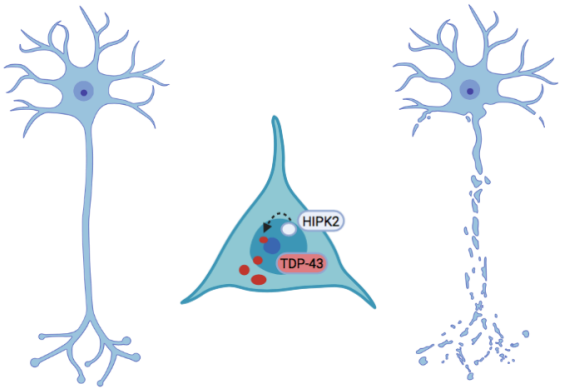
UNIVERSITY OF NAPLES FEDERICO II

DOCTORATE IN
MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXXIV CYCLE

Valeria Valente

"Unraveling the role of HIPK2 in neurodegeneration and pathogenesis of TDP-43-related ALS disease"



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"Unraveling the role of HIPK2 in neurodegeneration and pathogenesis of TDP-43-related ALS disease"

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Valeria Valente

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LIST OF ABBREVIATIONS

ALS	Amyotrophic Lateral Sclerosis
CA	Hippocampus, cornu ammonis
CBR	Cerebellum
CNS	Central Nervous System
CTX	Motor Cortex
FUS	fused in sarcoma/translocation in liposarcoma
HIPK	Homeodomain-Interacting Protein Kinase
HPP	Hippocampus
KD	Kinase dead
KO	Knock-out
L-BMAA	β-N-methylamino-L-alanine
MNs	Motor neurons
SC	Spinal cord
SOD1	Superoxide dismutase 1
STR	Striatum
TARDBP	TAR-DNA-binding protein
TDP-43	TAR-DNA-binding protein 43 kDa
WT	Wild Type

ABSTRACT

Homeodomain interacting protein kinase 2 (HIPK2) is a key protein involved in cell proliferation and apoptosis, during embryonic development and adult life. The disruption of HIPK2 activity has been correlated to developmental anomalies and several diseases. Recently, HIPK2 has been reported to exert a crucial role in the physiology of the nervous system. In fact, the neurological phenotype shown by *Hipk2* null mice is severely impaired and highly resembling a "motor neuron" disease such as ALS pathology. On the basis of these observations, we characterized the effects of *Hipk2* loss on neuromuscular physiology and on the pathogenesis of ALS using *in vivo* and *in vitro* model systems. Interestingly, we found that HIPK2-KO mice show diffuse neuronal necrosis throughout the nervous system and morphological alterations of the neuromuscular junctions associated with poor mitochondrial proliferation. Our results clearly indicate that the deletion of *Hipk2* is responsible for a myopathic phenotype in adult mice. Moreover, we found a strict interconnection between HIPK2 and TDP-43, which is one of the main pathological protein associated with both sporadic and familial ALS cases. Indeed, *Hipk2* null mice show a strong delocalization of TDP-43 protein from the nucleus that accumulates in the cytosol of spinal motor neurons, as demonstrated by immunofluorescence and biochemical approaches. Consistently, silencing of *Hipk2* expression in human derived neuroblastoma SH-SY5Y cells reproduces the same effect. Strikingly, the restoring of the expression of active *Hipk2*, but not of its kinase dead mutant, is responsible for the reestablishment of physiological TDP-43 subcellular distribution in HIPK2-interfered SH-SY5Y cells. Moreover, we found that *Hipk2* deficient mice are more susceptible to develop symptoms of neurodegeneration and mislocalization of TDP-43 protein upon treatment with the neurotoxin L-BMAA. Taken together, these data unravel the functional crosstalk existing between HIPK2 and TDP-43 proteins and shed light on the putative protective role exerted by HIPK2 on nervous system homeostasis.

1. INTRODUCTION

1.1 Homeodomain-Interacting Protein Kinases family

The Homeodomain-Interacting Protein Kinases (HIPKs) belong to a family of highly conserved nuclear serine/threonine kinases which includes HIPK1, HIPK2, HIPK3 and HIPK4. HIPKs proteins are involved in different cellular processes such as cell proliferation, apoptosis, DNA-damage response, oxidative stress and development, through the phosphorylation of many transcriptional regulators and chromatin modifiers (Rinaldo et al. 2008), (Saul and Schmitz 2013). HIPK1, HIPK2, and HIPK3 were originally identified through their interaction with homeobox-containing proteins, and show a conserved protein structure with more than 90% of homology in their kinase domains and about 70% in their homeobox-interacting domains (van der Laden, Soppa, and Becker 2015). HIPK4 protein was later identified during the human genome sequencing project (Manning et al. 2002), it is the less evolutionary conserved, and it is the most diverged member of the HIPK family, showing about 50% homology within the kinase domain with other HIPK family members (Li et al. 2007). HIPKs activity is mediated by phosphorylation of several important proteins. For example, HIPK1, 2 and 3 have been reported to phosphorylate the homeodomain protein NKx2.1 (Kim et al. 1998), HIPK2 phosphorylates tumor suppressor p53 (Sombroek and Hofmann 2009), and HIPK3 has been reported to phosphorylate Fas-associated protein with death domain (FADD), the adaptor protein that is critical in mediating extrinsic apoptosis signals (Rochat-Steiner et al. 2000). Several studies have shown that HIPKs act as integrators of stress signals mediating the functional interactions among different pathways, including Salvador-Warts-Hippo pathway, bone morphogenetic protein (BMP), Wnt/Wingless, Notch, TGFβ, p53 and mitogen-activated protein kinase (MAPK) signaling networks (D'orazi et al. 2012) HIPKs are not essential components of all of these pathways, but they modulate

