increase life span in mice (Bartke, 2005), although precisely how is as yet poorly understood. Moreover, inhibition of the mTOR pathway, either with rapamycin or deletion of ribosomal S6 protein kinase 1 (S6K1), increases mouse life span and also reducing incidence of age-related pathologies, including bone, immune, and motor dysfunction (Harrison *et al*, 2009; Selman *et al*, 2009). In the case of rapamycin it has been recently described that inhibition of mTORC1 is the responsible for the for the increased lifespan whereas inhibition of mTORC2 would be mediating the metabolic side effects of this drug like insulin resistance (Lamming *et al*, 2012)

The insulin/IGF signaling (IIS) pathway in worms and flies regulates body weight, ageing and is a life span determinant (Holzenberger *et al*, 2003; Cohen *et al*, 2009). Null mutations of the insect insulin-receptor substrate Chico, which acts downstream from IR, also extends lifespan (Clancy *et al*, 2001).

In vertebrates, the IR regulates energy metabolism whereas IGF-1R promotes growth (Lupu *et al*, 2001). Longevity increases in several mouse strains with low global activity of the growth hormone (GH)/IGF-1 axis. Life expectancy increases in mice that are heterozygous in terms of inactivation of the IGF-I receptor in whole body (Holzenberger *et al*, 2003). Longevity also increases in mouse models with reduced IGF-I bioavailability due to knockout of the IGF binding protein (IGFBP) specific protease PAPP-A (Conover & Bale, 2007), and in mice with defective IGF-1 signaling downstream of the IGF-1 receptor such as mice lacking the IGF-I receptor substrate p66shc (Migliaccio *et al*, 1999) and mice that overexpress KLOTHO (Kurosu *et al*, 2005), an hormone that actually also inhibit insulin signaling.

It seems clear then that IGF-1 is responsible for lifespan regulation in vertebrates, but the contribution of insulin signaling still remains unclear. Actually they share many of the downstream mediators, such as PI3K and Akt, and insulin and IGF-1 receptors share structural and functional homology to a large extent. They are activated by their own ligands, but at high concentrations of the ligands, they can also cross-react with each other's receptors. Therefore effects found when stimulating with high insulin

concentrations may actually be due to activation of the IGF-IRs (Johansson & Arnqvist, 2006). Moreover, there have been described hybrid receptors, consisting of an insulin receptor αβ-dimer and an IGF-IR αβ-dimer, in tissues where cells coexpress IRs and IGF-IRs (Bailyes *et al*, 1997; Moxhams *et al*, 1989).

Concerning insulin signaling, reduction of IR function in humans results in type 2-diabetes and homozygous loss of IR function results in leprechaunism, which usually leads to death within the first year of life (Wertheimer *et al*, 1993), and neonatal lethality in mice (Accele *et al*, 1996). However, complete loss of function of the insulin/IGF receptor also results in lethality in flies and worms (Gems & Partridge, 2001).

In the same line, mild insulin resistance or inhibited insulin sensitivity, either natural or rapamycin-induced, has been suggested to extend lifespan and also to prevent some typical diabetic diseases like retinopathy and nephropathy (Blagosklonny, 2012). Actually treatment with rapamycin also causes a reduction of insulin secretion, similarly to what happens with CR. Rapamycin is a known inhibitor to mTOR, which activates S6 kinase (S6K) and consequently causes degradation of insulin-receptor substrates (IRS), thus impairing insulin signaling (Harrison *et al*, 2009). However, there are pieces of the mechanism still to be elucidated because decreased mTORC1 signaling was sufficient to extend life span independently from changes in glucose homeostasis (Lamming *et al*, 2012).

Regarding JNK, worms with increased expression of JNK have extended lifespan under normal conditions (Oh *et al*, 2005). Actually one of the mechanisms that has been proposed is its interaction with the IIS/Insulin signaling pathway (Biteau *et al*, 2011). Similar consequences of JNK activation have been described in *D. melanogaster*. It has been described that moderate activation of JNK activation signaling results in increased stress tolerance and extended lifespan by inhibiting cellular and organism-wide responses to insulin signaling in flies (Wang *et al*, 2005). However nothing has been studied in mammals.

NUCLEAR RECEPTOR SUPERFAMILY

Nuclear Receptors (NRs)

Nuclear hormone receptors are ligand-regulated sequence-specific transcription factors that may activate or repress gene expression. Ligand-activated gene transcription is generally mediated by binding of NR to their cognate DNA elements (Beato *et al*, 1995). Though negative binding elements have been described, repression is mainly conducted by interference with other transcription factors, of which AP-1 and NF-κB are one of the most representative.

Glucocorticoid Receptor (GR)

Glucocorticoids play a key physiological role in development, cellular proliferation and differentiation. In addition, the prominent pharmacological actions of these hormones have prompted their widespread medical use to treat diverse pathological conditions such as asthma, allergic rhinitis, rheumatoid arthritis and leukemia (Barnes, 1998). Glucocorticoids exert most of their actions by binding to an intracellular regulator that belongs to the NR superfamily.

Hormone-activated GR regulates gene transcription, either positively or negatively, by two major modes of action. Essentially, it can bind to promoters of target genes or interfering with other transcriptional regulators. This mechanism is known as transrepression and are DNA-Independent protein to protein interactions. Important sets of genes transrepressed by GR are those that are under positive control of AP-1 and/or NF-κB. Actually, this is thought to be the mechanism by which glucocorticoids exert their anti-inflammatory and immune-suppressive actions (Herrlich, 2001).

In the case of AP-1, it exerts the inhibition by binding directly to c-Jun (Schüle *et al*, 1990) and attracting transcriptional correpressors like TIF2/GRIP1 (Rogatsky *et al*, 2001, 2002). In addition, GR can inhibit JNK activation, and therefore AP-1 activation, by a direct interaction (Caelles *et al*, 1997; Bruna *et al*, 2003). Moreover, JNK inhibition by

GR can also be transcriptionaly mediated, as GR can induce MKP-1 gene transcription (Kassel *et al*, 2001). In addition to that, inhibition of JNK by dexamethasone may also affect the activity of NF-κB through βTrCP; hence it is also contributing to transrepression of NF-κB by the Glucocorticoid Receptor (GR) (Arevalo, 2008).

Peroxisome proliferator-activated receptor γ (PPARγ)

The interaction between JNK and nuclear receptors has also been described for other members of the NR family like Peroxisome proliferator-activated receptor γ (PPAR-γ), which has been implicated in distinct physiological and pathological processes, such as adipogenesis, insulin sensitivity, type 2 diabetes, atherosclerosis, inflammation, and cancer (Rosen & Spiegelman, 2001).

As well as GR, PPARγ also negatively modulates the AP-1 and NF-κB pro-inflammatory pathways. It has been demonstrated that PPARγ inhibits activation of AP-1 and JNK activity and reduces activity of IKK and consequent degradation of IκBα, resulting in reduced activation of NF-κB (Zingarelli *et al*, 2003). In a similar manner, the hypoglycemic action of thiazolidinediones (TZDs), a group of synthetic PPARγ ligands with insulin-sensitizing activity, is mediated by the inhibition of JNK (Díaz-Delfín *et al*, 2007).

OBJECTIVES

The general objective of this thesis is the study of the JNK pathway cross talk with the NF-κB, nuclear receptors and insulin signaling pathways, respectively.

Detailed objectives:

1. Gain insight on the mechanism(s) by which JNK increases βTrCP mRNA and protein levels

2. Study the phenotype regarding the glucose homeostasis of a transgenic mouse with JNK specifically activated in the β-pancreatic cells in standard conditions.

3. Study the phenotype regarding glucose homeostasis of a transgenic mouse with JNK specifically activated in the pancreatic β-¬cells subjected to challenges such as high-fat high-carbohydrate induced obesity or ageing.

MATERIALS AND METHODS

Cell culture

HEK293T and Mice Embryonic Fibroblasts (MEFs) cells were cultured in a 5% CO_2 atmosphere and 37ºC in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS (Fetal Bovine Serum), L-glutamine (2 mM), penicillin and streptomycin (100 I.U./mL and 100 µg/mL, respectively).

Transfection

HEK293T cells were transfected using different expression plasmids (Table 1) with Polyethyleneimine (PEI) (Polyscience, Inc) (Boussif *et al*, 1995). Briefly, the plasmid DNA and the PEI were mixed in the wright proportion in 150 mM NaCl and after 30 minutes of incubation, the transfection mixture was added to the cells. After at least 5 hours of transfection and up to 24 hours, the medium was changed and cells were kept at least 24 hours to enable gene expression.

Insert	Tag	Species	Backbone
βTrCP	myc	Mouse	pcDNA3
βTrCP ΔF	myc	Mouse	pcDNA3
JNK	HA	Human	pCEFL-KZ
Skp1	flag	Human	pcDNA3.1
SKP2	myc	Human	pcDNA3
βTrCP	flag	Human	pcDNA3
MKK7D	-	Mouse	pCAGGS
ΔMEKK1	-	Mouse	pCEV29
TOP flash - Luciferase	-	Human - Firefly	Unknown
FOP flaso - Luciferase	-	Human - Firefly	Unknown
Renilla	-	Sea pansy	pRL-TK

Table 1: List of plasmids used.

Site-directed mutagenesis

To generate the βTrCP δmiR183 mutant, site-directed mutagenesis was performed according to manufacturer's instructions (Stratagene) using specific primers (with the appropriate reverse complement) (Table 2) and the expression vector pcDNA-hβTrCP as the template. All constructs were sequenced after generation with the commercial kit ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

Name	Sequence (5' ⟶ 3')
$\beta TrCP^{\delta miR183}$ FW	5' cacattggaatgcaagcggatactcactggacacacaggttcagtcctctg 3'
$\beta TrCP^{\delta miR183}$ RV	5' cagaggactgaacctgtgtgtccagtgagtatccgcttgcattccaatgtg 3'

Table 2: List of primers used.

Cell extracts, immunoprecipitation and immunoblot analysis

Cells were lysed with lysis buffer that contained 50 mM of Tris (pH 7.5), 100 mM NaCl, 50 mM NaF, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerolphosphate, 2 mM Na_3VO_4, 25 nM calyculin A, 1% TX100, 0.5 mM PMSF, 1 µg/L leupeptin and 1 µg/mL aprotinin. Whole cell extracts were obtained by centrifugation at 13.200 rpm for 10 minutes. Protein concentrations were determined using the Bradford reagent (BioRad). Immunoprecipitations were carried out with the indicated antibodies pre-bound to protein A/G dynabeds (Invitrogen). 1 mg of lysate was incubated for 1 hour at 4ºC and washed three times with lysis buffer. Immunoblotting was carried out after separation of proteins by SDS-PAGE and transfer to PVDF membranes (Immobilon-P Transfer Membrane, Millipore).

Membranes were probed with the following primary and secondary antibodies (Table 3 and 4) and were detected by ECL Chemiluminiscense (Thermo Scientific).

Antigen	Company / Reference
MKK7	Santa Cruz / sc-7104
JNK	Santa Cruz / sc-474

GFP	Roche / 11814460001
Akt	Cell Signaling / 9272S
Phospho-Akt	Cell Signaling / 4060P
Caspase 3	Cell Signaling / 9662
Pdx1	Upstate / 07-696
GLUT2	Chemicon / AB1342
Nek-9	(Roig *et al*, 2002)
ERK-1	Santa Cruz / sc-93
Flag	Sigma / F3165
βTrcP	Santa Cruz / sc-15354
myc	9e10 hybridoma
SKP1	Zymed / 32-3800
Cul1	Zymed / 71-8700
FoxO1	Cell Signaling / 9454
p27	Santa Cruz / sc-1641
Insulin	Dako / A0564
Glucagon	Dako / A0565

Table 3: Primary antibodies list.

Antigen	Conjugated	Company / Reference
Rabbit IgG	Peroxidase	Jackson / 115-095-003
Mouse IgG	Peroxidase	Jackson / 115-035-003
Goat IgG	Peroxidase	Jackson / 705-035-003
Mouse IgG	Alexa Fluor 594 (red)	Invitrogen / A11005
Mouse IgG	Alexa Fluor 488 (green)	Invitrogen/ A11001
Rabbit IgG	Alexa Fluor 488 (green)	Invitrogen / A11008
Rabbit IgG	Alexa Fluor 594 (red)	Invitrogen / A11012
Guinea pig IgG	Cy^{TM}2 (green)	Jackson / 706-225-148

Table 4: Secondary antibodies list.

Protein expression in bacteria

GST-fusion proteins were expressed in E. coli RossetaTM 2 (DE3) or E. coli BL21 (DE3) induced with isopropyl-β-D-thiogalactopyranoside (IPTG) for 16 hours at 18ºC or 3 hours at 30ºC (Table 4). They were purified with glutathione-sepharose (GE Healthcare) following standard protocols, and eluted with 25 mM reduced glutathione. All purified proteins were resolved in SDS-PAGE acrylamide gels and stained with Coomassie blue (Sigma) to check protein size and purity.

Insert	Tag	Species	Backbone
cJun	GST	Chicken	pGEX-4T1
JNK3β2	GST	Rat	pGEX-4T1

Table 4: List of plasmids used.

Luciferase assay

Alterations in transcriptional activity of β-catenin were measured by a reporter gene luciferase assay: TOP/FOPflash. The TOP reporter construct is specific for β-catenin transcriptional activity, comprising 3 tandem repeats of TCF binding sites. FOP is the negative control with a mutated TCF binding site. Renilla expression vector served as the transfection control.

Upon LiCl 30 mM treatment for 5 hours, cells were lysed using Passive Lysis Buffer and the assay was prepared using the Promega Dual Luciferase Assay Kit as per manufacturer's instructions. Luciferase activity was monitored using a Berthold Technologies Lumat LB 9507 Single Tube Luminometer.

Animals and *in vivo* studies

Transgenic mice C57BL/6J-Tg(Gfp^{loxP}-MKK7D)Ccf were generated by microinjection of oocytes with the transgen GFP^{loxP}-MKK7D (Fig. 15A), and two independent lines were selected for this study. All the experiments described in this study were performed in

both transgenic mouse lines, and comparable results were obtained. The RIP-Cre (C57BL/6-Tg(Ins2-Cre)25Mgn) and RIP-CreER (STOCK-Tg(Ins2-cre/ERT)1Dam/J) mouse strains were obtained from The Jackson Laboratory (Bar Harbor, Maine USA). The mice used in this study came from crosses of heterozygous Cre recombinase or Cre recombinase/estrogen receptor (ER)-expressing male mice with homozygous GFP^{loxP}-MKK7D female mice. To activate the Cre recombinase/ER fusion protein, 10 mg of tamoxifen (Sigma, T5648) diluted in corn oil was administered to 8-week-old mice by oral gavage every other day for 5 days. Experiments were performed two weeks after tamoxifen treatment.

The glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed in mice fasted for 6 h before intraperitoneal injection of glucose (2 mg/g body weight) or insulin (0.5 U/g body weight), respectively. Blood was collected from the tail at the indicated time points, and plasma insulin and glucose were determined by enzyme-linked immunosorbent assay (ELISA) (Mercodia 10-1249), and with an automatic glucometer (Elite, Bayer), respectively. Rosiglitazone (1 mg/kg body weight) or vehicle (PBS) was administered to 2-month-old mice by oral gavage once a day for 10 consecutive days. All animal procedures were approved by the Animal Care Research Committee of the University of Barcelona.

Islet isolation and insulin secretion

After pancreas digestion by intraductal injection of collagenase, isolated islets were purified by centrifugation in ficoll gradients. They were then washed twice with Hank's Balanced Salt Solution and cultured in RPMI 2% fetal bovine serum for 3 h. Afterwards, islets were handpicked, transferred to secretion vials in groups of six, and incubated for 60 min with a HEPES Krebs Ringer bicarbonate (HKRB) buffer with 2.8 mM glucose, unless otherwise indicated. Supernatants were recovered, and insulin secretion was analyzed by ELISA. JNKi-1, 3-isobutyl-1-methylxanthine (IBMX), KCl, succinate, KIC, rosiglitazone, and tolbutamide were used at 10 μM, 50 μM, 40 mM, 10 mM, 10 mM, 10 mM, and 250 mg/mL, respectively.

JNK immunocomplex assay

JNK was immunoprecipitated and the JNK activity associated with the immunoprecipitates was determined by its capacity to phosphorylate in vitro the substrate GST-cJun. Alternatively, possible targets of JNK were immunoprecipitated and incubated with GST-JNK to determine the capacity of JNK to phosphorylate them.

The cells were lysated with a buffer that contained 20 mM HEPES-Na (pH 7.5), 10 mM EGTA, 40 mM β-glycerolphosphate, 2.5 mM $MgCl_2$, 1% NP-40, 2 mM Na_3VO_4, 1 mM DTT, 0.5 mM PMSF and 1 µg/L aprotinin. 1 mg of cell extract was immunoprecipitated with the appropriated antibody and after three washes with the lysis buffer, the immunoprecipitate was incubated with either the substrate (GST-cJun) or the kinase (GST-JNK) for 30 minutes at 30ºC in presence of 20µM of ATP and 0.5µCi of [γ -^{32}P] ATP in a buffer containing 20 mM HEPES-Na (pH 7.5), 20 mM β-glycerolphosphate, 20 mM $MgCl_2$, 0.1 mM Na_3VO_4 and 2 mM DTT.

Reactions were terminated by addition of 5X Laemli buffer and boiling, and proteins were resolved by SDS-PAGE. Coomassie staining was used to visualize proteins and kinase activity was measured with a PhosphorImager system.

Immunohistochemistry

When relevant, mice were fasted for 6 h before intraperitoneal injection of glucose (2 mg/g body weight) or insulin (0.5 U/g body weight), respectively. The pancreas was surgically removed, fixed in 4% paraformaldehyde in PBS, dehydrated and embedded in paraffin. 5-µm sections were made with a manual microtome and stained with hematoxilin and eosin or immunostained with the indicated antibodies (Table 3 and 4) and DAPI (0.01 mg/mL). Slides were analyzed by fluorescence or confocal microscopy (Upright Microscope Nikon E1000 or Leica TCS SPE, respectively).

Quantitative real-time PCR (qRT-PCR)

Pancreatic islets were isolated as described above and they were stimulated either with insulin (2 µg/L for 3 hours) or glucose (24.4 mM for 16 hours). In the case of HEK293T cells, the lysis was performed 24 hours after the transfection. In both cases RNA was extracted with TRIzol reagent (Invitrogen) following Manufacturer's Instructions. RNA was reverse-transcribed with M-MLV reverse transcriptase (Invitrogen) and quantified by qRT-PCR using SYBR Green (Applied Biosystems). The pairs of primers thar were used are shown in Table 6.

Gene	Forward primer	Reverse primer
gapdh (mouse)	aagggctcatgaccaca	gatgcagggatgatgttctg
ins1 (mouse)	tgaccagctataatcagagacca	aggtggggaccacaaagat
ins2 (mouse)	aggcttttgtcaagcagcac	ggtctgaaggtcacctgctc
glut2 (mouse)	aatggtcgcctcattctttg	caagagggctccagtcaatg
gapdh (human)	agaaggctggggctcatttg	aggggccatccacagtcttc
βTrCP (human)	catacaacagctgtgccaga	tgcttgggcacaatcata

Table 6: List of genes and primers analyzed by quantitative real-time PCR.

Caspase 3/7 activity assay

Caspase3/7 activity was measured in extracts of pancreatic islets (10 µg) with the Caspase-Glo 3/7 Assay (Promega) according to the manufacturer's instructions.

Statistical analysis

Data were analyzed with a two-tailed unpaired Student's test. Values are presented as means + SE.

RESULTS

JNK EFFECT OVER βTrCP

As mentioned in the introduction it has been described that JNK has a stabilizing effect both on βTrCP mRNA and protein. Not only this, but both effects seem to be independent (Spiegelman *et al*, 2001; Arevalo, 2008). In the present thesis we have characterized the two mechanisms by which JNK is achieving the βTrCP accumulation and consequently increase NF-κB activation. Moreover, we have analyzed if the levels of other targets of βTrCP are also affected by the increase of its E3 ubiquitin ligase or if JNK induction also augments other $SCF^{F\text{-}Box}$ complexes.

Stabilization at a protein level

We tested the effects of JNK on βTrCP levels by overexpression of a constitutively activated form of MKK7 (MKK7D), which is a strictly specific activator of JNK. As a positive control was used a N-terminus truncated MEKK1 (ΔMEKK1), which is known as a very potent upstream activator of JNK pathway but also of IKK (Lee *et al*, 1997). We observed, as it was previously described, an increase of βTrCP protein levels in a MKK7D dose dependent manner, and thereby JNK-dependent activation (Fig. 10A).

As long as the $SCF^{\beta TrCP}$ ubiquitination ligase is a dynamic complex, we also analyzed if activating JNK other members of the complex were stabilized. As a result of this, we found that protein levels of SKP1 were also largely increased by activating JNK with MKK7D or ΔMEKK1 (Fig. 10B). In this case, however, the up-regulation of SKP1 protein levels by MKK7D was not strictly proportional to the JNK activity.