the signal integration through different effectors. Since their activity strongly depends on other proteins, all the HIPK family members are reported to exert contradictory effects on cell proliferation and tissue growth. For example, cell culture studies have shown that HIPKs can act to promote growth or inhibit cell proliferation in a context-dependent manner. In fact, depletion of HIPK2 strongly reduces proliferation in peripheral blood lymphocytes (Iacovelli et al. 2009) whereas loss of HIPK2 leads to increased proliferation of epidermal stem cells (Wei et al. 2007) and excessive endothelial cell growth in the yolk sacs of *Hipk1* $^{-/-}$ *Hipk2*$^{-/-}$ mice (Shang et al. 2013). Moreover, analysis of loss-of-function phenotypes in diverse cell types and organisms has shown that HIPK proteins are expressed in different and dynamic space/time patterns, highlighting their important role, mostly during development (Kondo et al. 2003). In particular, HIPK1 and HIPK2 are highly correlated and share the same functions during embryogenesis. Indeed, their functional redundancy is demonstrated by *in vivo* studies; single *Hipk1* $^{-/-}$ or *Hipk2* $^{-/-}$ mice are viable and fertile (Kondo et al. 2003) (Wiggins et al. 2004), whereas the double knock-out of *Hipk1* $^{-/-}$/*Hipk2* $^{-/-}$ results in embryonic lethality, caused by defective neural tube closure, defects in differentiation of the hematopoietic cell lineage, and defective vasculogenesis and angiogenesis (Kim et al. 1998, Isono et al. 2006, Rinaldo et al. 2007) These works demonstrate that HIPK family members are required for survival acting to fine-tune diverse signaling and mediate cellular crosstalk in context-specific biological processes.

1.2 HIPK2 protein

1.2.1 Structure and regulation

The best characterized member of HIPK family is HIPK2, a 130 kDa serine/threonine kinase of 1191 amino acids. As shown in Figure 1, HIPK2 structure comprises:

- an N-terminal region containing a small ubiquitin-related modifier (SUMO) binding site (K25), a conserved Ser/Thr kinase domain harboring the catalytic site (K221), and the so-called activation loop characterized by the 3 aa STY (352-354) (Kim et al. 1998, Hofmann et al. 2005).
- a central portion with a homeobox-interacting domain (HID) followed by a region rich in proline (P), glutamic acid (E), serine (S) and threonine (T) that acts as a signal peptide for protein degradation (PEST domain) overlapping with the speckle retention sequence (SRS) (Schmitz et al. 2014).
- a C-terminal region which includes an autoinhibitory domain (AID) and an ubiquitylation site (K1182).

HIPK2 kinase domain is a 330 amino-acid p38MAP-like domain highly conserved throughout evolution (Hofmann et al. 2000). The amino acid K221 is crucial for HIPK2 kinase activity due to its involvement in ATP binding (Kuwano et al. 2016). Indeed, the substitution of this lysine with an arginine or an alanine leads to the loss of the kinase activity: the mutant protein K221R (kinase dead, KD) retains the ability to interact with its target proteins, but it is unable to phosphorylate both target proteins and itself (Pierantoni et al. 2002, Hofmann et al. 2002). The interaction with the homeodomain transcription factors is mediated by the HID domain, showing 72% and 75% similarity between HIPK1–3 (Kim et al. 1998). Through this domain HIPK2 interacts with the transcription factors of the Nkx and HOX families, the proto-oncogene c-

Ski, and the transcriptional activators Smad1–4 (Kim et al. 1998, Harada et al. 2003, Isono et al. 2006, Zhang et al. 2007). Several *in vitro* studies converge on the recruitment of HIPK2 into speckled sub-nuclear structures, some of which are typical nuclear bodies containing the PML (pro-myelocytic leukemia protein) and nuclear antigen Sp100, as well as the tumor suppressor p53, one of HIPK2 main targets (D'Orazi et al. 2002, Hofmann et al. 2002, Möller et al. 2003). The recruitment of HIPK2 into the "HIPK bodies" is mediated by the speckle-retention signal (SRS) (Rinaldo et al. 2007), and it is involved in HIPK2-mediated transcriptional repression (Roscic et al. 2006).

HIPK2 is frequently unstable in the absence of specific stimuli and stress factors. However, several cellular stressors, like morphogenetic signals and DNA damage, are able to stabilize HIPK2 and activate its kinase activity. HIPK2 is extensively modulated by several post-translational modifications that can alter HIPK2 stability and localization as well as by miRNAs (Saul and Schmitz 2013, Conte and Pierantoni 2018). Autophosphorylation on conserved tyrosine residue in the kinase activation loop is the main modification which affects the degree of kinase activity, and the affinity for target substrates (Saul & Schmitz 2013, Schmitz et al. 2014). Two more post-translational modifications have been defined and characterized: sumoylation of Lys25 (Kim et al. 1999) and ubiquitylation of Lys1182 (Rinaldo et al. 2007). Sumoylation was found to regulate both HIPK2 stability and activity on target factors (Gresko et al. 2005, Hofmann et al. 2005, Sung et al. 2005). In particular, upon DNA damage, HIPK2 phosphorylates its own E3 SUMO ligase Pc2, which in turn triggers HIPK2 sumoylation through the addition of small ubiquitin-like modifier (SUMO) peptide, establishing a positive feedback loop that enhances HIPK2 ability to act as a transcriptional repressor (Roscic et al. 2006). Oppositely, ubiquitylation has been, thus far, involved in HIPK2 inactivation. Indeed, four different ubiquitin E3 ligases (MDM2, Siah, WSB-1, and Fbx3-formed SCF) mediate continuous HIPK2 ubiquitination, thus promoting its degradation via the proteasome, and guaranteeing low HIPK2 levels in unstressed cells (Rinaldo et al. 2007, Calzado

et al. 2007, Choi et al. 2014). Interestingly, the ubiquitylation site Lys1182 resides within the autoinhibitory region. HIPK2 autoinhibitory domain was firstly identified because of its interaction with Axin, an adaptor protein known from the Wnt signaling pathway: indeed, it has been reported that axin is able to stimulate HIPK2 activity occupying its autoinhibitory domain, and the removal of this region from HIPK2 results in the stimulation of p53 activity (Rui et al. 2004). Moreover, the existence of the autoinhibitory domain is further supported by the fact that this region has to be cleaved by caspases for full activation of HIPK2 (Gresko et al. 2006). Overall, HIPK2 protein contains multiple sites of interaction with target proteins and regions committed to regulate its localization, stability and activity.

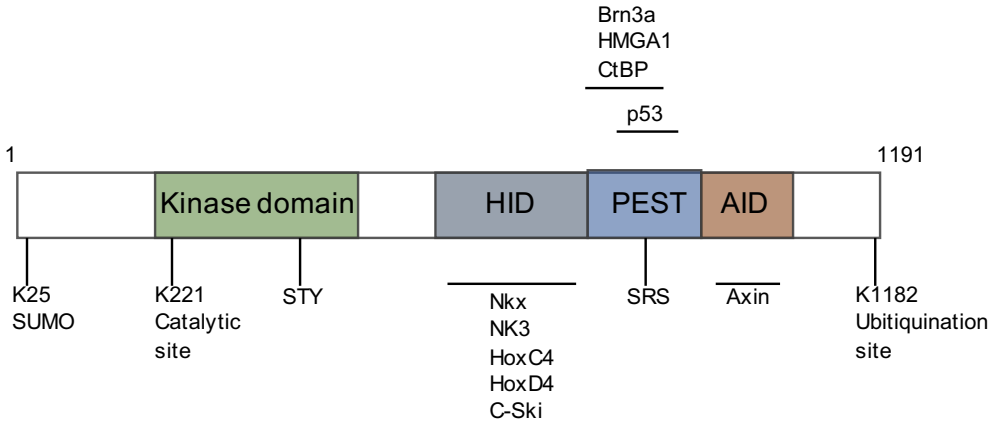

Figure 1. Schematic representation of HIPK2 protein.
K25 is the sumoylation site; kinase domain in which K221 is the catalytic site and STY is the activation loop; homeoprotein-interacting domain (HID); PEST domain with speckle retention signal (SRS); auto-inhibitory domain (AID); K1182 is the ubiquitylation site. Horizontal lines indicate the main HIPK2 interactors. *Adapted from Blaquiere & Verheyen 2017.*

1.2.2 Functions

HIPK2 protein is ubiquitously expressed in human and murine adult tissues; however, during the latest phases of embryogenesis, HIPK2 shows a preferential expression pattern in retina, muscle and neural tissues (Pierantoni et al. 2002). At subcellular level, HIPK2 peculiarly localizes to subnuclear HIPKs domains. However, a lower percentage of the kinase has been found more diffusely in both nucleoplasm and cytoplasm (Rinaldo et al. 2008). In the nucleus, HIPK2 was firstly shown to act as transcriptional corepressor for homeodomain-containing transcription factors (Kim et al. 1998). Subsequent works, conducted in several systems and cell types, have revealed that HIPK2 exerts a context-specific role in regulating gene expression, as it can repress or activate the transcription of many promoters, thus modulating many different cellular processes such as growth, development, morphogenesis, and cell death (D'Orazi et al. 2002, Hofmann et al. 2002, Calzado et al. 2007). The most extensively studied role for HIPK2 is the modulation of apoptosis during genotoxic stress conditions (Figure 2). Indeed, HIPK2 is a DNA damage sensitive kinase, activated by several kinds of genotoxic stimuli, including ultraviolet radiation and ionizing radiation, that exerts its effects by binding and/or phosphorylating a wide range of transcription factors and cofactors. The key step for HIPK2 to promote apoptosis is the phosphorylation of its first identified non-homeotic factor target, p53 tumor suppressor (D'Orazi et al. 2002, Hofmann et al. 2002, Rinaldo et al. 2007). Under physiological condition, p53 has a short half-life due to the binding with the E3-ubiquitin ligase MDM2 responsible for its proteasome degradation. Upon severe genotoxic stress, HIPK2 phosphorylates p53 at serine 46 disrupting its binding to MDM2 and allowing it to accumulate in the nucleus. Once in the nucleus, p53 activates the transcription of pro-apoptotic genes as such as *TP53, Noxa, Bax* and *Puma* leading to cell death (Oda et al. 2000, Iacovelli et al. 2009, Fritsche et al. 20015). Moreover, HIPK2 is also responsible for CBP-mediated p53 acetylation at Lys382 which, in combination with phosphorylation of Ser46, is necessary for