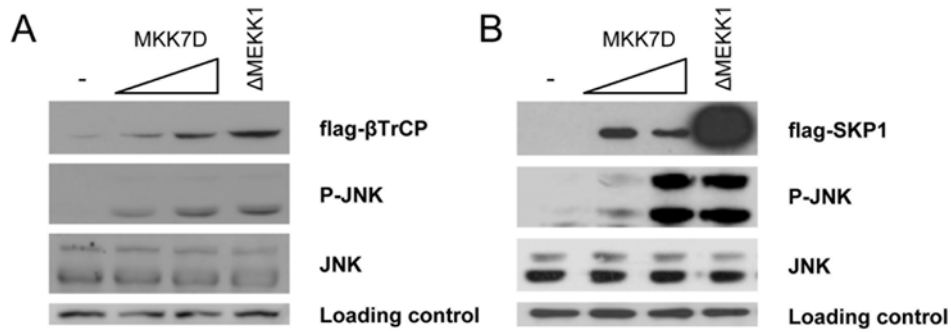

Figure 10. JNK activation leads to an increase of βTrCP and SKP1 protein levels. Immunoblot analysis of flag, phospho-JNK (P-JNK) and JNK in extracts from HEK293T cells transfected with equal amounts of flag-hβTrCP (A) or flag-hSKP1 (B) together with an empty vector (-), increasing doses of MKK7D or ΔMEKK1 expression vector, as indicated.

βTrCP and SKP1, whose levels are increased by JNK activation, form the core of the $SCF^{\beta TrCP}$ complex that will trigger the assembly of the rest of the complex (Bosu & Kipreos, 2008). Thus, the observed increases might obey to a cross-stabilization due to their interactions.

Results in Fig. 11A confirmed this hypothesis as βTrCP and SKP1 levels were higher when coexpressed. Moreover, this stabilization requires βTrCP-Skp1 interaction, as it was not achieved when using $\beta TrCP^{\Delta F}$, a mutant form of βTrCP that lacks the F-Box domain and therefore cannot interact with SKP1 (Fig. 11A). Actually, SKP1 levels were even decreased by $\beta TrCP^{\Delta F}$ in a dose dependent manner (Fig. 11A and B). As long as endogenous βTrCP was still present, these results suggest that the stability of SKP1 might be determined by the interaction with βTrCP and some other component of the complex that is being sequestered by the transfected $\beta TrCP^{\Delta F}$.

The stabilization of βTrCP by JNK activation was also depending on its interaction with SKP1. Even though $\beta TrCP^{\Delta F}$ protein levels were slightly increased by MKK7D overexpression (and that may be the result of mRNA stabilization), it was not in the same extend as the $\beta TrCP^{wt}$ (Fig. 11C). These results indicate that the interaction with SKP1 is needed to achieve the full JNK effect on βTrCP.

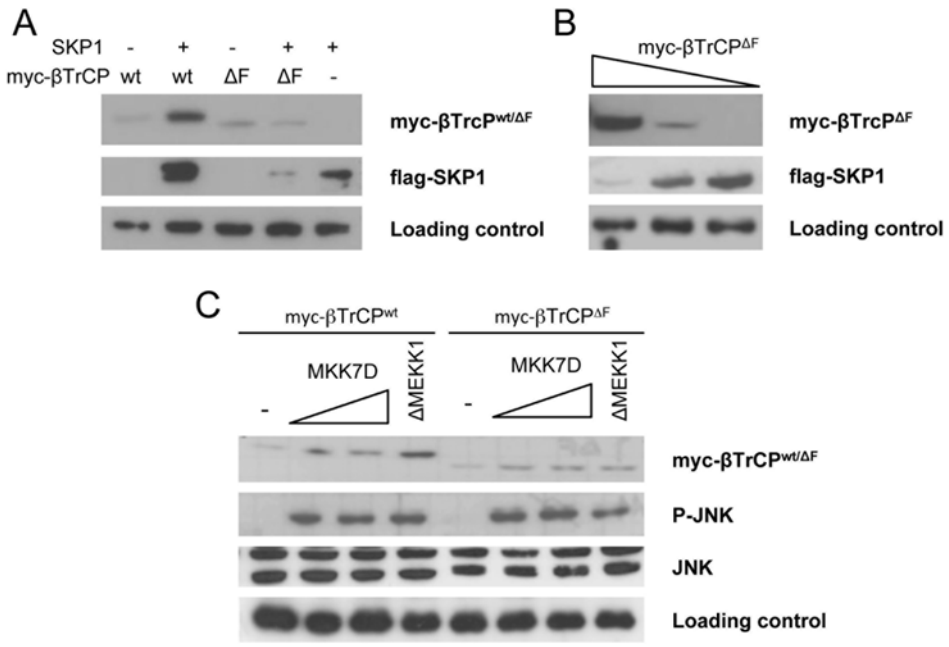

Figure 11. SKP1 and βTrCP interaction is needed for the JNK full effect on βTrCP. A: Myc and flag immunoblot analysis of extracts of HEK293T cells transfected with flag-SKP1 and either myc-βTrCP wild type or its truncated form myc-βTrCP$^{\Delta F}$. B: Myc and flag immunoblot analysis of extracts of HEK293T cells transfected with flag-SKP1 and increasing doses of myc-βTrCP$^{\Delta F}$. C: Myc, phospho-JNK (P-JNK) and JNK of extracts of HEK293T cells transfected with either myc-βTrCP wild type or myc-βTrCP ΔF and either MKK7D or ΔMEKK1.

JNK and the βTrCP mRNA - miR183 (microRNA 183) - CRD BP system

As long as βTrCP mRNA is known to be stabilized by JNK we decided to further investigate the mechanism. So far it has been described that this stabilization is transcription independent and using an expression vector containing βTrCP CDS is enough to achieve it. On the other hand, searching the literature, we found that the miR183 - CRDBP system (described in the introduction) stabilize βTrCP mRNA through interacting with its CDS region.

Firstly, we reproduced the mutant form of βTrCP (βTrCP$^{\delta miR\text{-}183}$), which was already described (Elcheva *et al*, 2009). We mutated six nucleotides in the miR-183-binding region of βTrCP in a way that it does not affect its amino acid sequence. These mutations was predicted to disrupt the formation of a nearly perfect duplex between

miR-183 and βTrCP mRNA (Fig. 12A). Although the efficiency of translation was not altered, transcripts of βTrCP$^{\delta miR\text{-}183}$ were more stable than their corresponding wild-type mRNAs (Elcheva *et al*, 2009).

To verify if the miR-183 was involved in the JNK transcription-independent stabilization of βTrCP mRNA we evaluated the capacity of JNK to induce βTrCP$^{\delta miR\text{-}183}$ mRNA. Actually, when miR-183 interaction was already inhibited, JNK did not further increase the levels of βTrCP mRNA, suggesting that the JNK pathway counteracts miR-183 to regulate βTrCP mRNA stability (Fig. 12B and C). This was not happening when using ΔMEKK1 (Fig. 12B and C), which also activates other pathways apart from JNK, indicating that there are other mechanisms within the CDS that can be affected by ΔMEKK1.

Given that the βTrCP$^{\delta miR\text{-}183}$ mRNA is not stabilized when selectively activating JNK, we further analyzed if the resulting protein was increased by cotransfection with MKK7D. Indeed, they were not only augmented after JNK activation, but the basal protein levels were already higher and this effect was maintained when activating JNK (Fig. 12D). These results indicate that there are several ways how JNK is enhancing βTrCP protein levels.

When studying the amino acidic sequence of CRD-BP, the protein that protects βTrCP mRNA from the attack of the miRNA-RISC system, we identified a putative JNK phosphorylation site that was conserved across the species (Fig. 13A). Actually, this site has been identified in many large-scale phosphoproteomic analysis as a phospho site (Olsen *et al*, 2006; Dephoure *et al*, 2008; Daub *et al*, 2008). However the kinase responsible to its phosphorylation it is unknown.

To evaluate CRD-BP as a JNK substrate, we performed an *in vitro* kinase assay with purified GST-JNK and immunoprecipitated CRDP. Apparently, JNK would be phosphorylating CRD-BP (Fig. 13B). Even though CRD-BP and GST-JNK have similar molecular weight (Fig. 13C) and JNK it is known to autophosphorylate itself *in vitro*

assays, this possibility is discarded because no phosphorylation was detected when there is only GST-JNK and no CRD-BP (Fig. 13B lane 3).

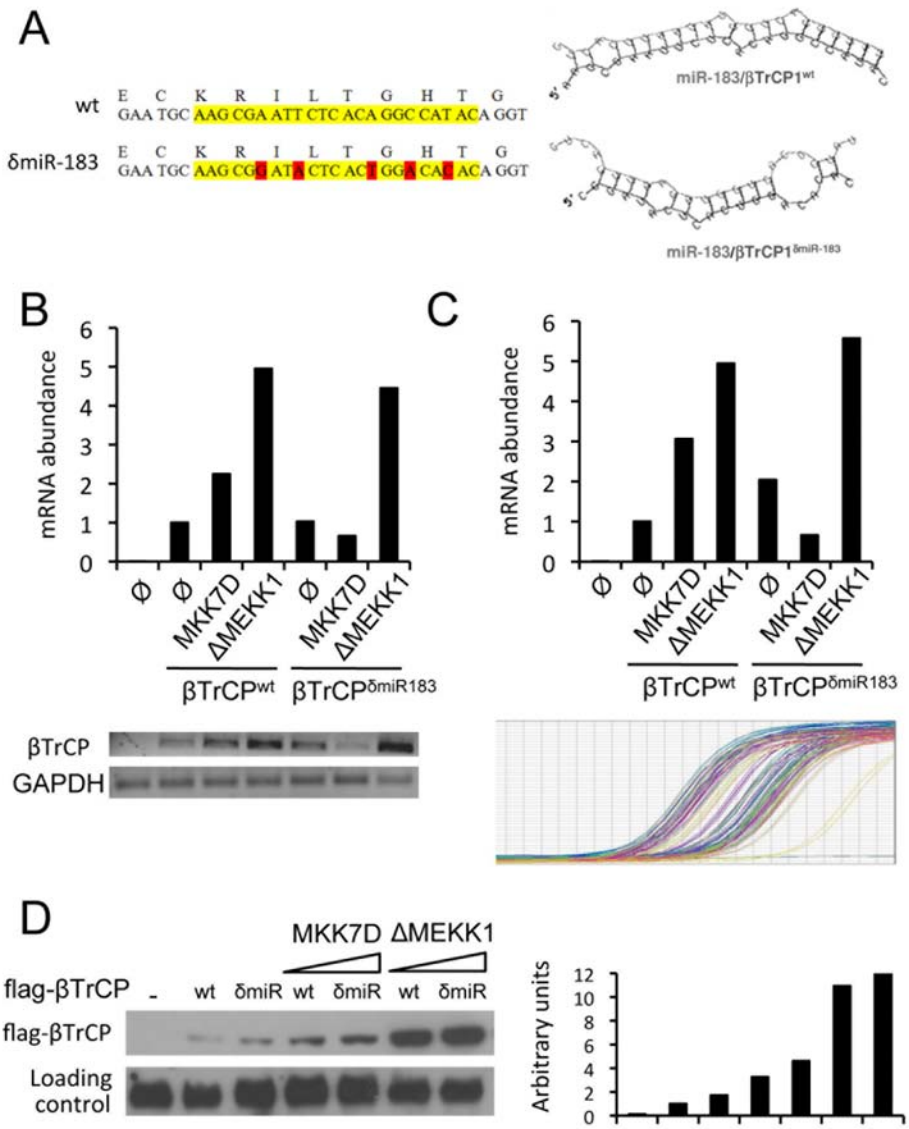

Figure 12. JNK is affecting the βTrCP - miR-183 interaction but still can increase the βTrCP^{δmiR183} mutant. A: schematic representation of the amino acidic and nucleotide sequence of the βTrCP^{wt} (wt) and the βTrCP^{δmiR183} (δmiR-183) and their predicted structure when bound to miR-183 (Adapted from Elcheva *et al*, 2009). In the right panel, yellow highlights the sequence where the miRNA binds and in red appear the mutations. Semiquantitative RT-PCR (B) and real time quantitative PCR (C) of RNAs extracted from HEK293T cells transfected with βTrCP^{wt} and βTrCP^{δmiR183} together with MKK7D or ΔMEKK1 as indicated. These samples were also analyzed by immunoblot against flag, the tag of both βTrCP^{wt} and βTrCP^{δmiR183} (D).

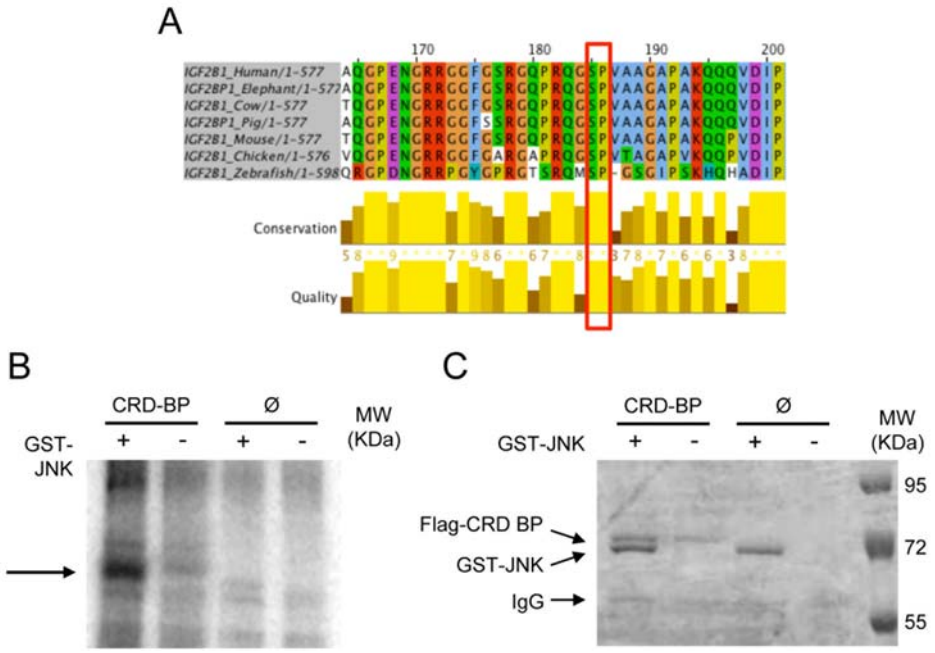

Figure 13. JNK phosphorylates CRD-BP *in vitro*. A: Schematic representation of the amino acidic sequence of the CRD-BP from different species and the graphic displaying the degree of conservation in between them and quality of the alignment. The red square highlights the putative phosphorylation site of JNK. Phosphoimager scan (B) and coomassie blue staining of an SDS-PAGE of immunoprecipitates of HEK293T cells transfected with CRD-BP or an empty vector (Ø) that were incubated with or without GST-JNK in presence of [γ -^{32}P] ATP.

JNK and other ubiquitination pathways

The activity of the SCF$^{\beta TrCP}$ ubiquitination complex is not limited to IκBα. There are many other known targets such as CDC25A (Guardavaccaro *et al*, 2003a), Claspin (Peschiaroli *et al*, 2006) or β-catenin (Latres *et al*, 1999). As long as βTrCP and the whole SCF$^{\beta TrCP}$ are augmented after JNK activation, we further studied what was the effect of JNK activation over other targets of βTrCP.

Regarding β-catenin, by activating JNK we were not able to detect a significant decrease in its protein level or activity in a steady state of the signaling pathway. However, when inhibiting GSK3, the kinase that phosphorylates β-catenin targeting it to degradation

(Willert & Nusset, 1998), we detected a 25% decrease of the TCF activity by activating JNK (Fig. 14A).

As long as other $SCF^{F\text{-}Box}$ complexes, like SCF^{SKP2}, share components like SKP1 and Cul1 and the F-Box mRNA also has binding sites for CRD-BP, we tested if their levels could be altered by JNK activity. In fact, SKP2 protein levels turned to be increased by both, JNK activity (Fig. 14B) and SKP1 overexpression (Fig. 14C). Accordingly, $p27^{Kip1}$ (p27), a *bona fide* target of SKP2, was found to be increase in two independently obtained lines of MEFs lacking JNK1/2 (Fig. 14D).

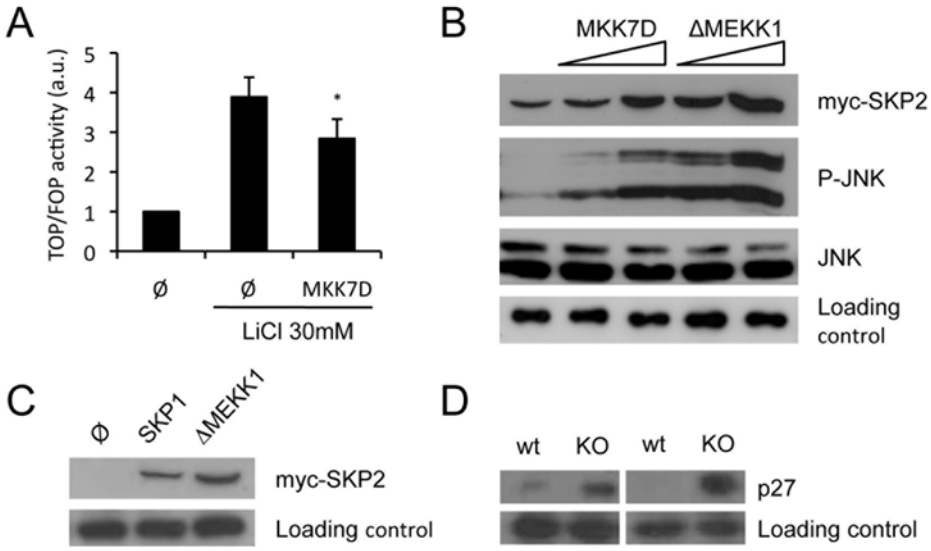

Figure 14. JNK affects other target of βTrCP and also other $SCF^{F\text{-}Box}$ complexes. A: TOP/FOP luciferase reporter assay in HEK293 transfected with or without MKK7D expression vector and with or without 5 hours induction with LiCl, as indicated. B: Immunoblot analysis of myc, P-JNK and JNK of HEK293T cells transfected with equal amounts of myc-SKP2 together with an empty vector or increasing doses of MKK7D or ΔMEKK1 expression vector, as indicated. C: Immunoblot analysis of myc of HEK293T cells transfected with equal amounts of myc-SKP2 together with an empty vector, SKP1 or ΔMEKK1 expression vector, as indicated. D: Immunoblot analysis of p27 of two independently obtained JNK1/2 KO MEFs.

IN VIVO JNK ACTIVATION IN PANCREATIC β-CELLS LEADS TO GLUCOSE INTOLERANCE CAUSED BY CENTRAL INSULIN RESISTANCE

MKK7D overexpression in pancreatic β-cells leads to JNK activation.

To gain insight into the effects of JNK activation on pancreatic β-cells and glucose homeostasis, we generated a transgenic mouse model that allows JNK activation in a Cre recombinase expression-dependent manner to study the effects of JNK activation in pancreatic β-cells regarding glucose homeostasis and β-cell function. We constructed a transgene (GFPloxP-MKK7D) in the context of the pCAG vector, which contains the CMV enhancer and chicken β-actin promoter sequences driving the constitutive expression of the GFP gene, which was flanked by two loxP sites and followed by a MKK7D expression unit (Fig. 15A).

The MKK7D sequence encodes the MAP2K of JNK, MKK7, which carries two mutations (S271D and T275D) that mimic the active form and has previously been used to trigger JNK activity in mice (Petrich *et al*, 2003). To activate JNK in pancreatic β-cells, mice harboring this transgene were crossed with RIP-Cre mice to obtain animals expressing either GFP (Control mice) or, due to the Cre recombinase–dependent excision of the GFP expression cassette, MKK7D (MKK7D mice).

Immunoblot analysis of extracts from pancreatic islets isolated from these animals demonstrated that Cre recombinase expression drastically reduced GFP expression while, concomitantly, increasing MKK7D expression, thereby resulting in augmented JNK phosphorylation and activity (Fig. 15B). MKK7D mice were born and developed normally, although they showed a slight decrease in body weight that was gender-independent (Fig. 15B).

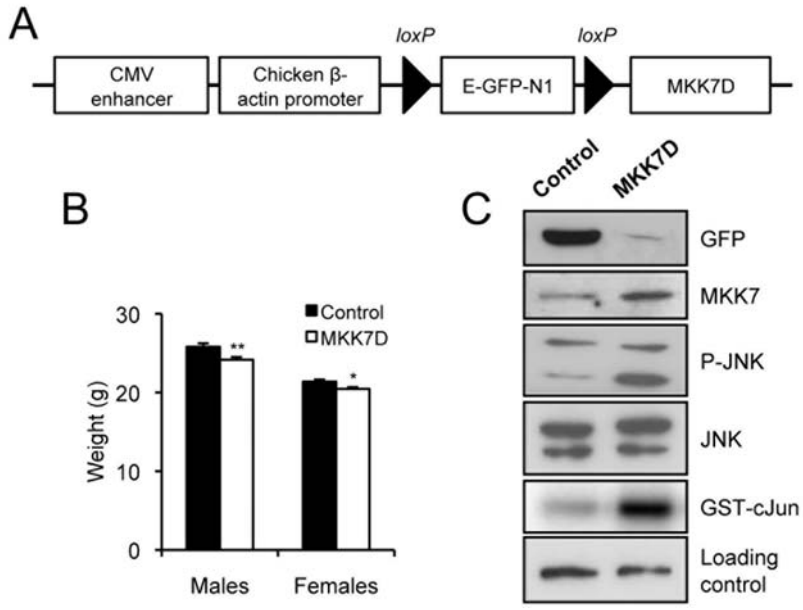

Figure 15. MKK7D overexpression in pancreatic β-cells leads to JNK activation and a slight body weight decrease. A: Diagram of the GFPloxP-MKK7D transgene. Transgenic mice constitutively express GFP under the control of the CMV enhancer/chicken β-actin promoter (Control mice). When crossed with RIP-Cre mice, the GFP cassette is floxed in pancreatic β-cells, leading to the constitutive expression of MKK7D in this cell type (MKK7D mice). B: Immunoblot analysis of GFP, MKK7, phospho-JNK (P-JNK) and JNK in extracts from isolated pancreatic islets of Control and MKK7D mice (first to fourth panels). Fifth panel shows JNK activity assessed by immunocomplex assay. C: Body weight comparison of Control and MKK7D 3-months old mice.