p53 pro-apoptotic activity (Hofmann et al. 2002). HIPK2 exerts pro-apoptotic effects also in a p53-independent manner, by promoting the expression of multiple target genes (Figure 2). Indeed, HIPK2 is able to sensitize p53-defective cells to apoptosis, through the interaction with the transcriptional corepressor C-terminal binding protein (CtBP) (Zhang et al. 2005) In fact, during UV-induced DNA damage conditions, HIPK2 phosphorylates the anti-apoptotic protein CtBP inducing its proteasome-dependent degradation, thus preventing CtBP-mediated downregulation of several pro-apoptotic genes such as PERP, p21, and Noxa (Chinnadurai et al. 2009).

Another relevant HIPK2 interactor is High Mobility Group AT-hook 1 protein (HMGA1), a chromatin-remodeling factor (Zhang et al. 2007). Several studies showed that HMGA1 and HIPK2 share various fields of interactions, such as regulation of cell proliferation, apoptosis, and regulation of p53 activity. Indeed, while HIPK2 potentiates p53 pro-apoptotic activity, HMGA1 antagonizes p53-driven transcription of apoptosis-related genes (Frasca et al. 2006, D'Orazi et al. 2012). In particular, it has been demonstrated that HIPK2 binds and phosphorylates HMGA1 at different Ser- and Thr- residues, decreasing its binding affinity to DNA, and thus altering the HMGA1-mediated regulation of gene expression (Pierantoni et al. 2001, Zhang et al. 2007). In addition to its well characterized pro-apoptotic activity, HIPK2 has also opposite functions in specialized cell types. For instance, HIPK2 is essential for the survival of murine enteric neurons during postnatal development. This pro-survival role of HIPK2 does not involve apoptosis but rather depends on other signal transduction pathways, like the bone morphogenetic proteins (BMPs) (Chalazonitis et al. 2011).

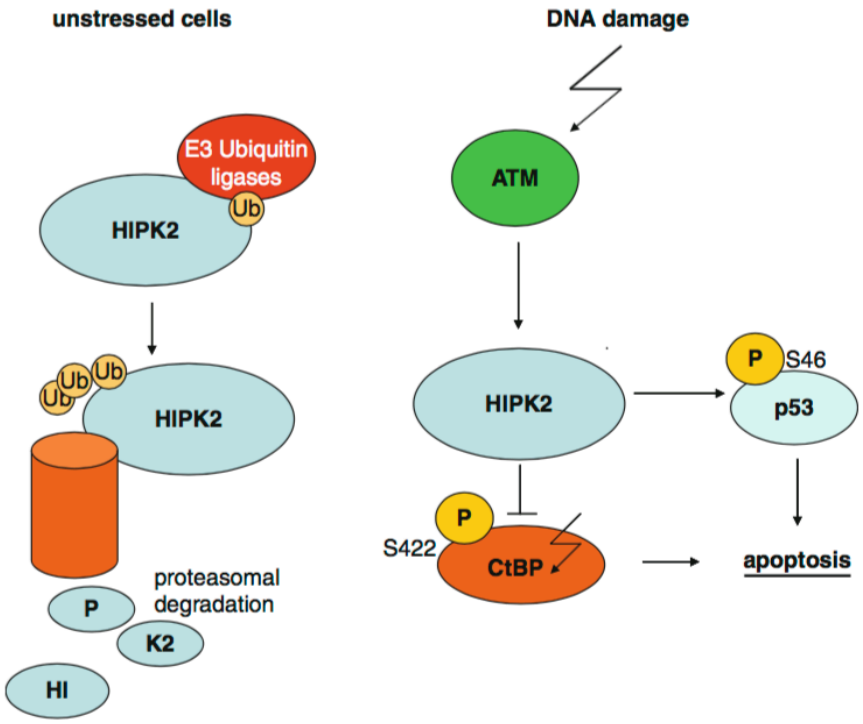

Figure 2. Schematic representation of HIPK2 role within the DNA damage response.
In unstressed cells, HIPK2 is almost completely degraded via the proteasomal pathway, whereas genotoxic stress causes HIPK2 stabilization and activation promoting apoptosis by phosphorylating p53 and/or CtBP proteins. *From Hofman & Krieghoff-Henning 2018*.

1.2.3 Role in embryonic development and nervous system homeostasis

HIPK2 function as regulator of cell proliferation and survival is closely linked to its contribution to cell differentiation (Blaquiere and Verheyen 2017). Embryonic development is characterized by a complex regulation of both the expression and activity of several homeobox proteins. In *Drosophila* it has been shown that the knockout of the only present HIPK gene (dHIPK) results in defective eye formation and frequent death during embryonal stages. Indeed, dHIPK regulates eye development antagonizing the global corepressor Groucho together with NK-3 homeoprotein and histone deacetylases (Lee et al. 2009). dHIPK-mediated phosphorylation of Groucho has been shown to attenuate its contact to DNA-bound transcription factors, thereby altering gene expression (Choi et al. 1999). Most prominently, HIPK2 developmental activity has been connected to the Wnt pathway which is important for axis specification, neural tube patterning and regulation of stem cell compartments. For instance, Wnt-1 induces the HIPK2- and NLK-dependent degradation of the transcription factor c-myb. Moreover, HIPKs have been shown to interact with and phosphorylate both β-catenin and transcription factors of the Lef/TCF-family in both human and *Drosophila* (Hikasa et al. 2010), thereby directly modulating the canonical Wnt signaling pathway. However, the link between HIPK2 and the canonical Wnt signaling pathway appears partially contradictory and requires further investigation to be fully elucidated. Furthermore, since HMGA1 is widely expressed during embryogenesis and plays key roles in chromatin architecture and transcriptional regulation (Reeves et al. 2001), its interaction with HIPK2 has a prominent role also in these contexts. Indeed, our research group had recently characterized the functional role of HIPK2 and HMGA1 interaction during development through the study of a mouse model in which both genes were disrupted (Gerlini et al. 2019). We reported that about 50% of HMGA1/HIPK2 double knock-out mice die within postnatal day 1 for respiratory failure due to incorrect pulmonary development. Indeed, both HMGA1 and HIPK2 positively regulate the expression of surfactant proteins

that are required for lung development. In addition, the surviving DKO mice show significant lower body dimension and thyroid abnormalities. This phenotype is due to the fact that both HMGA1 and HIPK2 proteins positively regulate the transcriptional activity of genes encoding thyroid-specific transcription factors involved in the differentiation of the gland. Consistently, HIPK2/HMGA1 cooperation results to be crucial for correct thyroid and lung development (Gerlini et al. 2019).

Extensive studies of genetically modified mice gave more detailed information about HIPK2 modulation of gene transcription during development. According to Isono (2006), analysis of *Hipk1/Hipk2* double KO mice revealed developmental defects suggesting an altered Hox gene regulation and an influence of HIPKs on the Sonic hedgehog (Shh) pathway via Pax1 and Pax3.

In particular, the fact that *Hipk1/Hipk2* double KO embryos fail to close the anterior neuropore, exhibit exencephaly and show defects in the closure of the dorsal neural tube, highlighted the relevant role of HIPK2 in the development and homeostasis of the nervous system (Zhang et al. 2007). For instance, HIPK2 might influence important morphogenetic processes, such as motor neuron formation interacting with both Pax and the Nkx family transcription factors. HIPK2 interaction with Pax may be responsible for the deficit in eye formation in *Hipk2* null mice which frequently have abnormally small eyes without a lens and with incorrect lamination and cell arrangement in the retina (Inoue et al. 2010). Furthermore, HIPK2 was recently shown to interact with the transcription factor cAMP-responsive element binding protein (CREB) (Sakamoto et al. 2011) and to increase its association with CBP, which also plays a role in neuron development and survival. It has also been reported that HIPK2 can interact with the corepressor c-ski and with SMAD1 thereby counteracting bone morphogenetic protein (BMP) signaling, which is implicated in embryo polarity, heart and central nervous system (CNS) development as well as bone formation (Harada et al. 2003) (Figure 3). Indeed, HIPK2 has been shown to induce pro-survival signals downstream of TGF-β and BMP in ventral midbrain

dopaminergic and enteric neurons (Zhang et al. 2007, Chalazonitis et al. 2011) , as well as it is required for TGF-β-mediated survival during programmed cell death in dopamine neurons (Zhang et al. 2007). On the contrary, HIPK2 was shown to suppress the transcription of pro-survival genes in sensory neurons through the interaction with the transcription factor Brn3a. In fact, HIPK2 promotes Brn3a binding to DNA, thus suppressing Brn3a-dependent transcription of TrkA, Bcl-xL and Brn3a itself (Wiggins et al. 2004). This contradictory role of HIPK2 is confirmed by the fact that its overexpression is able to induce apoptosis in cultured sensory neurons, meanwhile its deletion reduces apoptosis and increases neuron numbers in the trigeminal ganglion (Wiggins et al. 2004). Given these cell type-specific functions, loss of HIPK2 results in altered number of sensory, dopaminergic and enteric neurons during embryonic development and in early postnatal life, thus disrupting the physiological homeostasis of the nervous system.