JNK activation in pancreatic β-cells disrupts glucose homeostasis.

The JNK pathway has emerged as a main player in glucose homeostasis not only because of its physiological role as a negative-feedback mechanism of the insulin-signaling pathway (Lee *et al*, 2003), but also because of its contribution to insulin resistance in peripheral tissues (Hirosumi *et al*, 2002) as well as in pancreatic β-cells (Solinas *et al*, 2006) in diverse pathological scenarios. Therefore, we focused our attention on the effects of pancreatic β-cell-specific activation of JNK on glucose homeostasis in our MKK7D transgenic mice. Although we found no significant differences in glycemia and insulinemia between 6-h fasted Control and MKK7D mice (Fig. 16A and B), the GTT showed that the latter were glucose-intolerant (Fig. 16C). Furthermore, this defect in glucose homeostasis was not due to decreased peripheral

insulin sensitivity, as shown by the ITT (Fig. 16D), but to an impaired capacity to increase plasma insulin level in response to hyperglycemia (Fig. 16E). Actually in this experimental set up we failed to differentiate a first peak of insulin that occurs within the 5 first minutes after the induction with glucose.

Overall, these results indicated that MKK7D mice suffered from pancreatic dysfunction. This glucose-intolerant phenotype was repeated to a similar extent in males and females of both transgenic lines and was observed in animals as early as 1 month old.

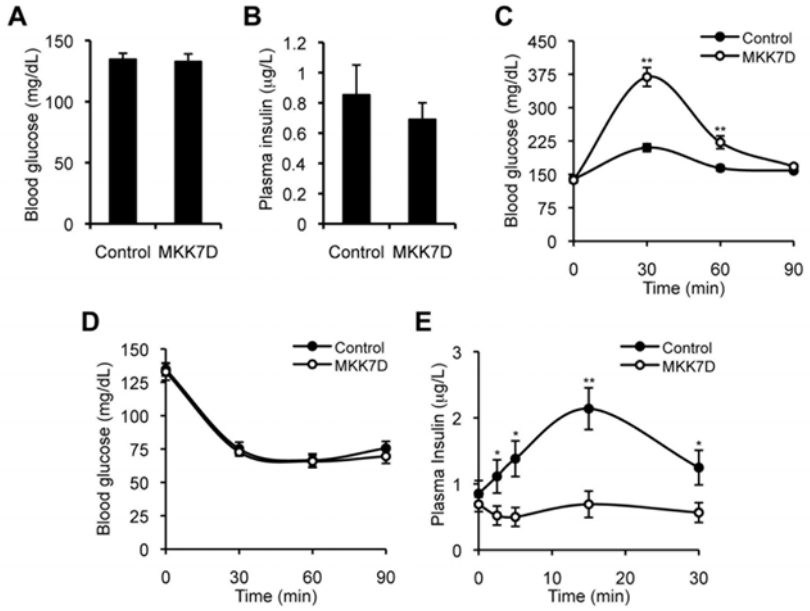

Figure 16. JNK activation in pancreatic β-cells leads to glucose intolerance as a result of an impaired capacity to increase insulinemia in response to hyperglycemia. Plasma glucose (A) and insulin (B) level in 6-h fasted Control and MKK7D mice. GTT (C), ITT (D) and glucose-stimulated insulin secretion (E) in Control and MKK7D 3 months-old mice.

JNK activation in pancreatic β-cells does not cause major morphostructural changes in islets.

The success of JNK inhibitors in increasing the survival of pancreatic islets or insulin-secreting cell lines subjected to transplantation protocols and/or exposed to pro-inflammatory cytokines, respectively, has led to the notion that the JNK pathway plays

a fundamental role in mediating β-cell death (Díaz-Delfín *et al*, 2007; Bonny *et al*, 2001; Aikin *et al*, 2004; Fornoni *et al*, 2008; Noguchi *et al*, 2005, 2007). This role might had been consistent with the phenotype we observed in the MKK7D mice. Therefore, we performed a histological analysis of the pancreatic islets by haematoxylin-eosin staining, and immunostaining using antibodies against insulin and glucagon.

In contrast to what we expected, the islet shape, number, size, and distribution and number of α- versus β-cells did not differ between Control and MKK7D mice (Fig. 17A-D) nor did the overall size of the pancreas (Fig. 17E). In addition, no differences regarding pancreas or islet insulin content were observed (Fig. 17F and G).

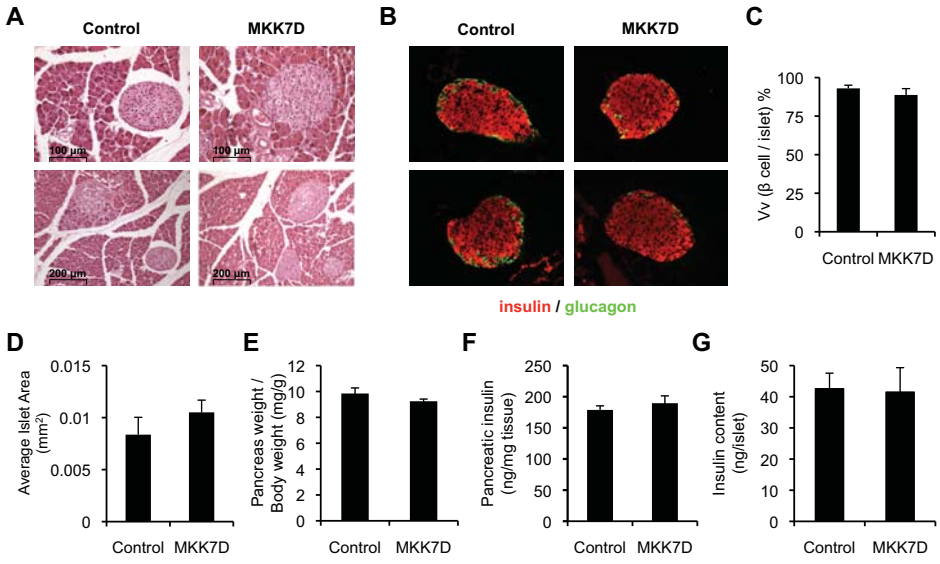

Figure 17. JNK activation in pancreatic β-cells affects neither the morphology nor the insulin content of the islets. Pancreatic histological sections of Control and MKK7D animals stained with haematoxilin and eosin (A) or analyzed by immunohistochemistry with antibodies against insulin and glucagon (B). Average β-cell percentage (C) and total islet area (D), pancreatic weight relative to body weight (E), insulin content of pancreas (F) and isolated islets (G) from Control and MKK7D 3-months old mice.

Data from the literature demonstrate that JNK-induced β-cell apoptosis is mediated by caspase 3 (Kim *et al*, 2005). In this regard, analysis of caspase 3 cleavage (Fig. 18A) and activity (Fig. 18B) in isolated islets showed that MKK7D-triggered activation of JNK did not lead to an activation of caspase 3. These results do not support the notion that major morphological or structural defects of pancreatic islets caused by massive β-cell

death were responsible for the glucose-intolerant phenotype observed in the MKK7D mice.

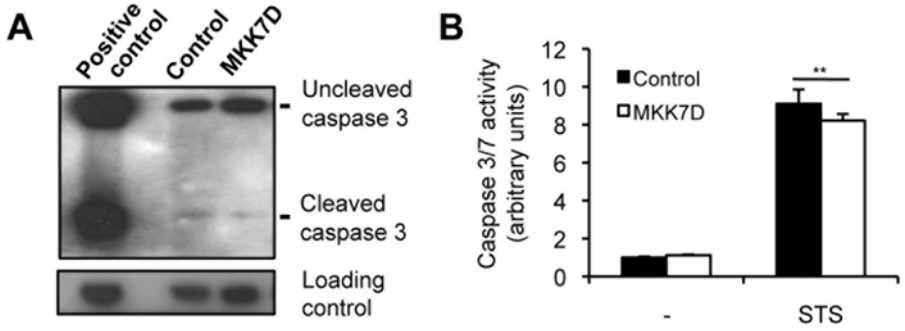

Figure 18. JNK activation in pancreatic β-cells does not activate Caspase 3. A: Immunoblot analysis of caspase 3 in extracts from isolated islets of Control and MKK7D mice. Positive control was an extract from neurons overnight treated with staurosporine. B: Caspase 3 activity in extracts from isolated islets untreated (-) or incubated for 20 hours with 10 μM staurosporine (STS) of Control and MKK7D mice.

JNK activation in pancreatic β-cells impairs insulin signaling.

As the morphology and structure of the pancreas and islets were apparently unaffected by the activation of JNK in β-cells, next we performed a series of functional assays addressed to analyze insulin secretion and signaling in response to glucose in isolated islets. First, we determined glucose-induced insulin secretion. In agreement with the studies in mice, we observed an impaired capacity of isolated islets from the MKK7D mice to secrete insulin in response to glucose when compared to Control mice (Fig. 19A). Moreover, this impairment was overcome when islets were incubated with a cell-permeable inhibitory peptide of JNK, JNKi-1 (Fig. 19B).

Afterwards, we confirmed that this defect was not caused by a reduction in overall amount or a subcellular mislocatization of the glucose transporter GLUT2 (Fig. 19C and D) or defects in the glucose signaling pathway, since insulin secretion of islets from MKK7D and Control mice was equally increased by a range of compounds, such as IBMX, KCl, succinate, KIC, and tolbutamide, which activate this pathway at different steps (Fig. 19E).

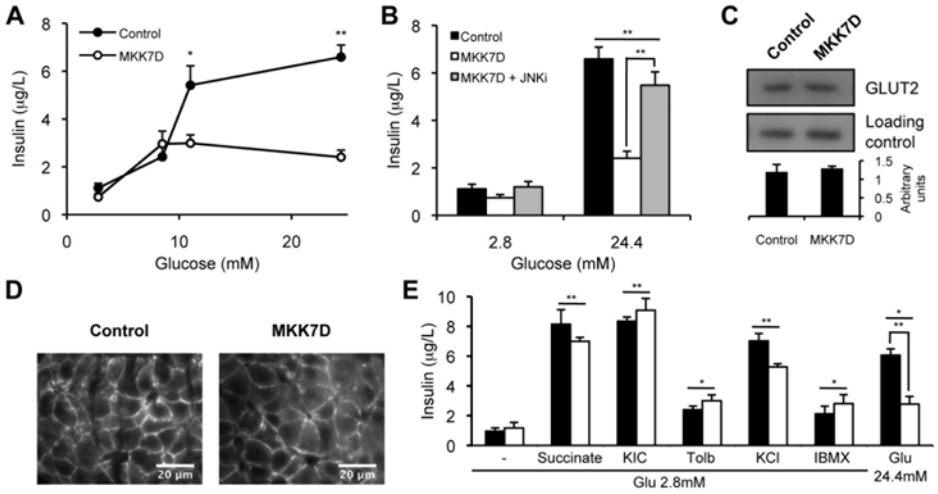

Figure 19. JNK activation in pancreatic β-cells impairs glucose-stimulated insulin secretion. Insulin secretion of pancreatic islets isolated from Control and MKK7D mice stimulated with the indicated concentrations of glucose (A) and in the presence or absence of JNKi-1 (JNKi), as indicated (B). Analysis of GLUT2 expression by immunoblot in extracts from pancreatic islets (C) and subcellular localization by immunohistochemistry in pancreatic sections (D) of Control and MKK7D mice. F: Insulin secretion of islets isolated from Control and MKK7D mice in response to 2.8 mM (-) and 24 mM glucose (Glu), succinate, KIC, tolbutamide (Tolb), KCl and IBMX, as indicated.

As long as insulin induces its own release, next we studied insulin-induced secretion of this hormone in isolated islets. We found that this autocrine action of insulin was impeded in islets derived from MKK7D mice and, again, this defect was overcome when these islets were incubated with JNKi-1 (Fig. 20A). These results strongly pointed to a JNK activation-dependent interference with insulin signaling. To test this hypothesis, we analyzed insulin-induced phosphorylation of Akt, a key protein in the insulin-signaling pathway (Kubota *et al*, 2012a). We found that this process was impaired in islets from MKK7D mice (Fig. 20B). Accordingly, insulin-induced expression of insulin target genes was also inhibited in these islets (Fig. 20C), regardless of their increased content in the transcriptional activator Pdx1 (Fig. 20E). Similar results were observed studying glucose-induced expression of this set of insulin target genes (Fig. 20D). Immunohistochemistry analysis of pancreatic sections of Control and MKK7D mice showed that Pdx1 is equally located in the nucleus in basal conditions but, in contrast to Control animals, MKK7D mice failed to induce Pdx1 nucleocytoplasmic shuttling in response to glucose or insulin (Fig. 4F)

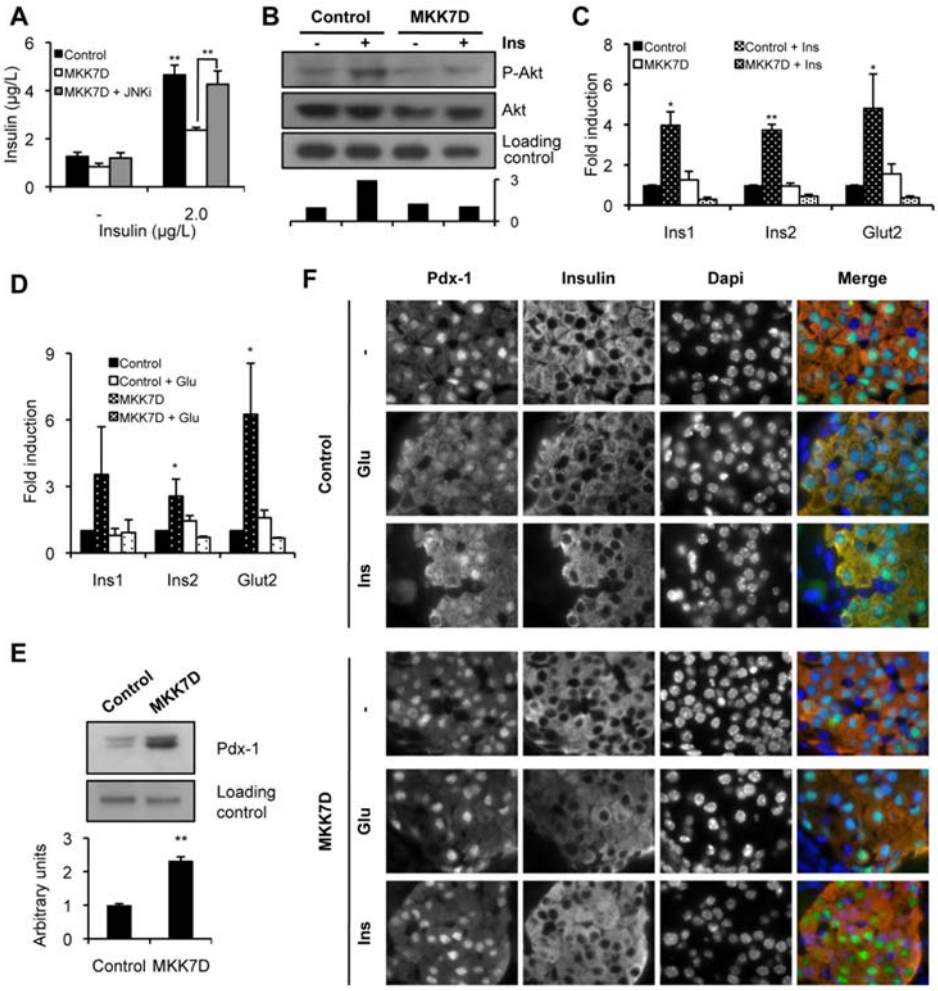

Figure 20. JNK activation in pancreatic β-cells impairs insulin signaling. A: Insulin-induced insulin secretion of pancreatic islets isolated from Control and MKK7D mice in the presence or absence of JNKi-1 (JNKi), as indicated. Secretions were performed in basal glucose concentration (2.8 mM). B: Immunoblot analysis of phospho-Akt (P-Akt) and Akt in extracts of islets from Control and MKK7D mice incubated with or without insulin (Ins), as indicated, for 20 min. The ratio of phospho-Akt versus total Akt is represented at the bottom panel. qRT-PCR analysis of the indicated insulin target genes in islets from Control and MKK7 mice incubated with or without insulin (Ins), as indicated, for 3 hours (C), or glucose, as indicated, for 16 hours (D). E: Immunoblot analysis of Pdx1 in extracts from pancreatic islets of Control and MKK7D mice. The ratio of Pdx1 versus loading control is represented at the bottom panel. F: Immunohistochemistry analysis of Pdx1 and insulin in pancreatic sections from Control and MKK7D mice untreated (-) or treated with glucose (Glu) or insulin (Ins) for 30 min.

Transient JNK activation in adulthood recapitulates the glucose-intolerant phenotype shown by MKK7D mice.

The experiments described above using the JNK inhibitory peptide showed that the JNK interference with the insulin-induced insulin secretion was reversible. Next, we addressed whether JNK activation is required early during development in order to achieve the phenotype or whether it occurs immediately after JNK activation, independently of the developmental stage.

Thus, transgenic mice were crossed with mice expressing a tamoxifen-inducible Cre recombinase/ER in pancreatic β-cells to obtain Control and MKK7DTam mice. Despite the overexpression of Cre-recombinase/ER in the β-cells, none of these mice were intolerant to glucose (Fig. 21A). The Cre-dependent recombination of the transgene was induced in adulthood by the administration of tamoxifen (see Research Design and Methods), and efficiency and concomitant JNK activation were confirmed by immunoblot analysis (Fig. 21B).

Tamoxifen-treated MKK7DTam mice (and Control animals) were subjected to a GTT and ITT. These tests revealed that these animals were glucose-intolerant (Fig. 21C), preserved peripheral insulin sensitivity (Fig. 21D), and were unable to increase insulinemia in response to hyperglycemia (Fig. 21E). Moreover, islets isolated from these mice did not secrete insulin in response to glucose or insulin (Fig. 21F). Therefore, upon tamoxifen treatment, MKK7DTam mice mimicked the phenotype previously observed in MKK7D mice.

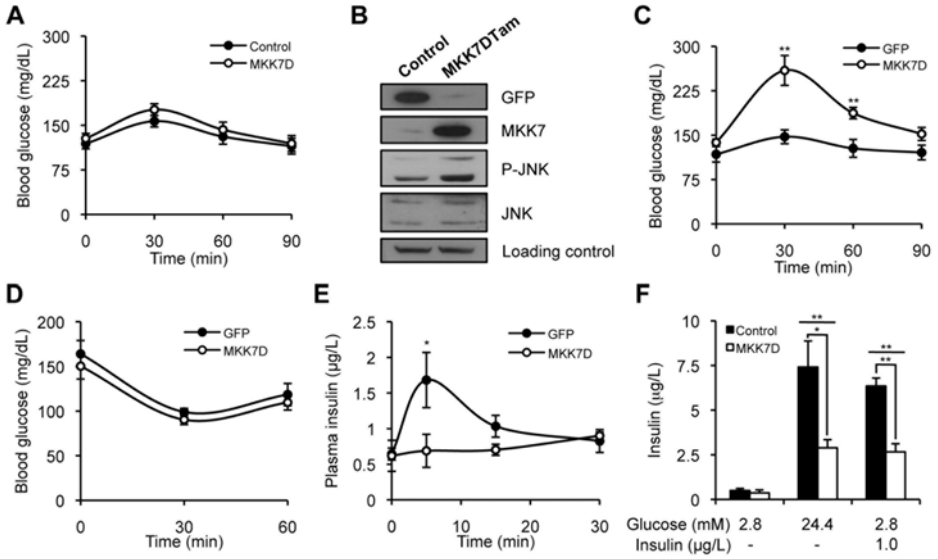

Figure 21. Tamoxifen-inducible JNK activation in adult mice causes glucose intolerance. A: GTT of Control and MKK7DTam 8-week-old mice before treatment with tamoxifen. B: Immunoblot analysis of GFP, MKK7, phospho-JNK (P-JNK) and JNK in extracts from isolated pancreatic islets of tamoxifen-treated Control and MKK7DTam mice. C, D and E: GTT, ITT and glucose-stimulated insulin secretion, respectively, in tamoxifen-treated Control and MKK7DTam mice. E: Insulin secretion of pancreatic islets isolated from tamoxifen-treated Control and MKK7DTam mice stimulated with glucose and insulin as indicated.