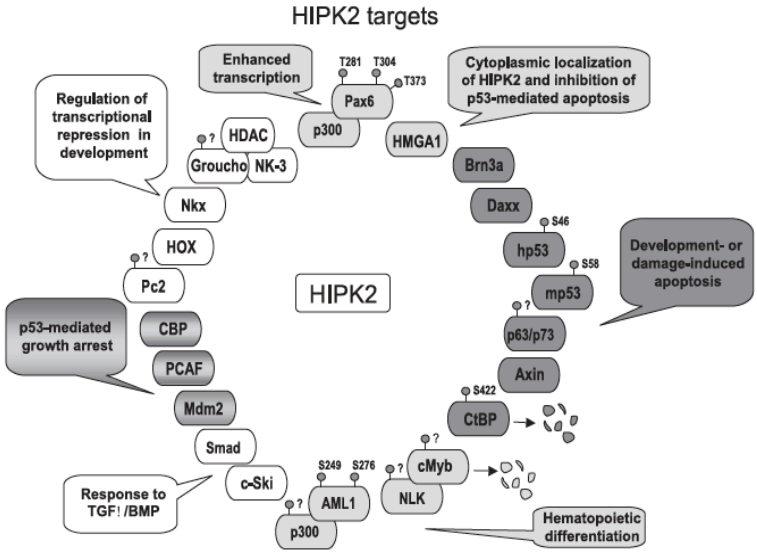

Figure 3. Scheme representing HIPK2 targets.
HIPK2 interactors and their respective biological activities. *From Rinaldo et al. 2007*

1.3 HIPK2 and neurodegenerative diseases

Since HIPK2 plays a crucial role in different signaling and proliferation pathways, alterations of its activity contribute to the development of diverse pathologies. In fact, dysregulation of HIPK2 functions has been found in many diseases, including cancer (Lavra et al. 2011), kidney fibrosis (Fan et al. 2014), hearth failure (Guo et al. 2019), and neurodegeneration (Lee et al. 2016). In particular, since HIPK2 exerts pro-survival and/or pro-apoptotic role in neurons, its deletion results in neuronal cell death and synaptic loss that are among the main hallmarks of neurodegenerative disease.

The first evidence of the involvement of HIPK2 in the homeostasis of the nervous system derived from the analysis of the phenotype of KO mice. The double knock-out *Hipk1*$^{-/-}$/*Hipk2* $^{-/-}$ mice, generated from Isono et al. die between 9.5 and 12.5 days *postcoitus,* showing neurological alterations, such as neural tube closure defect and exencephaly. These defects, caused by a reduction in proliferation rate of neural tube and the cephalic mesoderm cells, highlight the fact that HIPK1 and HIPK2 act in synergy to mediate growth regulation upon morphogenetic signals, even if HIPK2 may exert a slightly dominant role. Indeed, single *Hipk2*$^{-/-}$ mouse models generated by independent research groups, are all viable and fertile, but exhibit peculiar phenotypical and behavioral features (Figure 4): they show small body size, associated with head and snout crushed, curvature of the spine and distortion of the legs. Moreover, since the fundamental role of HIPK2 in the survival of midbrain dopamine neuron, KO mice also show Parkinson's-like movement impairments (Zhang et al. 2007). In addition, our research group has demonstrated that murine expression of HIPK2 is also required for Purkinje cells survival (Anzilotti et al. 2015). Indeed, HIPK2 null mice show a critical reduction in the number of cerebellar Purkinje neurons, due to the activation of an apoptotic process associated with a strong accumulation of ubiquitinated β-catenin which is a hallmark for several neurodegenerative diseases, like spinocerebellar ataxia or Parkinson's disease

(Lehman et al. 2009). Moreover, because of the cerebellar atrophy caused by Purkinje cells loss, KO mice also show severe psychomotor and behavioral abnormalities; these defects, including dystonia, impaired coordination, reduced motility, clasping of posterior limbs, poor motor coordination, and reduced responses to novelty are strongly indicative of an ataxic-like phenotype resembling psychomotor symptoms of neurodegenerative diseases (Anzilotti et al. 2015).

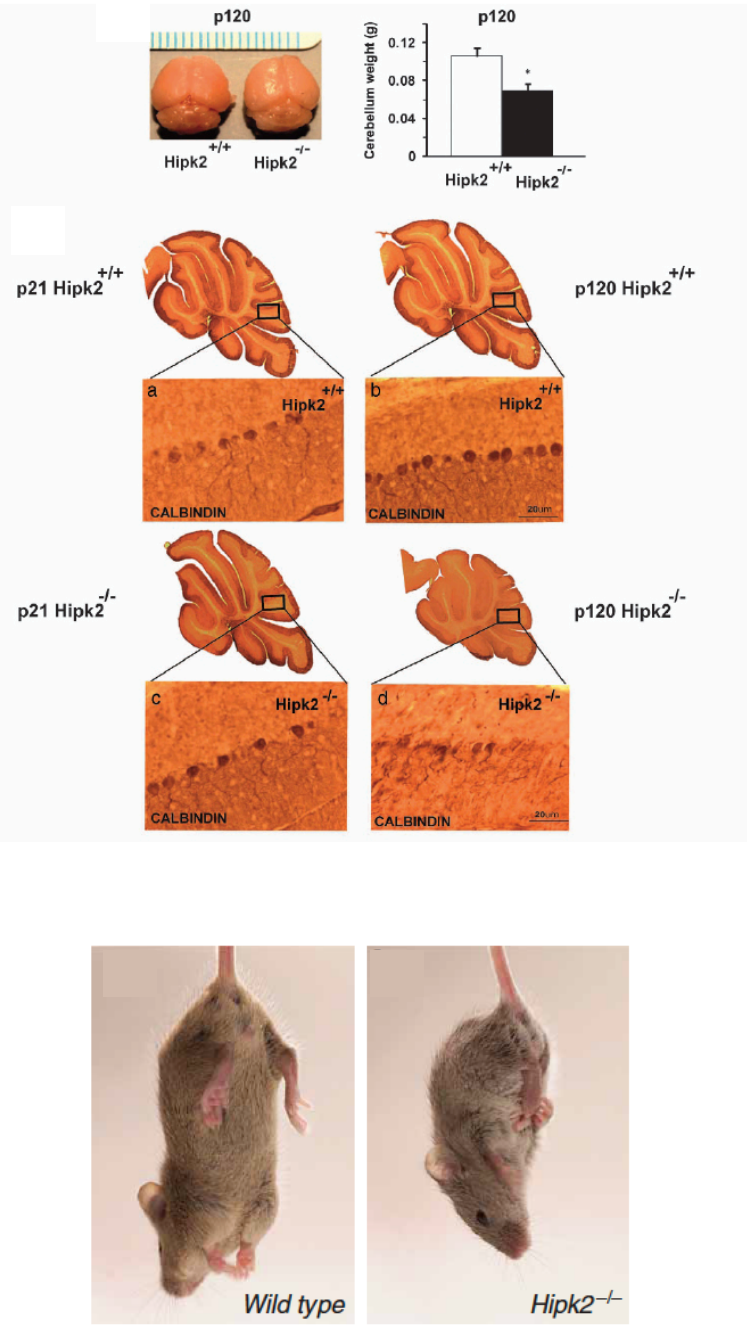

Figure 4. Cerebellar and motor behavioral abnormalities in *Hipk2*$^{-/-}$ mutants. The majority of *Hipk2* null mice show hindlimbs clasping when suspended by their tails due to cerebellar atrophy caused by Purkinje cell loss. *Adapted from Anzilotti et al. 2015, Zhang et al. 2007.*

1.4 Molecular mechanisms involved in Amyotrophic Lateral Sclerosis pathogenesis

Amyotrophic Lateral Sclerosis (ALS) is an adult-onset neurodegenerative disease that causes selective loss of both upper and lower motor neurons (MNs), leading to paralysis and death (Ewis et al. 2001). For this reason, the pathology is considered "motor neuron disease". Although ALS is the most common adult motor neuron disease, it is still classified as a rare pathology: its incidence is estimated to be 2 per 100,000 individuals, whereas its prevalence is 5.4 per 100,000 individuals (Chiò et al. 2013). The onset of ALS begins in late-adulthood, when patients show symptoms such as muscle atrophy and weakness. With the progression of the disease, different areas of CNS become involved, and consequently symptoms appear in other regions of the body, including cognitive impairment in up to 50% of patients (Tsermentseli et al. 2012). In the majority of the cases, the prominent effect on respiratory muscles limits the survival up to 2–4 years after disease onset (Chio et al. 2009). Even if the 90-95% of the ALS cases are sporadic (sALS), several gene mutations have been associated to this disease, and about 10% of the cases are inherited (fALS), indicating that both genetic and environmental factors contribute to risk (Byrne et al. 2011). In fALS, mutations in the C9orf72 gene represent 30–40% of cases, those in the SOD1 gene make up 15–20%, those in the FUS and TARDBP genes each represent approximately 5% of cases, and the remaining genes that have been associated with fALS represent a small proportion of cases (Blokhuis et al. 2013). Despite the considerable efforts made through the years, the pathogenic mechanism underlying MNs loss in ALS is still poorly understood. Furthermore, considering that many different cellular processes are involved in ALS progression, determining which ones may be causative remains challenging. The wide range of genetic and phenotypic heterogeneity between patients also make it difficult for studies in genetically similar animal models to translate to success in human clinical trials. Current treatment options are based on symptom management and respiratory support