Rosiglitazone reestablishes the glucose homeostasis and insulin signaling disrupted by JNK activation in pancreatic β-cells.

We have previously reported that TZDs, in a PPARγ-dependent manner, are able to inhibit obesity- and pro-inflammatory cytokine-induced activation of JNK and, in doing so, alleviate insulin resistance (Díaz-Delfín *et al*, 2007). In fact, JNK inhibition by TZDs, such as troglitazone and rosiglitazone, has been documented in a variety of cell types and circumstances (Díaz-Delfín *et al*, 2007; Khandoudi *et al*, 2002; Hernandez *et al*, 2004; Yoon *et al*, 2010).

Particularly relevant to this study is the reported inhibition of IL-1β-induced activation of JNK by rosiglitazone in insulin-secreting cell lines, where expression of PPARγ was

also documented (Díaz-Delfín *et al*, 2007). Therefore, we tested the effect of rosiglitazone treatment on the phenotype exhibited by MKK7D mice.

For this purpose, MKK7D and Control mice were treated with rosiglitazone for 10 consecutive days and, afterwards GTTs were performed. Rosiglitazone treatment significantly improved glucose tolerance in the MKK7D mice (Fig. 22A and B) and also restored normal insulin secretion in response to hyperglycemia in these animals (Fig. 22C and D), while no changes were observed in peripheral sensitivity to insulin in none of the animals tested, as assessed by ITT (Fig. 22E).

Moreover, the direct action of rosiglitazone on β-cells was further assessed by confirming the ability of this drug to restore insulin secretion in response to glucose, as well as to insulin, in islets isolated from rosiglitazone-treated MKK7D mice (Fig. 22F). As a result of JNK mediating the feedback inhibition of the insulin-signaling cascade (Lee *et al*, 2003), rosiglitazone treatment of islets from Control mice further augmented insulin-induced insulin secretion (Fig. 22F). This effect was not observed in response to glucose because experiments were conducted at an already saturating glucose dose (Fig. 19A). At molecular level, rosiglitazone inhibited MKK7D-induced activation of JNK and, accordingly, restored insulin-induced Akt phosphorylation (Fig. 22G).

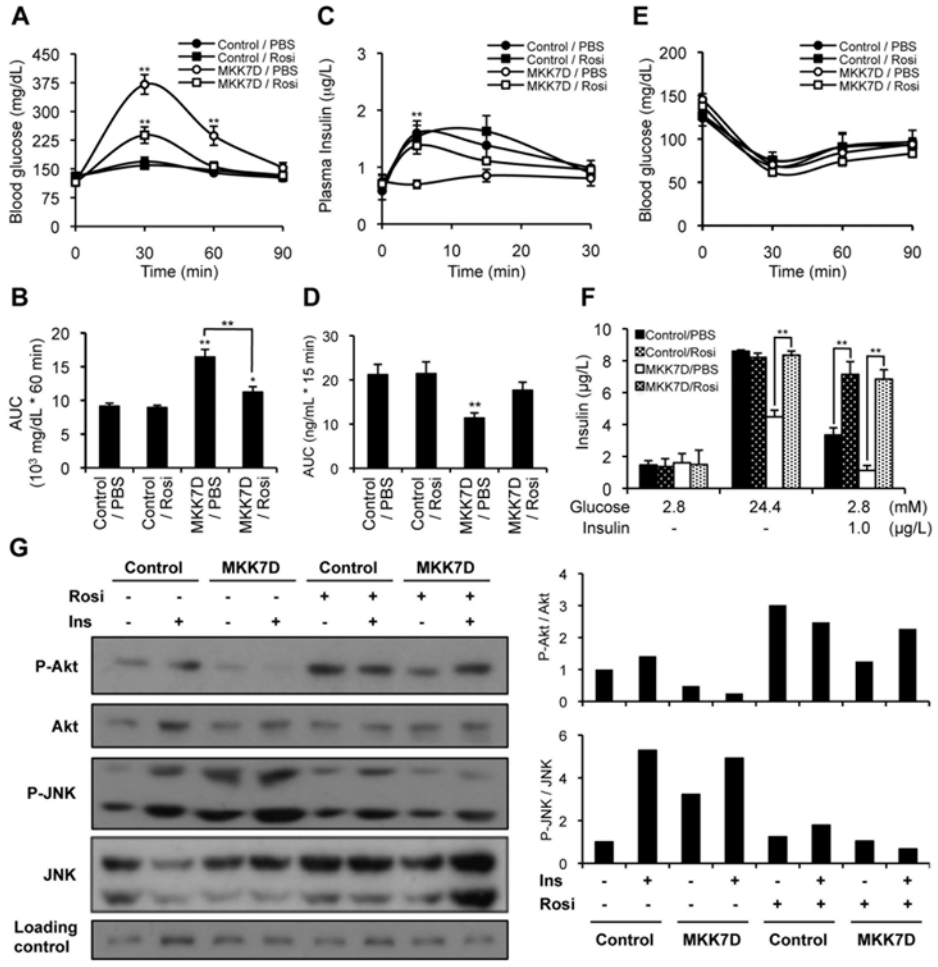

Figure 22. Rosiglitazone counteracts JNK-induced β-cell dysfunction. . GTT (A) and the corresponding area under the curve (AUC) for the first 60 min (B), glucose-stimulated insulin secretion (C) and the corresponding AUC for the first 15 min (D), and ITT (E) performed in Control and MKK7D 2-month-old mice treated for 10 days with rosiglitazone (Rosi) or PBS, as indicated. F: Insulin secretion of pancreatic islets isolated from Control and MKK7D mice treated with PBS or rosiglitazone. Islets were stimulated with glucose and insulin as indicated. G: Immunoblot analysis performed in extracts of pancreatic islets from Control and MKK7D mice treated with rosiglitazone and insulin (20 min), as indicated. The ratio of phospho-Akt and phospho-JNK versus AKT and JNK, respectively, are represented at the right side panels.

In vivo JNK activation in pancreatic β-cells protects mice from HFD-induced insulin resistance and hyperinsulinemia

As long as the MKK7D mice do not secrete insulin in response to glucose and exhibit central insulin resistance, we investigated what were the effects of feeding them with a high-fat and high-carbohydrate diet (HFD). Previous studies using mice with impaired insulin secretion have connected it with a protection from HFD-induced insulin resistance (Lo *et al*, 2010; Kebede *et al*, 2008; Uebele *et al*, 2009). However, the increased plasma level of FFA induce JNK activation and insulin resistance (Solinas & Karin, 2010) which may affect β-pancreatic cells performance in the MKK7D mice which already have high JNK activity. Actually, acute pancreatitis developed upon HFD feeding is protected by the treatment with rosiglitazone, a JNK inhibitor. Nevertheless, jnk1 deficiency relieves the inhibition of glucose-induced insulin transcription by FFA and enhances the obesity-inhibited and glucose-induced insulin secretion (Solinas *et al*, 2006; Kaneto *et al*, 2004; Bennett, 2003).

In fact, MKK7D mice resulted to be partially protected from obesity-induced HFD. They were leaner than the Control mice (Fig. 23A) and had normal fasted blood glucose after 13 weeks of HFD, whereas the Control mice start developing fasting hyperglycemia after 9 weeks of being fed with HFD, corresponding to 13 weeks of age (Fig. 23B).

Indeed, Control mice developed a standard HFD-induced type-2 diabetes. As they had higher basal blood glucose and insulin, developed insulin resistance, and, therefore were glucose intolerant (Fig. 23B to F). Remarkably, MKK7D mice, despite a lower but sufficient increase in weight due to HFD, remained insulin sensitive and had normal basal blood glucose and insulin levels (Fig. 23A, B, E and F). Moreover, their glucose intolerance was not worsened by the HFD (Fig. 23D).

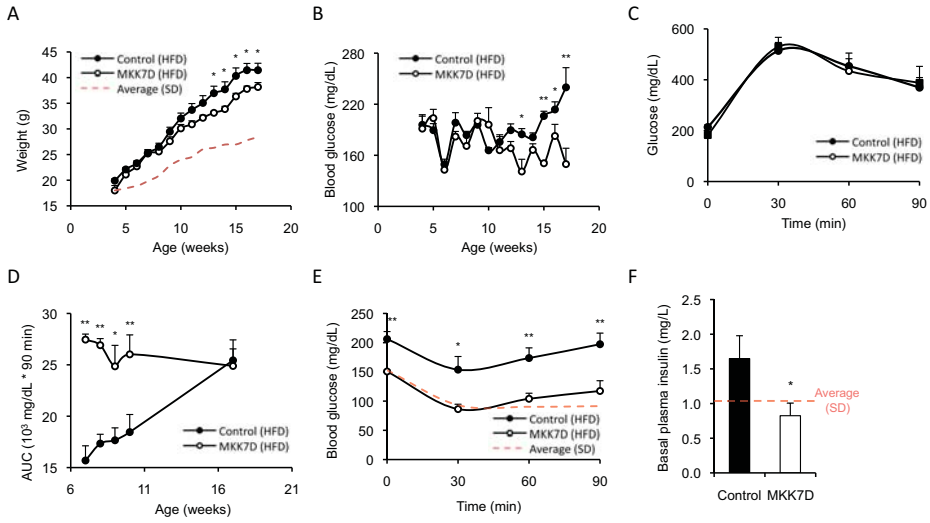

Figure 23. JNK activation in pancreatic β-cells protects from HFD-induced type-2 diabetes. A: Body weight curve of Control and MKK7D mice fed with HFD and average mice with standard diet (SD). B: Plasma glucose level in 6-h fasted Control and MKK7D mice fed with HFD. GTT (C) of 17-weeks old Control and MKK7D mice fed with high fat diet and the corresponding area under the curve (AUC) for the first 90 min at the indicated ages (D). ITT (E) and basal plasma insulin (F) of Control and MKK7D mice fed with HFD and average mice with SD. (Male mice; n = 6 for Control and n = 4 for MKK7D).

Increased life and health span in MKK7D mice

Glucose metabolism and insulin signaling have been also related to ageing. Specially IGF-1 is responsible to regulate lifespan (Holzenberger *et al*, 2003) but as long as insulin and IGF-1 share common downstream effectors and hybrid receptors have been described (Bailyes *et al*, 1997), insulin is also a focus of interest in ageing (Lamming *et al*, 2012). So far the studies using full IR KO have resulted in dramatic illness and early lethality (Wertheimer *et al*, 1993) but same results were obtained when using the IGF-1R KO (Gems & Partridge, 2001).

In this regard, MKK7D mice may be a good tool to study the impact of insulin in ageing as long as they have normal basal insulin levels but they lack the normal peaks of insulin in response to glucose. Actually, this phenotype resembles dietary restriction or the mutations in the nutrient sensing pathways, which also have been related to extended lifespan (Fontana *et al*, 2010).

MKK7D are protected from age induce obesity (Fig. 24A and B). Additionally, MKK7D mice had lower basal blood glucose than Control mice (Fig. 24C). In fact, glucose intolerance of MKK7D mice was progressively ameliorated (Fig. 24D) and their triglycerides were reduced to equal the levels of Control mice (Fig. 24E).

However, there were some age-symptoms that Control mice started to have like insulin resistance (Fig. 22F) and hyperinsulinemia (Fig. 22G) that the MKK7D mice not. These are indicators of initial diabetes before basal blood glucose increase and they become glucose intolerant. In this regard, MKK7D mice seem to be protected from them. On the top of that, general condition of Control mice was generally worse, having more infections, gastrointestinal problems and hyperplasias. Actually, at the age of 20 months 40% of lethality of Control mice whereas only a 10% of MKK7D mice was observed (Fig. 24H).

Therefore, according to these results overexpression of JNK in the pancreatic β-cells reduces insulin secretion by inhibiting insulin signaling, at least in youth, without affecting the general state of the mice. Actually this glucose intolerance and insulin secretion impairment seems to be beneficial in old ages by reducing diabetic traits in elderly and apparently extending lifespan. In spite of these, further analysis of the tissues and age markers are needed to clarify what the real state of the MKK7D mice is.

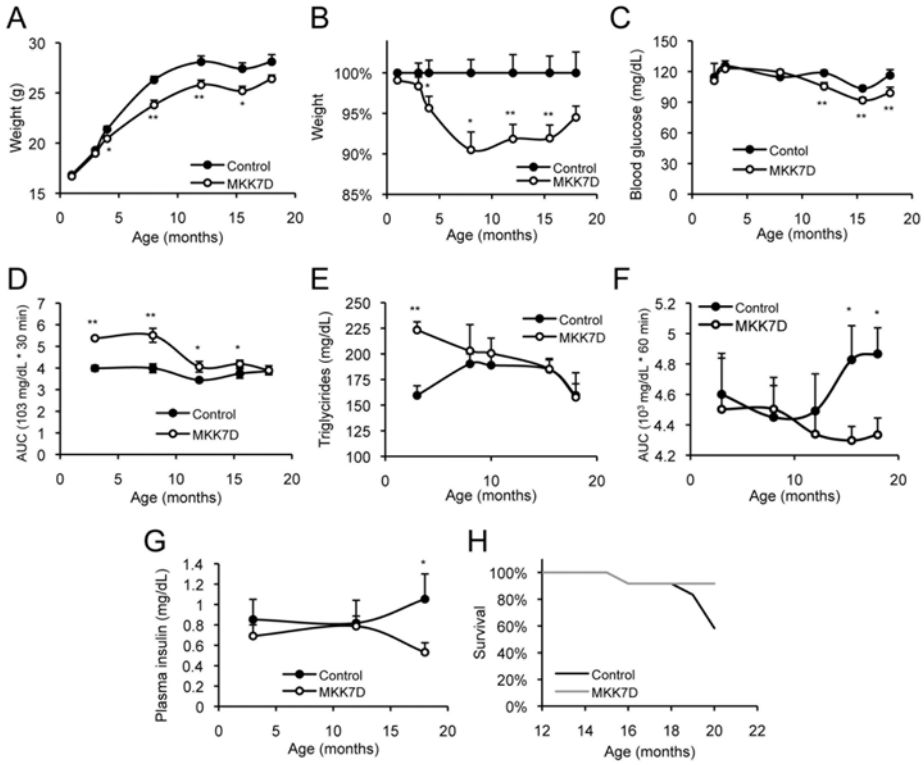

Figure 24. JNK activation in pancreatic β-cells protects against age-induced hyperinsulinemia, insulin resistance and extends lifespan. Body weight (A) and body weight percentage with respect the Control mice (B) comparison of Control and MKK7D mice. C: 6-h fasted plasma glucose level, area under de curve (AUC) of a GTT (D), 6-h fasted blood triglycerides levels (E), AUC of an ITT (F), 6-h fasted plasma insulin level (G) and survival percentage of Control and MKK7D mice at the indicated ages. (Female mice; n = 15/genotype).

DISCUSSION

JNK AND THE SCF$^{F\text{-}BOX}$ COMPLEXES

It is becoming more and more evident that signaling pathways cannot be studied as lineal and isolated cascades. Actually, it is the coordination of the different pathways what enables complex and tuned responses to a wide range of stimuli. In the case of inflammation, the two main pathways involved are JNK and NF-κB (Kyriakis & Avruch, 2012; Hayden & Ghosh, 2012).Essentially, the most known interaction in between them is the repressory effect of NF-κB over JNK in order to prevent apoptosis (Smaele *et al*, 2001; Tang *et al*, 2001). However, much little is known about the JNK activation needed to induce NF-κB in response to certain stimuli. Indeed, this positive effect of JNK on NF-κB is feasible to happen as long as they have common upstream activators (Lee *et al*, 1997). In the case of inflammation, for example, a joint labour could be clearly beneficial and could determine the correct functioning and the efficiency of the response.

Up until now, the main described effect of JNK on NF-κB has been the increase of the SCF$^{\beta TrCP}$ ubiquitination complex (Arevalo, 2008). We have tried to investigate separately the effects at βTrCP protein and mRNA level since they have been described to be independent (Spiegelman *et al*, 2001).

Regarding transcription independent stabilization, we have proved that protein-to-protein interaction is very important for the SCF$^{\beta TrCP}$ complex stability and therefore NF-κB activition. The core complex, formed by SKP1 and βTrCP, is increased just by overexpressing one of the members. This effect is lost when using the βTrCP$^{\Delta F}$, highlighting the importance of this interaction. Indeed, βTrCP$^{\Delta F}$ exerts a dominant negative action on SKP1 levels reinforcing the theory in which the interaction SKP1-βTrCP is crucial for the stability of the complex. Not only this but this result indicates that there might be some other protein needed that is being sequestered by βTrCP$^{\Delta F}$. However, the fact that βTrCP$^{\Delta F}$ can still be stabilized by JNK activation, supports the idea that there is more than one mechanism by which JNK is enhancing the levels of the complex.

Indeed, active JNK can be found bound to the SKP1-βTrCP dimmer (Arevalo, 2008) also supporting the idea that JNK could be acting as a scaffolding protein of the complex, enabling and/or enhancing its formation. JNK would not be the first kinase that acts in this way on an ubiquitination complex. The kinase DYRK2 was found to associate and serve as a molecular assembler for the EDV ubiquitination complex independently of its kinase activity (Maddika & Chen, 2009).

Another positive effect of JNK on βTrCP is the augmentation of the βTrCP mRNA in a transcription independent way. Results of this thesis suggest that this stabilization could be mediated by phosphorylation of CRD-BP by JNK. As long as the biochemical effect of phosphorylation on CRD-BP activity was not measured, it is not sure that this is the mechanism by which JNK induces βTrCP mRNA. Anyhow, the CRD-BP-miR183 system was clearly affected because the transcription of the mutant form of βTrCP, $\beta TrCP^{\delta miR183}$, was not induced anymore by activating specifically JNK. Hence, the JNK-mediated CRD-BP phosphorylation would be useless as long as mir183 cannot bind $\beta TrCP^{\delta miR183}$ anymore.

Nevertheless, $\beta TrCP^{\delta miR183}$, as well as $\beta TrCP^{\Delta F}$, retained the capacity of being stabilized at a protein level by JNK activation. All this results together indicate the complexity by which JNK is stabilizing βTrCP. In spite of this, all the results point towards an increase of βTrCP, and therefore an induction of NF-κB, upon JNK stimulation. This interaction may be critical for some responses such as IL-1β, were both JNK and NF-κB have a pro-apoptotic role (Cnop *et al*, 2005; Giannoukakis *et al*, 2000).

This interaction could be also very important in the cellular signaling induced by some anticancer drugs such as Doxorubicin. It has already been described that JNK-induced apoptosis is activated in response to this drug used in cancer chemotherapy (Panaretakis *et al*, 2005). Essentially, NF-κB activation has been related to cancer therapy-induced apoptosis resistance (Wang *et al*, 1996). However, some reports challenge this model and propose that NF-κB activity seen in response to DNA damage induced by ultraviolet radiation and chemotherapeutics can function to promote cell

death (Campbell *et al*, 2004; Ho *et al*, 2005; Ashikawa *et al*, 2004). These studies further suggest that NF-κB activation may be required for doxorubicin to induce cell death and therefore that combining targeted NF-κB inhibition could actually serve to counteract the desired cell killing effects of chemotherapy. Again the answer to this inconsistency may be explained by the differences in the cellular types and duration of the stimuli. Anyhow, it is clear that NF-κB activation in certain cases is relevant to induce apoptosis. Therefore, the joint response of the two pathways could be again essential for the efficiency of this anticarcinogenic agent.

In agreement with this first part of the results, β-catenin, another substrate for βTrCP was also affected. Indeed, Wnt signaling pathway also needed to be activated prior to see any difference in β-catenin activity. In addition, this is not the only mechanism by which JNK antagonizes Wnt/β-catenin signaling. Firstly, JNK can also phosphorylate and activate GSK3, what would trigger β-catenin phosphorylation and ubiquitination (Hu *et al*, 2008, 2009). Moreover, other studies indicate that JNK antagonizes canonical Wnt signaling by reducing nuclear β-catenin (Liao *et al*, 2006) In contrast, JNK can also cooperate with Wnt/β-catenin as JNK-induced phosphorylated c-Jun can bind TCF4/β-catenin and enhance the transcription of common target genes (Nateri *et al*, 2005).

Considering that $SCF^{F\text{-}Box}$ complexes share a great number of the proteins and regulators, the increase protein levels of SKP2 in response to JNK activation is not unexpected. Neither is it the consequent increase of its target p27 protein levels found in MEFs *jnk*1/2 KO. The similarities in between $SCF^{\beta TrCP}$ and SCF^{SKP2} can be found at different levels. First of all, they share components of the complex, like SKP1 and Cul1 for instance. Besides that, SKP2 mRNA also has predicted binding sites for CRD-BP, which we have suggested to be activated by JNK. Not only this but the final ensemble of the complex is regulated in both cases by COP2 (Denti *et al*, 2006; Wolf *et al*, 2003).

Interestingly, p27 is a cell cycle regulator that controls the cell cycle progression to G1 by inhibiting CDK2 and Cyclin E. It has been described that mutation or loss of p27 expression leads to an uncontrolled cellular proliferation and has been observed in

metastatic carcinomas (Fero *et al*, 1996; Nakayama *et al*, 1996; Kiyokawa *et al*, 1996). Accordingly, MEFs JNK1/2 KO, that had more p27, had a lower mitotic index and were arrested in G1 (unpublished data from the lab). This effect would link JNK to cell cycle progression by inducing SCF^{SKP2} levels and activity, what would induce p27 degradation and progression to S phase. Actually, JNK has already been proposed as a G1/S transition regulator even no targets were proposed (Nagata *et al*, 1998).

Together all these results suggest a major role of JNK in the regulation of $SCF^{F\text{-}Box}$ ubiquitination complexes. The possible implications of this interaction are very wide, as long as cellular functions, from inflammation to cell cycle, may be affected. The mechanism that we propose is complex, since it would be acting as a scaffold to trigger the $SCF^{F\text{-}Box}$ formation but also, through the phosphorylation of CRD-BP and subsequent stabilization of microRNA targeted F-Box mRNAs. Moreover, additional steps of the pathway could be affected in specific cases such as the F-Box target phosphorylation or the activation/inactivation of the kinase in charge of doing it.

JNK ACTIVATION IN THE β-PANCREATIC CELLS

Insulin resistance is an early trait in the development of type-2 diabetes; afterwards, pancreatic β-cell failure caused by an increased insulin demand is the major determinant of progression to hyperglycemia, the hallmark of diabetes. Compelling evidence has demonstrated that exacerbated JNK activity participates in promoting insulin resistance in peripheral insulin-target tissues, namely skeletal muscle, adipose tissue and liver (Hirosumi *et al*, 2002; Nakatani *et al*, 2004; Tuncman *et al*, 2006; Sabio *et al*, 2010; Zhang *et al*, 2011b; Solinas *et al*, 2007; Masharani *et al*, 2011).

In addition, a few studies indicate that the JNK pathway is also involved in the negative regulation of insulin sensitivity in pancreatic β-cells. In this regard, jnk1-deficient pancreatic islets show increased glucose-induced insulin secretion (Varona-Santos *et al*, 2008) and protection against FFA-induced inhibition of glucose-triggered insulin gene transcription (Solinas *et al*, 2006). Nonetheless, in vivo data supporting a regulatory role of JNK in insulin sensitivity in pancreatic β-cells has been obtained using genetic and/or chemical inhibition of JNK (Solinas *et al*, 2006; Kaneto *et al*, 2004; Bennett, 2003; Varona-Santos *et al*, 2008; Kaneto *et al*, 2002), approaches that allow the assessment of JNK requirement but not competence of this kinase to perform a particular role.

With this aim, we generated a transgenic mouse model that allows JNK activation in a Cre recombinase expression-dependent manner, and, using mice strains expressing the Cre recombinase or Cre recombinase/ER under the control of the Ins2 promoter, we achieved β-cell-specific activation of JNK in a constitutive and a tamoxifen-inducible manner, respectively.

Our results show that JNK activation in pancreatic β-cells produces a glucose-intolerant phenotype caused by impaired capacity to increase insulinemia in response to hyperglycemia. Although in vivo JNK activation by expression of a constitutively activated version of its immediately upstream activator MKK7D is a strategy previously shown to specifically induce JNK (Petrich *et al*, 2003), we confirmed that the observed defects were dependent on JNK activity as they were reverted by JNKi-1, a highly

specific JNK inhibitor peptide (Bonny *et al*, 2001), and/or by rosiglitazone, a treatment also known to inhibit JNK activation in vivo and in several cell types, including insulin-secreting cell lines (Díaz-Delfín *et al*, 2007), and also shown in the current study.

Moreover, the glucose intolerance phenotype shown by MKK7D mice was exhibited independently of gender and the post-natal age of the animals (though the experiments shown were performed in 3-month old mice, GTT performed on animals from 1 to 12 months of age gave similar results), and by all the animals tested so far (around 200 mice). These findings suggest that the penetrance of this phenotype is close to 100%. In addition, it is likely to be achieved immediately after JNK activation and independently of the developmental stage, as comparable results were obtained in MKK7D and tamoxifen-treated MKK7DTam mice, and reversible, as it was reverted in vivo by a relatively short treatment with rosiglitazone (the shortest time tested has been 4 days) and in isolated islets by incubation with JNKi-1.

Finally, although in both mice strains used the Cre recombinase expression is under the Ins2 promoter sequences, known to also drive expression to certain regions of the brain (Wicksteed *et al*, 2010; Chan & Sherwin, 2012), the finding that isolated pancreatic islets show defects in glucose-induced insulin secretion strongly suggest that the described phenotype is a β-cell autonomous effect caused by JNK activation specifically in this cell type.

According to our results, JNK activation in pancreatic β-cells results in glucose intolerance as a result of impaired capacity to increase blood insulin level in response to hyperglycemia. However, insulin secretion in response to glucose consists in two phases and in an in vivo setting only the second one can be observed. Importantly, first phase is only dependant in blood glucose whereas a more complex stimuli. Therefore, we cannot discard that MKK7D mice still have the ability to secrete some insulin.

Moreover, this insulin secretion-impairment does not correlate with any obvious morphological or structural abnormality in the pancreas or the islets or with lower

insulin content. Moreover, basal glycemia and insulinemia were normal in MKK7D and tamoxifen-treated MKK7DTam mice.

In contrast, static insulin secretions of pancreatic islets isolated from these animals were markedly impaired in response to glucose and insulin, a defect that was reversed by incubation of the islets with the JNK inhibitor peptide or with rosiglitazone. In contrast, these same islets were not defective for IBMX-, KCl-, succinate, KIC or tolbutamide-induced insulin release. Overall, these results indicate that insulin secretion in response to insulin, but not to glucose metabolism (Ashcroft & Rorsman, 2012), was affected by JNK activation. Accordingly, insulin-induced Akt phosphorylation, a key event in insulin signaling (Kubota *et al*, 2012b), and the induction of expression of downstream insulin-target genes was impaired in islets from MKK7 mice.

Defective insulin signaling in MKK7D mice was also supported by their inability to induce Pdx1 nucleocytoplasmic shuttling in response to glucose or insulin. In this regard, Pdx1 translocation from nucleus to cytoplasm has been reported to occur upon inhibition of insulin signaling by oxidative stress in cultured cells (Kawamori *et al*, 2003). Despite no differences were observed between Control and MKK7D mice in Pdx1 subcellular location in basal conditions, the later maintained Pdx1 nuclear localization even 30 min after stimulation with glucose or insulin, a time point by which insulin signaling might be in the process to be turned off as insulinemia almost returned to basal level, whereas in Control mice Pdx1 shuttled from nucleus to cytoplasm suggesting that deactivation of the insulin signaling pathway was taking place. Moreover, the increased Pdx1 content in MKK7D islets might be also indicative of defective insulin signaling as this pathway regulates the steady-state level of Pdx1 (Humphrey *et al*, 2010).

Overall, these results are consistent with the previously reported role of JNK in blocking InsR signaling through IRS-1/-2 phosphorylation on serine (Solinas *et al*, 2006; Aguirre *et al*, 2000).

TZD treatment improves the pancreatic condition in type 2 diabetes by alleviating insulin resistance in peripheral tissues in a PPARγ-dependent manner (Willson *et al*, 1996; Berger *et al*, 1996). Nonetheless, an insulin-sensitizing action of these drugs directly on the pancreas has not been reported previously. Supporting this notion is the documented expression of PPARγ in β-cells, where it regulates proliferation (Rosen *et al*, 2003). In addition, we had previously shown that the anti-diabetic action of rosiglitazone relies on the inhibition of the obesity-induced activation of the JNK pathway (Díaz-Delfín *et al*, 2007). Finally, we had also shown that rosiglitazone treatment improves the survival of insulin-secreting cells lines, such as Ins1-E and βTC3 cells, exposed to the pro-inflammatory cytokine IL-1β, concomitantly with the inhibition of the cytokine-induced activation of JNK (Díaz-Delfín *et al*, 2007).

Therefore, we considered that the MKK7D mouse was a suitable animal model in which to test whether rosiglitazone ameliorates pancreatic insulin resistance independently of its action on peripheral tissues. Remarkably, rosiglitazone almost completely reverted the in vivo (glucose intolerance) and in vitro (glucose and insulin-induced insulin secretion) phenotype induced by JNK activation in pancreatic β-cells. These actions correlated with the ability of rosiglitazone to inhibit MKK7D-induced activation of JNK and, concomitantly, the recovery of insulin-induced phosphorylation of Akt, hence the InsR signaling. In addition, since none of these animals exhibited peripheral insulin resistance, the ITTs were not affected by rosiglitazone treatment in any group of mice. These results indicate that, in addition to their insulin-sensitizing action in peripheral tissues (mainly in adipose tissue), TZDs also ameliorate pancreatic insulin resistance, and that in both actions the inhibition of exacerbated JNK activity plays a fundamental role.

JNK is involved in the loss of pancreatic β-cells induced by pro-inflammatory cytokines (Ammendrup *et al*, 2000; Kim *et al*, 2005), and jnk1 deficiency or treatment with JNK inhibitors prevents IL-1β-induced apoptosis of isolated islets and/or insulin-secreting cell lines (Díaz-Delfín *et al*, 2007; Bennett, 2003; Varona-Santos *et al*, 2008; Bonny *et al*,

2001). Moreover, JNK inhibition enhances the survival of islets subjected to transplantation protocols (Aikin *et al*, 2004; Noguchi *et al*, 2005). While all these data support a role of JNK in promoting β-cell death, they do not distinguish whether JNK is sufficient or merely required to attain this process.

In this regard, our results support the notion that JNK activation is not sufficient to promote β-cell death as, contrary to what would be expected if JNK activation was sufficient, no morphological or structural abnormalities were found in the pancreas or islets of MKK7D mice. Moreover, the only activation of JNK did not induce cleavage and concomitant activation of caspase 3, a mediator of β-cell death in response to stimuli that also trigger JNK activity (Kim *et al*, 2005). Therefore, involvement of additional pathways deregulated in obesity and type 2 diabetes due to the chronic inflammatory state, increased FFAs and/or induction of endoplasmic reticulum stress (Gregor & Hotamisligil, 2011; Ozcan *et al*, 2004), such as the NFκB cascade, might be required to effectively induce β-cell death (Eldor *et al*, 2006).

Not only this, but when we forced the engine by feeding the animals with HFD or letting them age, in both challenges MKK7D mice performed better than the Control mice. In both situations they were more sensible to insulin and had lower basal blood concentrations of glucose and insulin. These results also remark that JNK activation in β-cell was not as dramatic as previous results would suggest even in an extreme setting, even though some adaptation event seems to happen in old MKK7D mice.

In contrast to our findings, other studies have enhanced protection of HFD-induced diabetes by inducing insulin signaling in the β-cells, like the PTEN KO mice (Wang *et al*, 2010). However, in that model the protection was at a pancreatic level and the mice developed peripheral insulin resistance normally. PTEN KO mice enhance β-cell function by regulating negatively insulin signaling and increasing β-cell mass so they can overcome the increased insulin demand. In comparison, MKK7D mice were insulin sensitive until the end of the experiment even though they were glucose intolerant. Actually, from all the measured parameters, only the weight was increased even tough

it was not increased as much as Control mice. It is especially relevant that MKK7D mice did not develop HFD-induced insulin resistance.

Hence, the development of peripheral insulin resistance seems to require not only high circulating levels of FFA but also repeated peaks of insulin. Correspondingly, mice with induced hyperinsulinemia develop insulin resistance without having hyperglycemia or increased weight (Shanik *et al*, 2008). Actually, hyperinsulinemia is often preceding insulin resistance (Gray *et al*, 2010) and many patients with sustained hyperinsulinemia induced by an insulinoma have reduced responsiveness to administered insulin (Nankervis *et al*, 1985).

Additionally, it has been proposed that inhibition of JNK could be beneficial to prevent insulin resistance, specially in obese subjects (Nakatani *et al*, 2004; Henstridge *et al*, 2012). Actually, JNK1-deficient mice are protected against HFD-induced obesity and insulin resistance (Hirosumi *et al*, 2002). In the case of muscle or adipose tissue specific ablation of JNK1, the protection is only seen at the level of insulin sensitivity (Sabio *et al*, 2008, 2010). In contrast, inhibiting JNK in adipose by depletion of JIP1 is protective against HFD-induced obesity and insulin resistance (Jaeschke *et al*, 2004).

Here we provide further information about the impact of JNK activation/inactivation and insulin resistance. We therefore reprove systemic inhibition of JNK to prevent insulin resistance since as a result central insulin resistance would be diminished. Not only we have proved that it is beneficial for the glucose homeostasis in a HFD scenario but also β-cells would secrete more insulin and consequently enhancing insulin resistance.

Regarding the ageing experiments, one striking result is that MKK7D mice become glucose tolerant. Firstly they had a small increase in peripheral insulin sensitivity and in the other hand they started to secrete a little bit of insulin in response to glucose. However, both effects were very slight and not enough to explain the change in the glucose clearance. More studies should be performed to address this question but one

of the options is that their renal tubes have lowered the glucose concentration threshold and it is being excreted through the urine. Alternatively, peripheral tissues may have got used to the lack of insulin peaks and they have increased their capacity to internalize glucose, for example, by increasing or changing the type of glucose transporters. This last hypothesis would be supported by the fact that the weight difference gap is becoming smaller. It should be also taken into consideration as well that recombination efficiency is about 85% and the remaining 15% may have repopulated the islets. However this theory cannot be addressed with alive mice.

Whatever the explanation is, MKK7D mice have shown to have an extended lifespan and better ageing both in terms of metabolic performance and overall physiology. Actually, the lack of insulin peaks, at least in youth, has resulted in an increase of a 30% in the survival at the age of 20 months. It needs to be pointed out that the mortality of Control mice is a bit higher than it would be expected for wild-type mice. One possible reason is that they have GFP expression in all the tissues what may induce cellular stress. Nevertheless, if that were the case, it would be the same for MKK7D mice except in β-cells, where Cre recombinase expression drastically reduces GFP expression while, concomitantly, increasing MKK7D expression. However, ageing markers like telomerase length or N-glycomics still need to be performed.

This lifespan increase is in agreement with the experiments with reduced IGF-1 levels or reduced IGF-1 signaling (Holzenberger *et al*, 2003; Cohen *et al*, 2009; Conover & Bale, 2007). In contrast reduction of function of the IR in humans results in type 2-diabetes and homozygous loss of IR function results in leprechaunism, which usually leads to death within the first year of life (Wertheimer *et al*, 1993), and neonatal lethality in mice (Accele *et al*, 1996). Nevertheless, complete loss of function of the insulin/IGF receptor also results in lethality in flies and worms (Gems & Partridge, 2001). However, some mice models with reduced insulin signaling also point to be protective against ageing (Harrison *et al*, 2009; Wang *et al*, 2005).

Actually, IGF-1 and Insulin signaling have a common ancestral origin in flies and worms, IIS (Fontana *et al*, 2010). As a consequence, they share many downstream effectors, there exist hybrid receptors (Bailyes *et al*, 1997; Moxhams *et al*, 1989) and they can cross-react with each other's receptors (Johansson & Arnqvist, 2006). Therefore is likely to happen that the lack of insulin peaks is also reducing the IGF-1-like signaling induced by insulin, what would explain the results.

In fact, MKK7D mimic somehow caloric restriction as long as there is less amount of plasma insulin. As a consequence, in the two scenarios there is less insulin/IGF-1 signaling and indeed both have extended lifespan (Fontana *et al*, 2010). Accordingly, mutations in the nutrient sensing pathways also recapitulate the phenotype (Dilova *et al*, 2007).

In summary, our data support the relevance of JNK in the regulation of insulin sensitivity in pancreatic β-cells by showing that mere activation of this kinase is sufficient to inhibit IR signaling and hence promote insulin resistance in pancreatic β-cells in vivo. However, in this same experimental setting, JNK activation does not seem to elicit β-cell death, thereby suggesting that JNK activity may be required but is not sufficient for this process to occur. Moreover, we also provide the first evidence of insulin-sensitizing action of TZDs directly on pancreatic β-cells. Our observations therefore contribute to a better understanding of the molecular mechanisms that mediate the anti-diabetic action of these drugs.

At the same time, results with the experiments with HFD and ageing have shown that MKK7D mice didn't perform worse than the Control mice in these situations. Not only this but they had a better metabolic status and overall physiology. The experiments with HFD have denoted that insulin itself it is required for the development of peripheral insulin resistance and other traits of HFD-induced diabetes, while JNK activation in β-cells helped to maintain a normal condition. In the case of ageing, MKK7D overexpression also denoted the importance that insulin itself and not only IGF-1 can have in the extension of lifespan.

It is also remarkable that, even though JNK activity has already been shown that it is implicated in *C. elegans* and *D. melanogaster* life span (Wang *et al*, 2005; Lee *et al*, 2009; Neumann-Haefelin *et al*, 2008), this is the first mice model to demonstrate JNK involvement in ageing.

CONCLUSIONS

JNK effect on βTrCP

1. JNK targets miRNA183/CRD-BP system to stabilize βTrCP mRNA
2. SPK1-βTrCP complex formation is required for JNK-dependent SKP1 and βTrCP protein stabilization
3. The βTrCP substrate β-catenin is down regulated by the JNK-dependent increase of βTrCP.
4. The protein level of SKP2 and its substrate p27 are oppositely regulated by JNK

JNK activation in β-pancreatic cells and glucose homeostasis

1. JNK activation in pancreatic β-cells leads to glucose intolerance as a result of an impaired capacity to increase insulinemia in response to hyperglycemia.
2. JNK activation in pancreatic β-cells affects neither the morphology nor the insulin content of the islets.
3. JNK activation in pancreatic β-cells impairs insulin signaling and consequently inhibits insulin induced-insulin secretion and gene transcription.
4. Rosiglitazone counteracts JNK-induced β-cell dysfunction.
5. JNK activation in pancreatic β-cells is protective against HFD- and age-induced hyperinsulinemia and insulin resistance.
6. JNK activation in pancreatic β-cells extends lifespan in mice.

REFERENCES

Abdelli S, Ansite J, Roduit R, Borsello T, Matsumoto I, Sawada T, Allaman-pillet N, Henry H, Beckmann JS, Hering BJ & Bonny C (2004) Intracellular Stress Signalin Pathways Activated During Human Islet Preparation and Following Acute Cytokine Exposure. *Diabetes* 53: 2815–2823

Accele D, Drago J, Lee E, Johnson M, Cool M, Salvatore P, Asico L, José P, Taylor S & Westphal H (1996) Early neonatal death in mice homozygus for a null allele of the insulin receptor gene. *Nature genetics* 12: 106–10

Adjei a a (2001) Blocking oncogenic Ras signaling for cancer therapy. *Journal of the National Cancer Institute* 93: 1062–74

Adler V, Polotskaya a, Wagner F & Kraft a S (1992) Affinity-purified c-Jun amino-terminal protein kinase requires serine/threonine phosphorylation for activity. *The Journal of biological chemistry* 267: 17001–5

Aguirre V, Uchida T, Yenush L, Davis R & White MF (2000) The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). *The Journal of biological chemistry* 275: 9047–54

Aikin R, Maysinger D & Rosenberg L (2004) Cross-talk between phosphatidylinositol 3-kinase/AKT and c-jun NH2-terminal kinase mediates survival of isolated human islets. *Endocrinology* 145: 4522–31

Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P & Hemmings BA (1996) Mechanism of activation of protein kinase B by insulin and IGF-1. *The EMBO journal* 15: 6541–6551

Ammendrup A, Maillard A, Nielsen K, Andersen NA, Serup P, Madsen OD, Mandrup-poulsen T & Bonny C (2000) The c-Jun Amino-Terminal Kinase Pathway Is Preferentially Activated by Interleukin-1 and Controls Apoptosis in Differentiating Pancreatic Æ-Cells. *Diabetes* 49: 1468–1475

Arevalo M (2008) La Ruta de JNK: interacción con la vía de NF-κB y diseño de un ratón transgénico para su activación selectiva in vivo.

Ashcroft FM & Rorsman P (2012) Diabetes mellitus and the β cell: the last ten years. *Cell* 148: 1160–71

References

Ashikawa K, Shishodia S, Fokt I, Priebe W & Aggarwal BB (2004) Evidence that activation of nuclear factor-κB is essential for the cytotoxic effects of doxorubicin and its analogues. *Biochemical Pharmacology* 67: 353–364

Baeuerle P a & Baltimore D (1988) I kappa B: a specific inhibitor of the NF-kappa B transcription factor. *Science* 242: 540–6

Bailyes EM, Navé BT, Soos M a, Orr SR, Hayward a C & Siddle K (1997) Insulin receptor/IGF-I receptor hybrids are widely distributed in mammalian tissues: quantification of individual receptor species by selective immunoprecipitation and immunoblotting. *The Biochemical journal* 327 (Pt 1: 209–15

Barkett M & Gilmore TD (1999) Control of apoptosis by Rel / NF-kB transcription factors. *Oncogene* 18: 6910–6924

Barnes PJ (1998) Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clinical science* 94: 557–72

Barr RK & Bogoyevitch MA (2001) The c-Jun N-terminal protein kinase family of mitogen-activated protein kinases (JNK MAPKs). *The international journal of biochemistry & cell biology* 33: 1047–63

Bartke A (2005) Minireview: role of the growth hormone/insulin-like growth factor system in mammalian aging. *Endocrinology* 146: 3718–23

Beato M, Herrlich P & Schütz G (1995) Steroid hormone receptors: many actors in search of a plot. *Cell* 83: 851–7

Bennett B (2003) JNK: a new therapeutic target for diabetes. *Current Opinion in Pharmacology* 3: 420–425

Berger J, Bailey P, Biswas C, Cullinan C a, Doebber TW, Hayes NS, Saperstein R, Smith RG & Leibowitz MD (1996) Thiazolidinediones produce a conformational change in peroxisomal proliferator-activated receptor-gamma: binding and activation correlate with antidiabetic actions in db/db mice. *Endocrinology* 137: 4189–95

Besnard-Guerin C, Belaïdouni N, Lassot I, Segeral E, Jobart A, Marchal C & Benarous R (2004) HIV-1 Vpu sequesters beta-transducin repeat-containing protein (betaTrCP) in the cytoplasm and provokes the accumulation of beta-catenin and other SCFbetaTrCP substrates. *The Journal of biological chemistry* 279: 788–95

Biesma DH, Hannema AJ, Velzen-blad H Van, Mulder L, Zwieten R Van, Kluijt I & Roos D (2001) A family with complement factor D deficiency. *The Journal of Clinical Investigation* 108: 233–240

Biondi RM & Nebreda AR (2003) Signalling specificity of Ser/Thr protein kinases through docking-site-mediated interactions. *The Biochemical journal* 372: 1–13

Biteau B, Karpac J, Hwangbo D & Jasper H (2011) Regulation of Drosophila lifespan by JNK signaling. *Experimental Gerontology* 46: 349–354

Black R, Rauch C, Kozlozky C, Peschon J, Slack J, Wolfson M, Castner F, Stocking K, Reddy P, Srinivasan S, Nelson N, Boiani N, Schooley K, Gerhart M, Davis R, Fitzner J, Johnson R, Paxton R, March C & Cerretti C (1977) A metalloproteinase disintegrin that releases tumor-necrosis factor-a from cells. *Nature* 385: 729–733

Blackwell K, Zhang L, Thomas GS, Sun S, Nakano H & Habelhah H (2009) TRAF2 phosphorylation modulates tumor necrosis factor alpha-induced gene expression and cell resistance to apoptosis. *Molecular and cellular biology* 29: 303–14

Blagosklonny M V (2012) Once again on rapamycin-induced insulin resistance and longevity: despite of or owing to. *Aging* 4: 350–8

Bonny C, Oberson A, Negri S, Sauser C & Schorderet DF (2001) Cell-Permeable Peptide Inhibitors of JNK. *Diabetes* 50: 77–82

Bosu DR & Kipreos ET (2008) Cullin-RING ubiquitin ligases: global regulation and activation cycles. *Cell division* 3: 7

Bour S, Perrin C, Akari H & Strebel K (2001) The human immunodeficiency virus type 1 Vpu protein inhibits NF-kappa B activation by interfering with beta TrCP-mediated degradation of Ikappa B. *The Journal of biological chemistry* 276: 15920–8

Boussif O, Lezoualc'h F, Zanta M a, Mergny MD, Scherman D, Demeneix B & Behr JP (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proceedings of the National Academy of Sciences of the United States of America* 92: 7297–301

Brown K, Park SUN, Kanno T, Franzoso G & Siebenlist U (1993) Mutual regulation of the transcriptional activator NF-kB and its Inhibitor, IkB-

alpha. *Proceedings of the National Academy of Sciences of the United States of America* 90: 2532–2536

Bruna A, Nicolàs M, Muñoz A, Kyriakis JM & Caelles C (2003) Glucocorticoid receptor-JNK interaction mediates inhibition of the JNK pathway by glucocorticoids. *The EMBO journal* 22: 6035–44

Bruning JC, Dinesh G, Burks D, Gillette J, Schubert M, Orban P, Klein R, Krone W, Müller-Wieland D & Kahn C (2000) Role of Brain Insulin Receptor in Control of Body Weight and Reproduction. *Science* 289: 2122–2125

Bubici C, Papa S, Dean K & Franzoso G (2006) Mutual cross-talk between reactive oxygen species and nuclear factor-kappa B: molecular basis and biological significance. *Oncogene* 25: 6731–48

Bunting M, Harris ES, McIntyre TM, Prescott SM & Zimmerman G a (2002) Leukocyte adhesion deficiency syndromes: adhesion and tethering defects involving beta 2 integrins and selectin ligands. *Current opinion in hematology* 9: 30–5

Caelles C, González-Sancho JM & Muñoz a (1997) Nuclear hormone receptor antagonism with AP-1 by inhibition of the JNK pathway. *Genes & development* 11: 3351–64

Campbell KJ, Rocha S & Perkins ND (2004) Active repression of antiapoptotic gene expression by RelA(p65) NF-kappa B. *Molecular cell* 13: 853–65

Cao Z, Xiong J, Takeuchi M, Kurama T & Goeddel D V (1996) TRAF6 is a signal transducer for interleukin-1. *Nature* 383: 443–6

Castrillo A, Trave PG, Martín-sanz P, Parkinson S, Parker PJ & Bosca L (2003) Potentiation of Protein Kinase C z Activity by 15-Deoxy- D 12 , 14 - Prostaglandin J 2 Induces an Imbalance between Mitogen-Activated Protein Kinases and NF-kB That Promotes Apoptosis in Macrophages. *Molecular and Cellular Biology* 23: 1196–1208

Cenciarelli C, Chiaur DS, Guardavaccaro D, Parks W, Vidal M, Pagano M & F-box SE (1999) Identification of a family of human F-box proteins F-box proteins are an expanding family of eukaryotic proteins characterized by an approximately 40 amino- acid motif , the F box (so named because cyclin F was SCFs . The other three subunits are the Skp. *Current biology* 9: 1177–1179

Chan O & Sherwin RS (2012) Hypothalamic regulation of glucose-stimulated insulin secretion. *Diabetes* 61: 564–5

Chang L & Karin M (2001) Mammalian MAP kinase signalling cascades. *Nature* 410: 37–40

Chen FE & Ghosh G (1999) Regulation of DNA binding by Rel / NF-kB transcription factors: structural views. *Oncogene* 18: 6845–6852

Chu W-M (2012) Tumor necrosis factor. *Cancer letters* 328: 222–225

Clancy DJ, Gems D, Harshman LG, Oldham S, Stocker H, Hafen E, Leevers SJ & Partridge L (2001) Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein. *Science* 292: 104–6

Cnop M, Welsh N, Jonas J, Jo A, Lenzen S & Eizirik D (2005) Mechanisms of Pancreatic ÆB-Cell Death in Type 1 and Type 2 Diabetes. *Diabetes* 54: 97–107

Cohen E, Paulsson JF, Blinder P, Burstyn-Cohen T, Du D, Estepa G, Adame A, Pham HM, Holzenberger M, Kelly JW, Masliah E & Dillin A (2009) Reduced IGF-1 signaling delays age-associated proteotoxicity in mice. *Cell* 139: 1157–69

Conover C a & Bale LK (2007) Loss of pregnancy-associated plasma protein A extends lifespan in mice. *Aging cell* 6: 727–9

Cope G a & Deshaies RJ (2006) Targeted silencing of Jab1/Csn5 in human cells downregulates SCF activity through reduction of F-box protein levels. *BMC biochemistry* 7: 1

Cope G a, Suh GSB, Aravind L, Schwarz SE, Zipursky SL, Koonin E V & Deshaies RJ (2002) Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1. *Science* 298: 608–11

Cross D a, Alessi DR, Cohen P, Andjelkovich M & Hemmings B a (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378: 785–9

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y & Greenberg ME (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 91: 231–41

Daub H, Olsen J V, Bairlein M, Gnad F, Oppermann FS, Körner R, Greff Z, Kéri G, Stemmann O & Mann M (2008) Kinase-selective enrichment enables quantitative phosphoproteomics of the kinome across the cell cycle. *Molecular cell* 31: 438–48

Davis M, Hatzubai A, Andersen JS, Ben-shushan E, Fisher GZ, Yaron A, Bauskin A, Mercurio F, Mann M & Ben-neriah Y (2002) Pseudosubstrate regulation of the SCF B-TrCP ubiquitin ligase by hnRNP-U. *Genes & Development* 16: 439–451

Davis RJ (2000) Signal transduction by the JNK group of MAP kinases. *Cell* 103: 239–52

Deng Y, Ren X, Yang L, Lin Y & Wu X (2003) A JNK-dependent pathway is required for TNFalpha-induced apoptosis. *Cell* 115: 61–70

Denti S, Fernandez-Sanchez ME, Rogge L & Bianchi E (2006) The COP9 signalosome regulates Skp2 levels and proliferation of human cells. *The Journal of biological chemistry* 281: 32188–96

Dephoure N, Zhou C, Villén J, Beausoleil S a, Bakalarski CE, Elledge SJ & Gygi SP (2008) A quantitative atlas of mitotic phosphorylation. *Proceedings of the National Academy of Sciences of the United States of America* 105: 10762–7

Derijard B, Hibi M, Wu I, Barrett T, Su B, Deng T, Karin M & Davis RJ (1994) JNK1: A protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* 76: 1025–1037

Dilova I, Easlon E & Lin S-J (2007) Calorie restriction and the nutrient sensing signaling pathways. *Cellular and molecular life sciences* 64: 752–67

Dérijard B, Raingeaud J, Barrett T, Wu IH, Han J, Ulevitch RJ & Davis RJ (1995) Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. *Science* 267: 682–5 Available at: http://www.ncbi.nlm.nih.gov/pubmed/7839144

Díaz-Delfín J, Morales M & Caelles C (2007) Hypoglycemic Action of Thiazolinediones/Peroxisome Proliferator-Activated Receptor gamma by Inhibition of the c-Jun NH2-Terminal Kinase Pathway. *Diabetes* 56: 1865–1871

Eferl R & Wagner EF (2003) AP-1: a double-edged sword in tumorigenesis. *Nature reviews* 3: 859–68

Elcheva I, Goswami S, Noubissi FK & Spiegelman VS (2009) CRD-BP protects the coding region of betaTrCP1 mRNA from miR-183-mediated degradation. *Molecular cell* 35: 240–6

Eldor R, Yeffet a, Baum K, Doviner V, Amar D, Ben-Neriah Y, Christofori G, Peled a, Carel JC, Boitard C, Klein T, Serup P, Eizirik DL & Melloul D (2006) Conditional and specific NF-kappaB blockade protects pancreatic beta cells from diabetogenic agents. *Proceedings of the National Academy of Sciences of the United States of America* 103: 5072–7

Fan M, Goodwin M, Vu T, Brantley-Finley C, Gaarde W a & Chambers TC (2000) Vinblastine-induced phosphorylation of Bcl-2 and Bcl-XL is mediated by JNK and occurs in parallel with inactivation of the Raf-1/MEK/ERK cascade. *The Journal of biological chemistry* 275: 29980–5

Fero ML, Rivkin M, Tasch M, Porter P, Carow CE, Firpo E, Polyak K, Tsai LH, Broudy V, Perlmutter RM, Kaushansky K & Roberts JM (1996) A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. *Cell* 85: 733–44

Fong A & Sun S-C (2002) Genetic evidence for the essential role of beta-transducin repeat-containing protein in the inducible processing of NF-kappa B2/p100. *The Journal of biological chemistry* 277: 22111–4

Fontana L, Partridge L & Longo VD (2010) Extending healthy life span-from yeast to humans. *Science* 328: 321–6

Fornoni a, Pileggi a, Molano RD, Sanabria NY, Tejada T, Gonzalez-Quintana J, Ichii H, Inverardi L, Ricordi C & Pastori RL (2008) Inhibition of c-jun N terminal kinase (JNK) improves functional beta cell mass in human islets and leads to AKT and glycogen synthase kinase-3 (GSK-3) phosphorylation. *Diabetologia* 51: 298–308

Fuchs SY, Adler V, Buschmann T, Yin Z, Wu X, Jones SN & Ronai Z (1998) JNK targets p53 ubiquitination and degradation in nonstressed cells. *Genes & development* 12: 2658–2663

Fuchs SY, Chen a, Xiong Y, Pan ZQ & Ronai Z (1999) HOS, a human homolog of Slimb, forms an SCF complex with Skp1 and Cullin1 and targets the phosphorylation-dependent degradation of IkappaB and beta-catenin. *Oncogene* 18: 2039–46

Fuchs SY, Spiegelman VS & Kumar KGS (2004) The many faces of beta-TrCP E3 ubiquitin ligases: reflections in the magic mirror of cancer. *Oncogene* 23: 2028–36

Fukuda K, Tesch GH & Nikolic-Paterson DJ (2008) c-Jun amino terminal kinase 1 deficient mice are protected from streptozotocin-induced islet injury. *Biochemical and biophysical research communications* 366: 710–6

Gao M & Karin M (2005) Regulating the regulators: control of protein ubiquitination and ubiquitin-like modifications by extracellular stimuli. *Molecular cell* 19: 581–93

Gao M, Labuda T, Xia Y, Gallagher E, Fang D, Liu Y-C & Karin M (2004) Jun turnover is controlled through JNK-dependent phosphorylation of the E3 ligase Itch. *Science* 306: 271–5

Gems D & Partridge L (2001) Insulin/IGF signalling and ageing: seeing the bigger picture. *Current opinion in genetics & development* 11: 287–92

Ghosh S, Karin M & Haven N (2002) Missing Pieces in the NF-kB Puzzle. *Cell* 109: 81–96

Giannoukakis N, Rudert W a, Trucco M & Robbins PD (2000) Protection of human islets from the effects of interleukin-1beta by adenoviral gene transfer of an Ikappa B repressor. *The Journal of biological chemistry* 275: 36509–13

Gilmore TD (2006) Introduction to NF-kappaB: players, pathways, perspectives. *Oncogene* 25: 6680–4

Gray SL, Donald C, Jetha A, Covey SD & Kieffer TJ (2010) Hyperinsulinemia precedes insulin resistance in mice lacking pancreatic beta-cell leptin signaling. *Endocrinology* 151: 4178–86

Gregor MF & Hotamisligil GS (2011) Inflammatory mechanisms in obesity. *Annual review of immunology* 29: 415–45

Guardavaccaro D, Ganoth D & Dorrello NV (2003a) Degradation of Cdc25A by b -TrCP during S phase and in response to DNA damage. *Nature* 426: 87–91

Guardavaccaro D, Kudo Y, Boulaire J, Barchi M, Busino L, Donzelli M, Margottin-Goguet F, Jackson PK, Yamasaki L & Pagano M (2003b)

Control of meiotic and mitotic progression by the F box protein beta-Trcp1 in vivo. *Developmental cell* 4: 799–812

Gupta S, Barrett T, Whitmarsh a J, Cavanagh J, Sluss HK, Dérijard B & Davis RJ (1996) Selective interaction of JNK protein kinase isoforms with transcription factors. *The EMBO journal* 15: 2760–70

Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors M a, Fernandez E & Miller R a (2009) Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature* 460: 392–5

Hayden MS & Ghosh S (2004) Signaling to NF-kappaB. *Genes & development* 18: 2195–224

Hayden MS & Ghosh S (2012) NF-kB , the first quarter-century : remarkable progress and outstanding questions. *Genes & Development* 26: 203–234

Henstridge DC, Bruce CR, Pang CP, Lancaster GI, Allen TL, Estevez E, Gardner T, Weir JM, Meikle PJ, Lam KSL, Xu a, Fujii N, Goodyear LJ & Febbraio M a (2012) Skeletal muscle-specific overproduction of constitutively activated c-Jun N-terminal kinase (JNK) induces insulin resistance in mice. *Diabetologia* 55: 2769–78

Hernandez R, Teruel T, De Alvaro C & Lorenzo M (2004) Rosiglitazone ameliorates insulin resistance in brown adipocytes of Wistar rats by impairing TNF-alpha induction of p38 and p42/p44 mitogen-activated protein kinases. *Diabetologia* 47: 1615–24

Herrlich P (2001) Cross-talk between glucocorticoid receptor and AP-1. *Oncogene* 20: 2465–75

Hershko a & Ciechanover a (1998) The ubiquitin system. *Annual review of biochemistry* 67: 425–79

Hibi M, Lin a, Smeal T, Minden a & Karin M (1993) Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes & Development* 7: 2135–2148

Hirano M, Osada S, Aoki T, Hirai S, Hosaka M, Inoue J & Ohno S (1996) MEK Kinase Is Involved in Tumor Necrosis Factor a-Induced NF-kB Activation and Degradation of IkBa. *The Journal of biological chemistry* 271: 13234–13238

Hirosumi J, Tuncman G, Chang L, Görgün CZ, Uysal KT, Maeda K, Karin M & Hotamisligil GS (2002) A central role for JNK in obesity and insulin resistance. *Nature* 420: 333–6

Ho WC, Dickson KM & Barker P a (2005) Nuclear factor-kappaB induced by doxorubicin is deficient in phosphorylation and acetylation and represses nuclear factor-kappaB-dependent transcription in cancer cells. *Cancer research* 65: 4273–81

Hoffmann A, Levchenko A, Scott ML & Baltimore D (2002) The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. *Science* 298: 1241–5

Holland PM, Suzanne M, Campbell JS, Noselli S & Cooper J a (1997) MKK7 is a stress-activated mitogen-activated protein kinase kinase functionally related to hemipterous. *The Journal of biological chemistry* 272: 24994–8

Holzenberger M, Dupont J, Ducos B, Leneuve P, Géloën A, Even PC, Cervera P & Le Bouc Y (2003) IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. *Nature* 421: 182–7

Hotamisligil GS (2006) Inflammation and metabolic disorders. *Nature* 444: 860–7

Hu D, Bi X, Fang W, Han A & Yang W (2009) GSK3beta is involved in JNK2-mediated beta-catenin inhibition. *PloS one* 4: e6640

Hu D, Fang W, Han A, Gallagher L, Davis RJ, Xiong B & Yang W (2008) c-Jun N-terminal kinase 1 interacts with and negatively regulates Wnt/beta-catenin signaling through GSK3beta pathway. *Carcinogenesis* 29: 2317–24

Huang G, Shi LZ & Chi H (2009) Regulation of JNK and p38 MAPK in the immune system: Signal integration, propagation and termination. *Cytokine* 48: 161–169

Humphrey RK, Yu S-M, Flores LE & Jhala US (2010) Glucose regulates steady-state levels of PDX1 via the reciprocal actions of GSK3 and AKT kinases. *The Journal of biological chemistry* 285: 3406–16

Ikeda F, Deribe YL, Skånland SS, Stieglitz B, Grabbe C, Franz-Wachtel M, Van Wijk SJL, Goswami P, Nagy V, Terzic J, Tokunaga F, Androulidaki A,

Nakagawa T, Pasparakis M, Iwai K, Sundberg JP, Schaefer L, Rittinger K, Macek B & Dikic I (2011) SHARPIN forms a linear ubiquitin ligase

Ito CY, Kazantsevl AG & Jr ASB (1994) Three NF-xB sites in the IxB-oa promoter induction of gene expression by TNFcg are required for. *Nulceic Acids Research* 22: 3787–3792

Jaeschke A, Czech MP & Davis RJ (2004) An essential role of the JIP1 scaffold protein for JNK activation in adipose tissue. : 1976–1980

Joazeiro C a & Weissman a M (2000) RING finger proteins: mediators of ubiquitin ligase activity. *Cell* 102: 549–52

Johansson GS & Arnqvist HJ (2006) Insulin and IGF-I action on insulin receptors, IGF-I receptors, and hybrid insulin/IGF-I receptors in vascular smooth muscle cells. *American journal of physiology. Endocrinology and metabolism* 291: E1124–30

Kallunki T, Su B, Tsigelny I, Sluss HK, Derijard B, Moore G, Davis R & Karin M (1994) JNK2 contains a specificity-determining region responsible for efficient c-Jun binding and phosphorylation. *Genes & Development* 8: 2996–3007

Kamata H, Honda S-I, Maeda S, Chang L, Hirata H & Karin M (2005) Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell* 120: 649–61

Kanakaraj P, Schafer PH, Cavender DE, Wu Y, Ngo K, Grealish PF, Wadsworth S a, Peterson P a, Siekierka JJ, Harris C a & Fung-Leung WP (1998) Interleukin (IL)-1 receptor-associated kinase (IRAK) requirement for optimal induction of multiple IL-1 signaling pathways and IL-6 production. *The Journal of experimental medicine* 187: 2073–9

Kaneto H, Nakatani Y, Miyatsuka T, Kawamori D, Matsuoka T, Matsuhisa M, Kajimoto Y, Ichijo H, Yamasaki Y & Hori M (2004) Possible novel therapy for diabetes with cell-permeable JNK-inhibitory peptide. *Nature medicine* 10: 1128–32

Kaneto H, Xu G, Fujii N, Kim S, Bonner-Weir S & Weir GC (2002) Involvement of c-Jun N-terminal kinase in oxidative stress-mediated suppression of insulin gene expression. *The Journal of biological chemistry* 277: 30010–8

Karin M, Liu ZG & Zandi E (1997) AP-1 function and regulation. *Current opinion in cell biology* 9: 240–6

Kassel O, Sancono a, Krätzschmar J, Kreft B, Stassen M & Cato a C (2001) Glucocorticoids inhibit MAP kinase via increased expression and decreased degradation of MKP-1. *The EMBO journal* 20: 7108–16

Kato T, Delhase M, Hoffmann A & Karin M (2003) CK2 Is a C-Terminal IkappaB Kinase Responsible for NF-kappaB Activation during the UV Response. *Molecular cell* 12: 829–39

Kawakami T, Chiba T, Suzuki T, Iwai K, Yamanaka K, Minato N, Suzuki H, Shimbara N, Hidaka Y, Osaka F, Omata M & Tanaka K (2001) NEDD8 recruits E2-ubiquitin to SCF E3 ligase. *The EMBO journal* 20: 4003–12

Kawamori D, Kajimoto Y, Kaneto H, Umayahara Y, Fujitani Y, Miyatsuka T, Watada H, Leibiger IB, Yamasaki Y & Hori M (2003) Oxidative Stress Induces Nucleo-Cytoplasmic Translocation of Pancreatic Transcription Factor PDX-1 Through Activation of c-Jun NH 2 -terminal Kinase. *Diabetes* 52: 2896–2904

Kebede M, Alquier T, Latour MG, Semache M & Tremblay C (2008) The Fatty Acid Receptor GPR40 Plays a Role in Insulin Secretion In Vivo After High-Fat Feeding. *Diabetes* 57: 2432–2437

Khandoudi N, Delerive P, Berrebi-bertrand I, Buckingham RE, Staels B & Bril A (2002) Rosiglitazone, a Peroxisome Proliferator-Activated Receptor-g, Inhibits the Jun NH2-Terminal Kinase/Activatin Protein 1 Pathway and Protects the Heart From Ischemia/Reperfusion Injury. *Diabetes* 51: 1507–1514

Kim WH, Lee JW, Gao B & Jung MH (2005) Synergistic activation of JNK/SAPK induced by TNF-alpha and IFN-gamma: apoptosis of pancreatic beta-cells via the p53 and ROS pathway. *Cellular signalling* 17: 1516–32

Kiyokawa H, Kineman RD, Manova-Todorova KO, Soares VC, Hoffman ES, Ono M, Khanam D, Hayday a C, Frohman L a & Koff a (1996) Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27(Kip1). *Cell* 85: 721–32

Kondoh K & Nishida E (2007) Regulation of MAP kinases by MAP kinase phosphatases. *Biochimica et biophysica acta* 1773: 1227–37

Kroll M, Margottin F, Kohl A, Renard P, Concordet J, Arenzana-seisdedos F & Benarous R (1999) Inducible Degradation of IkBa by the Proteasome Requires Interaction with the F-box Protein h-BTrCP. *The Journal of biological chemistry* 274: 7941–7945

Kubota H, Noguchi R, Toyoshima Y, Ozaki Y, Uda S, Watanabe K, Ogawa W & Kuroda S (2012a) Temporal Coding of Insulin Action through Multiplexing of the AKT Pathway. *Molecular Cell*: 1–13

Kubota H, Noguchi R, Toyoshima Y, Ozaki Y-I, Uda S, Watanabe K, Ogawa W & Kuroda S (2012b) Temporal Coding of Insulin Action through Multiplexing of the AKT Pathway. *Molecular cell* 46: 820–32

Kurosu H, Yamamoto M, Clark JD, Pastor J V, Nandi A, Gurnani P, McGuinness OP, Chikuda H, Yamaguchi M, Kawaguchi H, Shimomura I, Takayama Y, Herz J, Kahn CR, Rosenblatt KP & Kuro-o M (2005) Suppression of Aging in Mice by the Hormone Klotho. *Science* 309: 1829–33

Kyriakis J, Banerjee P, Nikolakaki E, Dai T, Rubie E, Ahmad M, Avruch J & Woodgett J (1994) The stress-activated protein kinase subfamily of c-Jun kinases. *Nature* 369: 156–60

Kyriakis JM & Avruch J (2012) Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update. *Physiological reviews* 92: 689–737

Kyriakis M & Avruch J (1990) pp54 Microtubule-associated Protein 2 Kinase. *The Journal of biological chemistry* 265: 17355–17363

Lamming DW, Ye L, Katajisto P, Goncalves MD, Saitoh M, Stevens DM, Davis JG, Salmon AB, Richardson A, Ahima RS, Guertin D a, Sabatini DM & Baur J a (2012) Rapamycin-induced insulin resistance is mediated by mTORC2 loss and uncoupled from longevity. *Science* 335: 1638–43

Lang V, Janzen J, Fischer GZ, Beinke S, Salmeron A, Allen H, Hay RT, Ben-neriah Y, Ley SC & Soneji Y (2003) Requires Phosphorylation of p105 Serines 927 and 932 BTrCP-Mediated Proteolysis of NF-kB1 p105 Requires Phosphorylation of p105 Serines 927 and 932. *Molecular and Cellular Biology* 23: 402–413

Lassot I, Segeral E, Berlioz-torrent C, Durand H, Groussin L, Hai T, Benarous R & Margottin-goguet F (2001) ATF4 Degradation Relies on a Phosphorylation-Dependent Interaction with the SCF BTrCP Ubiquitin Ligase. *Molecular and Cellular Biology* 21: 2192–2202

Latres E, Chiaur DS & Pagano M (1999) The human F box protein beta-Trcp associates with the Cul1/Skp1 complex and regulates the stability of beta-catenin. *Oncogene* 18: 849–54

Lawler S, Fleming Y, Goedert M & Cohen P (1998) Synergistic activation of SAPK1/JNK1 by two MAP kinase kinases in vitro. *Current biology* 8: 1387–90

Lee FS, Hagler J, Chen ZJ & Maniatis T (1997) Activation of the IkappaB alpha kinase complex by MEKK1, a kinase of the JNK pathway. *Cell* 88: 213–22

Lee K-S, Iijima-Ando K, Iijima K, Lee W-J, Lee JH, Yu K & Lee D-S (2009) JNK/FOXO-mediated neuronal expression of fly homologue of peroxiredoxin II reduces oxidative stress and extends life span. *The Journal of biological chemistry* 284: 29454–61

Lee YH, Giraud J, Davis RJ & White MF (2003) c-Jun N-terminal kinase (JNK) mediates feedback inhibition of the insulin signaling cascade. *The Journal of biological chemistry* 278: 2896–902

Leitges M, Sanz L, Martin P, Duran a, Braun U, García JF, Camacho F, Diaz-Meco MT, Rennert PD & Moscat J (2001) Targeted disruption of the zetaPKC gene results in the impairment of the NF-kappaB pathway. *Molecular cell* 8: 771–80

Li X, Commane M, Jiang Z & Stark GR (2001) IL-1-induced NFkB and c-Jun N-terminal kinase (JNK) activation diverge at IL-1 receptor-associated kinase (IRAK). *Proceedings of the National Academy of Sciences of the United States of America* 98: 4461–4465

Liao G, Tao Q, Kofron M, Chen J-S, Schloemer A, Davis RJ, Hsieh J-C, Wylie C, Heasman J & Kuan C-Y (2006) Jun NH2-terminal kinase (JNK) prevents nuclear beta-catenin accumulation and regulates axis formation in Xenopus embryos. *Proceedings of the National Academy of Sciences of the United States of America* 103: 16313–8

Lin A (2003) Activation of the JNK signaling pathway: breaking the brake on apoptosis. *BioEssays : news and reviews in molecular, cellular and developmental biology* 25: 17–24

Lin A, Minden A, Martinetto H, Claret FX, Lange-Carter C, Mercurio F, Johnson GL & Karin M (1995) Identification of a dual specificity kinase that activates the Jun kinases and p38-Mpk2. *Science* 268: 286–90

Livingstone C, Patel G & Jones N (1995) ATF-2 contains a phosphorylation-dependent transcriptional activation domain. *The EMBO journal* 14: 1785–97

Lo C-M, King A, Samuelson LC, Kindel TL, Rider T, Jandacek RJ, Raybould HE, Woods SC & Tso P (2010) Cholecystokinin knockout mice are resistant to high-fat diet-induced obesity. *Gastroenterology* 138: 1997–2005

Lo C-M, Obici S, Dong HH, Haas M, Lou D, Kim DH, Liu M, D'Alessio D, Woods SC & Tso P (2011) Impaired insulin secretion and enhanced insulin sensitivity in cholecystokinin-deficient mice. *Diabetes* 60: 2000–7

Loetscher H, Pan Y-CE, Lahm H-W, Gentz R, Brockhaus M, Tabuchi H & Lessauer W (1990) Molecular cloning and expression of the human 55 kd tumor necrosis factor receptor. *Cell* 61: 351–359

Lupu F, Terwilliger JD, Lee K, Segre G V & Efstratiadis a (2001) Roles of growth hormone and insulin-like growth factor 1 in mouse postnatal growth. *Developmental biology* 229: 141–62

Lyapina S, Cope G, Shevchenko A, Serino G, Tsuge T, Zhou C, Wolf DA, Ning W & Deshaies RJ (2001) Promotion of NEDD8-CUL1 Conjugate Cleavage by COP9 Signalosome. *Science* 292: 1382–1385

Maddika S & Chen J (2009) Protein kinase DYRK2 is an E3-ligase specific molecular assembler. *Nature Cell Biology* 11: 409–419

Manning AM & Davis RJ (2003) Targeting JNK for therapeutic benefit: from junk to gold? *Nature reviews* 2: 554–65

Margottin-goguet F, Hsu JY, Loktev A, Hsieh H, Reimann JDR & Jackson PK (2003) Prophase Destruction of Emi1 by the SCF BTrCP / Slimb Ubiquitin Ligase Activates the Anaphase Promoting Complex to Allow Progression beyond Prometaphase. *Developmental Cell* 4: 813–826

Masharani UB, Maddux B a, Li X, Sakkas GK, Mulligan K, Schambelan M, Goldfine ID & Youngren JF (2011) Insulin resistance in non-obese subjects is associated with activation of the JNK pathway and impaired insulin signaling in skeletal muscle. *PloS one* 6: e19878

Matsuzawa A, Saegusa K, Noguchi T, Sadamitsu C, Nishitoh H, Nagai S, Koyasu S, Matsumoto K, Takeda K & Ichijo H (2005) ROS-dependent activation of the TRAF6-ASK1-p38 pathway is selectively required for TLR4-mediated innate immunity. *Nature immunology* 6: 587–92

Mayo MW & Baldwin a S (2000) The transcription factor NF-kappaB: control of oncogenesis and cancer therapy resistance. *Biochimica et biophysica acta* 1470: M55–62

Medzhitov R (2008) Origin and physiological roles of inflammation. *Nature* 454: 428–35

Migliaccio E, Giorgio M, Mele S, Pelicci G, Reboldi P, Pandolfi PP, Lanfrancone L & Pelicci PG (1999) The p66shc adaptor protein controls oxidative stress response and life span in mammals. *Nature* 402: 309–13

Moriguchi T, Toyoshima F, Masuyama N, Hanafusa H, Gotoh Y & Nishida E (1997) A novel SAPK/JNK kinase, MKK7, stimulated by TNFalpha and cellular stresses. *The EMBO journal* 16: 7045–53

Morrison DK & Davis RJ (2003) Regulation of MAP kinase signaling modules by scaffold proteins in mammals. *Annual review of cell and developmental biology* 19: 91–118

Moxhams CP, Duronioo V, Jacobs S & Carolina N (1989) Insulin-like Growth Factor I Receptor B -Subunit Heterogeneity. *The Journal of biological chemistry* 264: 13238–13244

Muller D, Huang GC, Amiel S, Jones PM & Persaud SJ (2006) Identification of insulin signaling elements in human beta-cells: autocrine regulation of insulin gene expression. *Diabetes* 55: 2835–42

Musti AM, Treier M & Bohamann D (1997) Reduced Ubiquitin-Dependent Degradation of c-Jun After Phosphorylation by MAP Kinases. *Science* 275: 400–402

Nagata K, Driessens M, Lamarche N, Gorski JL & Hall a (1998) Activation of G1 progression, JNK mitogen-activated protein kinase, and actin filament assembly by the exchange factor FGD1. *The Journal of biological chemistry* 273: 15453–7

Nakano H, Nakajima a, Sakon-Komazawa S, Piao J-H, Xue X & Okumura K (2006) Reactive oxygen species mediate crosstalk between NF-kappaB and JNK. *Cell death and differentiation* 13: 730–7

Nakatani Y, Kaneto H, Kawamori D, Hatazaki M, Miyatsuka T, Matsuoka T-A, Kajimoto Y, Matsuhisa M, Yamasaki Y & Hori M (2004) Modulation of the JNK pathway in liver affects insulin resistance status. *The Journal of biological chemistry* 279: 45803–9

Nakayama K, Hatakeyama S, Maruyama S, Kikuchi A, Onoé K, Good R a & Nakayama KI (2003) Impaired degradation of inhibitory subunit of NF-kappa B (I kappa B) and beta-catenin as a result of targeted disruption of the beta-TrCP1 gene. *Proceedings of the National Academy of Sciences of the United States of America* 100: 8752–7

Nakayama K, Ishida N, Shirane M, Inomata a, Inoue T, Shishido N, Horii I & Loh DY (1996) Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* 85: 707–20

Nankervis a, Proietto J, Aitken P & Alford F (1985) Hyperinsulinaemia and insulin insensitivity: studies in subjects with insulinoma. *Diabetologia* 28: 427–31

Nateri AS, Riera-Sans L, Da Costa C & Behrens A (2004) The ubiquitin ligase SCFFbw7 antagonizes apoptotic JNK signaling. *Science* 303: 1374–8

Nateri AS, Spencer-Dene B & Behrens A (2005) Interaction of phosphorylated c-Jun with TCF4 regulates intestinal cancer development. *Nature* 437: 281–5

Natoli G & Chiocca S (2008) Nuclear ubiquitin ligases, NF-kappaB degradation, and the control of inflammation. *Science signaling* 1: pe1

Neumann-Haefelin E, Qi W, Finkbeiner E, Walz G, Baumeister R & Hertweck M (2008) SHC-1/p52Shc targets the insulin/IGF-1 and JNK signaling pathways to modulate life span and stress response in C. elegans. *Genes & development* 22: 2721–35

Niu J, Shi Y, Iwai K & Wu Z-H (2011) LUBAC regulates NF-κB activation upon genotoxic stress by promoting linear ubiquitination of NEMO. *The EMBO journal* 30: 3741–53

Noguchi H, Nakai Y, Matsumoto S, Kawaguchi M, Ueda M, Okitsu T, Iwanaga Y, Yonekawa Y, Nagata H, Minami K, Masui Y, Futaki S & Tanaka K (2005) Cell permeable peptide of JNK inhibitor prevents islet apoptosis immediately after isolation and improves islet graft function. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 5: 1848–55

Noguchi H, Nakai Y, Ueda M, Masui Y, Futaki S, Kobayashi N, Hayashi S & Matsumoto S (2007) Activation of c-Jun NH2-terminal kinase (JNK)

pathway during islet transplantation and prevention of islet graft loss by intraportal injection of JNK inhibitor. *Diabetologia* 50: 612–9

Oh SW, Mukhopadhyay A, Svrzikapa N, Jiang F, Davis RJ & Tissenbaum H a (2005) JNK regulates lifespan in Caenorhabditis elegans by modulating nuclear translocation of forkhead transcription factor/DAF-16. *Proceedings of the National Academy of Sciences of the United States of America* 102: 4494–9

Olsen J V, Blagoev B, Gnad F, Macek B, Kumar C, Mortensen P & Mann M (2006) Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell* 127: 635–48

Owens DM & Keyse SM (2007) Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases. *Oncogene* 26: 3203–13

Ozcan U, Cao Q, Yilmaz E, Lee A-H, Iwakoshi NN, Ozdelen E, Tuncman G, Görgün C, Glimcher LH & Hotamisligil GS (2004) Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* 306: 457–61

Panaretakis T, Laane E, Pokrovskaja K, Bjo A, Moustakas A, Zhivotovsky B, Heyman M, Shoshan MC & Grande D (2005) Doxorubicin Requires the Sequential Activation of Caspase-2 , Protein Kinase C ϛ , and c-Jun NH 2 - terminal Kinase to Induce Apoptosis □. *Molecular Biology of the Cell* 16: 3821–3831

Pansuria M, Xi H, Li L, Yang X & Wang H (2012) Insulin resistance, metabolic stress and atherosclerosis. *Front Biosci*: 916–931

Perkins ND (2006) Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway. *Oncogene* 25: 6717–30

Peschiaroli A, Dorrello NV, Guardavaccaro D, Venere M, Halazonetis T, Sherman NE & Pagano M (2006) SCFbetaTrCP-mediated degradation of Claspin regulates recovery from the DNA replication checkpoint response. *Molecular cell* 23: 319–29

Petrich BG, Molkentin JD & Wang Y (2003) Temporal activation of c-Jun N-terminal kinase in adult transgenic heart via cre-loxP-mediated DNA recombination. *FASEB journal* 17: 749–51

Pham CG, Bubici C, Zazzeroni F, Papa S, Jones J, Alvarez K, Jayawardena S, De Smaele E, Cong R, Beaumont C, Torti FM, Torti S V & Franzoso G (2004)

Ferritin heavy chain upregulation by NF-kappaB inhibits TNFalpha-induced apoptosis by suppressing reactive oxygen species. *Cell* 119: 529–42

Pini M, Rhodes DH, Castellanos KJ, Cabay RJ, Grady EF & Fantuzzi G (2012) Rosiglitazone improves survival and hastens recovery from pancreatic inflammation in obese mice. *PloS one* 7: e40944

Pomerantz JL & Baltimore D (2002) Two Pathways to NF-κB. *Molecular Cell* 10: 693–695

Potapova O, Gorospe M, Bost F, Dean NM, Gaarde W a, Mercola D & Holbrook NJ (2000) c-Jun N-terminal kinase is essential for growth of human T98G glioblastoma cells. *The Journal of biological chemistry* 275: 24767–75

Potapova O, Haghighi A, Liu C, Birrer MJ, Gjerset R & Mercola D (1997) Functions to Regulate DNA Pathway Sensitizes Tumor Cells to Cisplatin. *The Journal of Biological Chemistry*: 14041–14044

Pullen N, Dennis P, Andrejelkovic M, Dufner A, Kozma S, Hemmings A & Thomas G (1998) Phosphorylation and Activation of p70s6k by PDK1. *Science* 279: 707–710

Pulverer B, Kyriakis J, Avruch J, Nikolakaki E & Woodgett J (1991) Phosphorylation of c-jun mediated by MAP kinases. *Nature* 353: 670–4

Putters J, Slotman J a, Gerlach JP & Strous GJ (2011) Specificity, location and function of βTrCP isoforms and their splice variants. *Cellular signalling* 23: 641–7

Rana A, Gallo K, Godowski P, Hirai S, Ohno S, Zon L, Kyriakis JM & Avruch J (1996) The Mixed Lineage Kinase SPRK Phosphorylates and Activates the Stress-activated Protein Kinase Activator, SEK-1. *The Journal of biological chemistry* 1: 19025–19028

Read M a, Whitley MZ, Gupta S, Pierce JW, Best J, Davis RJ & Collins T (1997) Tumor necrosis factor alpha-induced E-selectin expression is activated by the nuclear factor-kappaB and c-JUN N-terminal kinase/p38 mitogen-activated protein kinase pathways. *The Journal of biological chemistry* 272: 2753–61

Rogatsky I, Luecke HF, Leitman DC & Yamamoto KR (2002) Alternate surfaces of transcriptional coregulator GRIP1 function in different glucocorticoid

receptor activation and repression contexts. *Proceedings of the National Academy of Sciences of the United States of America* 99: 16701–6

Rogatsky I, Zarember K a & Yamamoto KR (2001) Factor recruitment and TIF2/GRIP1 corepressor activity at a collagenase-3 response element that mediates regulation by phorbol esters and hormones. *The EMBO journal* 20: 6071–83

Roig J, Alexei M, Christopher B & Jul AJ (2002) Nercc1, a mammalian NIMA-family kinase, binds the Ran GTPase and regulates mitotic progression. *Genes & development* 16: 12101123

Rosen ED, Kulkarni RN, Sarraf P, Ozcan U, Okada T, Hsu C, Eisenman D, Magnuson MA, Gonzalez FJ, Kahn CR & Spiegelman BM (2003) Targeted Elimination of Peroxisome Proliferator-Activated Receptor ⫽ in NL Cells Leads to Abnormalities in Islet Mass without Compromising Glucose Homeostasis. *Molecular and Cellular Biology* 23: 7222–7229

Rosen ED & Spiegelman BM (2001) PPARgamma : a nuclear regulator of metabolism, differentiation, and cell growth. *The Journal of biological chemistry* 276: 37731–4

Sabio G, Cavanagh-Kyros J, Ko HJ, Jung DY, Gray S, Jun JY, Barrett T, Mora A, Kim JK & Davis RJ (2009) Prevention of steatosis by hepatic JNK1. *Cell metabolism* 10: 491–8

Sabio G, Das M, Mora A, Zhang Z, Jun JY, Ko HJ, Barrett T, Kim JK, Davis RJ, Zhao HY & Deng XW (2008) A Stress Signaling Pathway in Adipose Tissue Regulates Hepatic Insulin Resistance. *Science* 322: 1539–1543

Sabio G, Kennedy NJ, Cavanagh-Kyros J, Jung DY, Ko HJ, Ong H, Barrett T, Kim JK & Davis RJ (2010) Role of muscle c-Jun NH2-terminal kinase 1 in obesity-induced insulin resistance. *Molecular and cellular biology* 30: 106–15

Sanjo H, Zajonc DM, Braden R, Norris PS & Ware CF (2010) Allosteric regulation of the ubiquitin:NIK and ubiquitin:TRAF3 E3 ligases by the lymphotoxin-beta receptor. *The Journal of biological chemistry* 285: 17148–55

Schulla V, Renström E, Feil R, Feil S, Franklin I, Gjinovci A, Jing X-J, Laux D, Lundquist I, Magnuson M a, Obermüller S, Olofsson CS, Salehi A, Wendt A, Klugbauer N, Wollheim CB, Rorsman P & Hofmann F (2003) Impaired

insulin secretion and glucose tolerance in beta cell-selective Ca(v)1.2 Ca2+ channel null mice. *The EMBO journal* 22: 3844–54

Schüle R, Rangarajan P, Kliewer S, Ransone LJ, Bolado J, Yang N, Verma IM & Evans RM (1990) Functional antagonism between oncopro

Selman C, Tullet JM a, Wieser D, Irvine E, Lingard SJ, Choudhury AI, Claret M, Al-Qassab H, Carmignac D, Ramadani F, Woods A, Robinson IC a, Schuster E, Batterham RL, Kozma SC, Thomas G, Carling D, Okkenhaug K, Thornton JM, Partridge L, *et al* (2009) Ribosomal protein S6 kinase 1 signaling regulates mammalian life span. *Science* 326: 140–4

Sen R & Baltimore D (1986) Multiple nuclear factors interact with the immuoglobulin enhancer sequences. *Cell* 46: 705–716

Serhan CN & Savill J (2005) Resolution of inflammation: the beginning programs the end. *Nature immunology* 6: 1191–7

Shanik MH, Xu Y, Skrha J, Dankner R, Zick Y & Roth J (2008) Insulin resistance and hyperinsulinemia: is hyperinsulinemia the cart or the horse? *Diabetes care* 31 Suppl 2: S262–8

Shirane M, Hatakeyama S, Hattori K, Nakayama K & Nakayama K (1999) Common Pathway for the Ubiquitination of IkBa, IkBb, and IkBe Mediated by the F-Box Protein FWD1. *The Journal of biological chemistry* 274: 28169–28174

Shirogane T, Jin J, Ang XL & Harper JW (2005) SCFbeta-TRCP controls clock-dependent transcription via casein kinase 1-dependent degradation of the mammalian period-1 (Per1) protein. *The Journal of biological chemistry* 280: 26863–72

Skowyra D, Craig KL, Tyers M, Elledge SJ & Harper JW (1997) F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. *Cell* 91: 209–19

Smaele E De, Zazzeroni F, Papa S, Nguyen DU, Jin R, Jones J, Cong R & Franzoso G (2001) Induction of gadd45b by NF-kB downregulates pro-apoptotic JNK signalling. *Nature* 414: 308–313

Smith C, Davis T, Anderson D, Solam L, Beckmann MP, Jerzy R, Dower SK, Cosman D & Goodwin RG (1990) A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. *Science* 248: 1019–23

Smith C, Farrah T & Goodwin RG (1994) The TNF receptor superfamily of cellular and viral proteins: Activation, costimulation, and death. *Cell* 76: 959–962

Solinas G & Karin M (2010) JNK1 and IKKbeta: molecular links between obesity and metabolic dysfunction. *FASEB journal* 24: 2596–611

Solinas G, Naugler W, Galimi F, Lee M-S & Karin M (2006) Saturated fatty acids inhibit induction of insulin gene transcription by JNK-mediated phosphorylation of insulin-receptor substrates. *Proceedings of the National Academy of Sciences of the United States of America* 103: 16454–9

Solinas G, Vilcu C, Neels JG, Bandyopadhyay GK, Luo J-L, Naugler W, Grivennikov S, Wynshaw-Boris A, Scadeng M, Olefsky JM & Karin M (2007) JNK1 in hematopoietically derived cells contributes to diet-induced inflammation and insulin resistance without affecting obesity. *Cell metabolism* 6: 386–97

Song HY, Régnier CH, Kirschning CJ, Goeddel D V & Rothe M (1997) Tumor necrosis factor (TNF)-mediated kinase cascades: bifurcation of nuclear factor-kappaB and c-jun N-terminal kinase (JNK/SAPK) pathways at TNF receptor-associated factor 2. *Proceedings of the National Academy of Sciences of the United States of America* 94: 9792–6

De Souza CT, Araujo EP, Bordin S, Ashimine R, Zollner RL, Boschero AC, Saad MJ a & Velloso L a (2005) Consumption of a fat-rich diet activates a proinflammatory response and induces insulin resistance in the hypothalamus. *Endocrinology* 146: 4192–9

Spiegelman VS, Slaga TJ, Pagano M, Minamoto T, Ronai Z & Fuchs SY (2000) Wnt B-Catenin Signaling Induces the Expression and Activity of BTrCP Ubiquitin Ligase Receptor. *Molecular cell* 5: 877–882

Spiegelman VS, Stavropoulos P, Latres E, Pagano M, Ronai Z, Slaga TJ & Fuchs SY (2001) Induction of beta-transducin repeat-containing protein by JNK signaling and its role in the activation of NF-kappaB. *The Journal of biological chemistry* 276: 27152–8

Steneberg P, Rubins N, Bartoov-Shifman R, Walker MD & Edlund H (2005) The FFA receptor GPR40 links hyperinsulinemia, hepatic steatosis, and impaired glucose homeostasis in mouse. *Cell metabolism* 1: 245–58

Sun S-C (2011) Non-canonical NF-κB signaling pathway. *Cell research* 21: 71–85

Suzuki H, Chiba T, Suzuki T, Fujita T, Ikenoue T, Omata M, Furuichi K, Shikama H & Tanaka K (2000) Homodimer of two F-box proteins betaTrCP1 or betaTrCP2 binds to IkappaBalpha for signal-dependent ubiquitination. *The Journal of biological chemistry* 275: 2877–84

Symons A, Beinke S & Ley SC (2006) MAP kinase kinase kinases and innate immunity. *Trends in immunology* 27: 40–8

Tang G, Minemoto Y, Dibling B, Purcell NH, Li Z, Karin M & Lin A (2001) Inhibition of JNK activation through NF-kB target genes. *Nature* 414: 313–317

Taniguchi CM, Emanuelli B & Kahn CR (2006) Critical nodes in signalling pathways: insights into insulin action. *Nature reviews. Molecular cell biology* 7: 85–96

Tanoue T & Nishida E (2003) Molecular recognitions in the MAP kinase cascades. *Cellular Signalling* 15: 455–462

Tanti JF, Grillo S, Grémeaux T, Coffer PJ, Van Obberghen E & Le Marchand-Brustel Y (1997) Potential role of protein kinase B in glucose transporter 4 translocation in adipocytes. *Endocrinology* 138: 2005–10

Tergaonkar V, Bottero V, Ikawa M, Li Q & Verma IM (2003) IkB Kinase-Independent IkBa Degradation Pathway: Functional NF-kB Activity and Implications for Cancer Therapy. *Molecular and Cellular Biology* 23: 8070–8083

Thomas GS, Zhang L, Blackwell K & Habelhah H (2009) Phosphorylation of TRAF2 within its RING domain inhibits stress-induced cell death by promoting IKK and suppressing JNK activation. *Cancer research* 69: 3665–72

Tiwari VK, Stadler MB, Wirbelauer C, Paro R, Schübeler D & Beisel C (2012) A chromatin-modifying function of JNK during stem cell differentiation. *Nature genetics* 44: 94–100

Tobiume K, Matsuzawa a, Takahashi T, Nishitoh H, Morita K, Takeda K, Minowa O, Miyazono K, Noda T & Ichijo H (2001) ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. *EMBO reports* 2: 222–8

Tournier C (2000) Requirement of JNK for Stress- Induced Activation of the Cytochrome c-Mediated Death Pathway. *Science* 288: 870–874

Tournier C, Dong C, Turner TK, Jones SN, Flavell RA & Davis RJ (2001) MKK7 is an essential component of the JNK signal transduction pathway activated by proinflammatory cytokines. *Genes & Development* 15: 1419–1426

Tournier C, Whitmarsh a J, Cavanagh J, Barrett T & Davis RJ (1997) Mitogen-activated protein kinase kinase 7 is an activator of the c-Jun NH2-terminal kinase. *Proceedings of the National Academy of Sciences of the United States of America* 94: 7337–42

Tsuruta F, Sunayama J, Mori Y, Hattori S, Shimizu S, Tsujimoto Y, Yoshioka K, Masuyama N & Gotoh Y (2004) JNK promotes Bax translocation to mitochondria through phosphorylation of 14-3-3 proteins. *The EMBO journal* 23: 1889–99

Tuncman G, Hirosumi J, Solinas G, Chang L, Karin M & Hotamisligil GS (2006) Functional in vivo interactions between JNK1 and JNK2 isoforms in obesity and insulin resistance. *Proceedings of the National Academy of Sciences of the United States of America* 103: 10741–6

Uchida S, Watanabe N, Kudo Y, Yoshioka K, Matsunaga T, Ishizaka Y, Nakagama H, Poon RYC & Yamashita K (2011) SCFβ(TrCP) mediates stress-activated MAPK-induced Cdc25B degradation. *Journal of cell science* 124: 2816–25

Uebele VN, Gotter AL, Nuss CE, Kraus RL, Doran SM, Garson SL, Reiss DR, Li Y, Barrow JC, Reger TS, Yang Z, Ballard JE, Tang C, Metzger JM, Wang S, Koblan KS & Renger JJ (2009) Antagonism of T-type calcium channels inhibits high-fat diet – induced weight gain in mice. *The Journal of Clinical Investigation* 119: 1659–1667

Varfolomeev E, Goncharov T, Maecker H, Zobel K, Kömüves LG, Deshayes K & Vucic D (2012) Cellular inhibitors of apoptosis are global regulators of NF-κB and MAPK activation by members of the TNF family of receptors. *Science signaling* 5: ra22

Varfolomeev EE & Ashkenazi A (2004) Tumor necrosis factor: an apoptosis JuNKie? *Cell* 116: 491–7

Varona-Santos JL, Pileggi a, Molano RD, Sanabria NY, Ijaz a, Atsushi M, Ichii H, Pastori RL, Inverardi L, Ricordi C & Fornoni a (2008) c-Jun N-terminal

kinase 1 is deleterious to the function and survival of murine pancreatic islets. *Diabetologia* 51: 2271–80

Velloso L a, Araújo EP & De Souza CT (2008) Diet-induced inflammation of the hypothalamus in obesity. *Neuroimmunomodulation* 15: 189–93

Ventura J-J, Hübner A, Zhang C, Flavell R a, Shokat KM & Davis RJ (2006) Chemical genetic analysis of the time course of signal transduction by JNK. *Molecular cell* 21: 701–10

Wang C, Mayo M, Korneluk R, Goeddel D & Jr AB (1998) NF-B Antiapoptosis: Induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to Suppress Caspase-8 Activation. *Science* 281: 1680–1683

Wang CY, Mayo MW & Baldwin a S (1996) TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. *Science* 274: 784–7

Wang L, Liu Y, Lu SY, Nguyen KT, Schroer SA, Suzuki A, Mak TW, Gaisano H & Woo M (2010) Deletion of Pten in Pancreatic ÆB-Cells Protects Against Deficient ÆB-Cell Mass and Function in Mouse Models of Type 2 Diabetes. *Diabetes* 59: 3117–3126

Wang M, Bohmann D & Jasper H (2005) JNK extends life span and limits growth by antagonizing cellular and organism-wide responses to insulin signaling. *Cell* 121: 115–25

Watanabe N, Arai H, Nishihara Y, Taniguchi M, Watanabe N, Hunter T & Osada H (2004) M-phase kinases induce phospho-dependent ubiquitination of somatic Wee1 by SCFbeta-TrCP. *Proceedings of the National Academy of Sciences of the United States of America* 101: 4419–24

Wee S, Geyer RK, Toda T & Wolf DA (2005) CSN facilitates Cullin – RING ubiquitin ligase function by counteracting autocatalytic adapter instability. *Nature Cell Biology* 7: 387–391

Weeren PC Van, Bruyn KMT De, Vries-smits AMM De, Lint J Van & Burgering BMT (1998) Essential Role for Protein Kinase B (PKB) in Insulin-induced Glycogen Synthase Kinase 3 Inactivation. *The Journal of biological chemistry* 273: 13150–13156

Weiss C, Schneider S, Wagner EF, Zhang X, Seto E & Bohmann D (2003) JNK phosphorylation relieves HDAC3-dependent suppression of the transcriptional activity of c-Jun. *The EMBO journal* 22: 3686–95

Wertheimer E, Lu S, Backeljauw P, Davenport M & Taylor S (1993) Homozygous deletion of the human insulin receptor gene results in leprechaunism. *Nature genetics* 5: 71–73

Westbrook TF, Hu G, Ang XL, Mulligan P, Pavlova NN, Liang A, Leng Y, Maehr R, Shi Y, Harper JW & Elledge SJ (2008) SCFbeta-TRCP controls oncogenic transformation and neural differentiation through REST degradation. *Nature* 452: 370–4

Weston CR & Davis RJ (2007) The JNK signal transduction pathway. *Current opinion in cell biology* 19: 142–9

Whitmarsh AJ, Kuan C, Kennedy NJ, Kelkar N, Haydar TF, Mordes JP, Appel M, Rossini AA, Jones SN, Flavell RA, Rakic P & Davis RJ (2001) Requirement of the JIP1 scaffold protein for stress-induced JNK activation. : 2421–2432

Wicksteed B, Brissova M, Yan W, Opland DM, Plank JL, Reinert RB, Dickson LM, Tamarina NA, Philipson LH, Shostak A, Bernal-mizrachi E, Elghazi L, Roe MW, Labosky PA, Jr MGM, Gannon M, Powers AC & Dempsey PJ (2010) Analysis of Ectopic Cre Transgene Expression in the Brain. *Diabetes* 59:

Willert K & Nusset R (1998) B-catenin: a key mediator of Wnt signaling. *Current opinion in genetics & development* 8: 95–102

Willson TM, Cobb JE, Cowan DJ, Wiethe RW, Correa ID, Prakash SR, Beck KD, Moore LB, Kliewer S a & Lehmann JM (1996) The structure-activity relationship between peroxisome proliferator-activated receptor gamma agonism and the antihyperglycemic activity of thiazolidinediones. *Journal of medicinal chemistry* 39: 665–8

Winston JT, Strack P, Beer-Romero P, Chu CY, Elledge SJ & Harper JW (1999) The SCFbeta-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IkappaBalpha and beta-catenin and stimulates IkappaBalpha ubiquitination in vitro. *Genes & development* 13: 270–83

Winter-Vann AM & Johnson GL (2007) Integrated activation of MAP3Ks balances cell fate in response to stress. *Journal of cellular biochemistry* 102: 848–58

Wolf D a, Zhou C & Wee S (2003) The COP9 signalosome: an assembly and maintenance platform for cullin ubiquitin ligases? *Nature cell biology* 5: 1029–33

Wu G, Xu G, Schulman B a, Jeffrey PD, Harper JW & Pavletich NP (2003) Structure of a beta-TrCP1-Skp1-beta-catenin complex: destruction motif binding and lysine specificity of the SCF(beta-TrCP1) ubiquitin ligase. *Molecular cell* 11: 1445–56

Xiao G, Harhaj EW & Sun S (2001) NF-kB-Inducing Kinase Regulates the Processing of NF-kB2 p100. *Molecular cell* 7: 401–409

Yamamoto K, Ichijo H & Korsmeyer SJ (1999) BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M. *Molecular and cellular biology* 19: 8469–78

Yang R & Trevillyan JM (2008) c-Jun N-terminal kinase pathways in diabetes. *The international journal of biochemistry & cell biology* 40: 2702–6

Yang Z, Costanzo M, Golde DW & Kolesnick RN (1993) Tumor necrosis factor activation of the sphingomyelin pathway signals nuclear factor kappa B translocation in intact HL-60 cells. *The Journal of biological chemistry* 268: 20520–3

Yao Z, Diener K, Wang XS, Zukowski M, Matsumoto G, Zhou G, Mo R, Sasaki T, Nishina H, Hui CC, Tan TH, Woodgett JP & Penninger JM (1997) Activation of stress-activated protein kinases/c-Jun N-terminal protein kinases (SAPKs/JNKs) by a novel mitogen-activated protein kinase kinase. *The Journal of biological chemistry* 272: 32378–83

Yaron A, Hatzubai A, Davis M, Lavron I, Amir S, Manning AM, Andersen JS, Mann M, Mercurio F & Ben-Neriah Y (1998) Identification of the receptor component of the IkBa-ubquitin ligase. *Nautre* 396: 590–594

Yasuda J, Whitmarsh a J, Cavanagh J, Sharma M & Davis RJ (1999) The JIP group of mitogen-activated protein kinase scaffold proteins. *Molecular and cellular biology* 19: 7245–54

Yoon S-Y, Park J-S, Choi J-E, Choi J-M, Lee W-J, Kim S-W & Kim D-H (2010) Rosiglitazone reduces tau phosphorylation via JNK inhibition in the hippocampus of rats with type 2 diabetes and tau transfected SH-SY5Y cells. *Neurobiology of disease* 40: 449–55

Yu C, Minemoto Y, Zhang J, Liu J, Tang F, Bui TN, Xiang J & Lin A (2004) JNK suppresses apoptosis via phosphorylation of the proapoptotic Bcl-2 family protein BAD. *Molecular cell* 13: 329–40

Zhang L, Blackwell K, Altaeva A, Shi Z & Habelhah H (2011a) TRAF2 phosphorylation promotes NF-κB-dependent gene expression and inhibits oxidative stress-induced cell death. *Molecular biology of the cell* 22: 128–40

Zhang S, Lin Z-N, Yang C-F, Shi X, Ong C-N & Shen H-M (2004) Suppressed NF-kappaB and sustained JNK activation contribute to the sensitization effect of parthenolide to TNF-alpha-induced apoptosis in human cancer cells. *Carcinogenesis* 25: 2191–9

Zhang X, Xu A, Chung SK, Cresser JHB, Sweeney G, Wong RLC, Lin A & Lam KSL (2011b) Selective inactivation of c-Jun NH2-terminal kinase in adipose tissue protects against diet-induced obesity and improves insulin sensitivity in both liver and skeletal muscle in mice. *Diabetes* 60: 486–95

Zingarelli B, Sheehan M, Hake PW, Connor O, Denenberg A & Cook JA (2003) Peroxisome Proliferator Activator Receptor-g Lignads, 15-Deoxy-D12,14-Prostaglandin J2 and Ciglitazone, reduce systemic inflammation on polymicrobial sepsis by modulation of signal transduction pathways. *The Journal of Immunology* 171: 6827–37

LIST OF ABREVIATIONS

AP	Acute Pancreatitis AP
ANK	Ankyrin repeat domain
AP-1	Activator Protein-1
APC	Anaphase-Promoting Complex
ASK	Apoptosis signal-regulating kinase
βTrCP	β-Transducin Repeat-Containing protein
CC	Coiled-coil domain
CSN	COP9 signalosome
CR	Caloric restriction
DAMPS	Danger-associated molecular patterns
DD	Death domain
DUSP	Dual-specificity phosphatase
ERK	Extracellular signal-regulated kinase
GH	Growth hormone
GR	Glucocorticoid Receptor
GRR	Glycine-rich region
HECT	Homologous to E-AP C-terminus domain
IBD/DimD	IKK-binding domain/dimerization domain
IFN	Interferon
IκB	Inhibitor of NF-κB
IKK	IκBα Kinase
IL	Interleukin
IR	Insulin Receptor
IRS	Insulin receptor substrate
JIP	JNK interacting protein
JNK	c-Jun N-Terminal Kinase
KO	Knockout
LPS	Lipopolysaccharide
LZ	Leucine zipper domain
MAPK	Mitogen activated protein kinase
MEKK	MEK kinase
MKK	MAPK kinases
MKP	MAPK phosphatase
MLK	Mixed-lineage kinase
MOD	Minimal oligomerization domain
NBD	NEMO-binding domain
NEMO	NF-κB Essential Modifier
NF-κB	Nuclear Factor-κB
NLK	Nemo-like kinase
NLS	Nuclear Localization Sequence
PAMP	Pathogen-associated molecular pattern
PEST	Proline-rich, glut. acid-rich, serine-rich, and threonine-rich

PRR	Pattern recognition receptors
POSH	Plenty of SH_3
Rbx	RING box protein
RHD	Rel Homology Domain
RING	Really Interesting New Gene Domain
RIP	Receptor-interacting protein
Roc	Regulator of cullins
ROS	Reactive oxygen species
SAPK	Stress Activated Protein Kinase
SDD	Scaffolding and dimerization domain
Skp1	S-phase kinase associated protein 1
TAB1/2	TAK1 binding protein 1 and 2
TACE	TNF alpha converting enzyme
TAD	Transactivation domain
TAK1	TGFβ-activated kinase 1
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TNFR	TNF receptor
TNF-RSC	TNF receptor signaling complex
TRADD	TNFR1-Associated Death Domain
TRAF	TNFR Associated factor
UBD	Ubiquitin-binding domain
ULD	Uubiquitin-like domain
UV	Ultraviolet