with the only approved medications in widespread use Riluzole and Edaravone (a glutamate antagonist and a radical oxygen species scavenger, respectively) that provide only modest benefits and only in some patients (Petrov et al. 2017, Sawada et al. 2017). Several molecular mechanisms have been linked to neurodegeneration in ALS including glutamate neurotoxicity mitochondrial dysfunction (Chung et al. 2002) impaired axonal transport (Ikenaka et al. 2012), and abnormal protein accumulations (Blokhuis et al. 2013). In particular, the central pathological hallmark of ALS is the presence of cytoplasmic proteins aggregation in the soma of MNs. Almost all (97%) ALS patients display a common phenotype in disease-affected tissues, namely the deposition of the TAR-DNA binding protein "TDP-43" (Figure 5) which is also the major feature of frontotemporal dementia (FTD) (Neumann et al. 2006, Arai et al. 2006, Mori et al. 2008, Ling et al. 2013). The presence of cytoplasmic aggregation of ALS proteins leads to neurodegeneration through multiple mechanisms, including the loss of normal function of these proteins, and the sequestration of multiple interactors that are essential for neuronal survival. Moreover, it is still unknown why mutations in proteins that are ubiquitously expressed, result in pathological alterations selectively in the MNs. Considering the importance of protein aggregation in the pathogenesis of ALS, a further understanding of its molecular basis, may aid the development of novel therapeutic strategies for this disease.

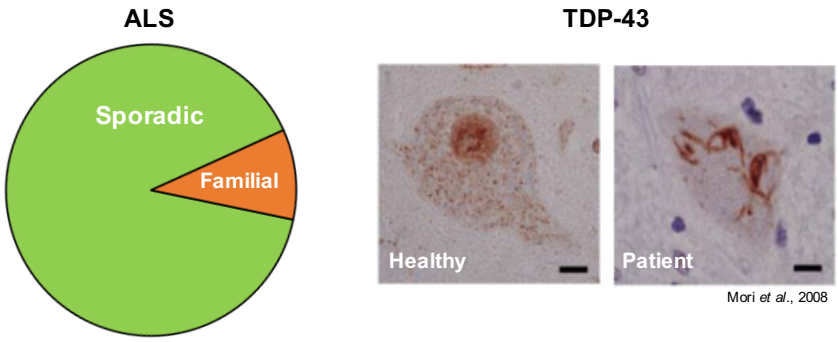

Figure 5. Deposition of TDP-43 protein in ALS disease.
Representative TDP-43 immunohistochemistry in the spinal cord motor neurons of patient with amyotrophic lateral sclerosis and healthy subject. Scale bar = 10 µm. *From Mori et al. 2008.*

1.4.1 TDP-43 proteinopathy

TDP-43 is the major component of cytoplasmic insoluble inclusions in the neurons of ALS and FTD patients (Arai et al. 2006, Neumann et al. 2006, Le et al. 2016). Indeed, this pathological feature is considered a hallmark of ALS, as it is observed in approximately 97% of all ALS patients, regardless of the sporadic or familiar nature of the disease. The only exceptions are represented by fALS cases caused by mutations in SOD1 and FUS (Mackenzie et al. 2007) Wales et al. 2011, Hardiman et al. 2017). Neuropathological studies have also revealed that the clinical course of ALS reflects the presence of TDP-43 pathology, from its deposition at an initial site of onset, to its spread to contiguous regions of the CNS (Brettschneider et al. 2013).

TDP-43, encoded by the *TARDBP* gene, is a ubiquitously expressed DNA/RNA-binding protein, involved in several RNA processing events, including splicing, transcription, and translation. Since the UG-rich sequences recognized by TDP-43 are present within approximately one third of all transcribed genes, this protein is able to influence the processing of hundreds to thousands of transcripts (Polymenidou and Cleveland 2011, Tollervey et al. 2011, Sephton et al. 2011). As suggested by its fundamental functions, the level and localization of TDP-43 are tightly regulated and critical for cell health. TDP-43 knockout mice die early in embryogenesis, and partial or conditional knockout animals exhibit neurodegeneration and behavioral deficits (Kraemer et al. 2010, Iguchi et al. 2013). Additionally, sustained TDP-43 overexpression results in neurodegeneration in several animal models, including mice (Swarup et al. 2011), rats (Dayton et al. 2013), fruit flies (Li et al. 2010), zebrafish (Schmid et al. 2013), and primates (Jackson et al. 2015). All these data provide convincing evidence that either excess or a deficit of TDP-43 can be lethal. Even if TDP-43 shows a canonical nuclear localization (Ayala et al. 2005), in motor neurons it has been also detected in the cytosol where it was supposed to exert a role in the formation of new dendritic trees and branches to create new synapses (Wang et al. 2008). In neurons of ALS patients TDP-43 nuclear localization is partially

lost and aggregated in the cytoplasm, suggesting that loss of TDP-43 nuclear function could be an important event in ALS pathogenesis (Neumann et al. 2006, Van Deerlin et al. 2008). The effects of cytosolic TDP-43 aggregation in ALS pathogenesis remains still unclear.

1.4.2 β-N-methylamino-L-alanine role in neurodegeneration

β-N-methylamino-L-alanine (L-BMAA) is a cyanobacteria-produced non-protein amino acid with genotoxic/neurotoxic activity, which has been hypothesized to cause ALS and possibly other neurodegenerative diseases. L-BMAA-producing cyanobacteria are present in many ecosystems, and it has been found concentrated in the tissues of organisms at higher trophic levels, such as fish and mollusks, that are consumed by humans (Jonasson et al. 2010). Dietary consumption of cycad seeds highly enriched in L-BMAA became a concern during the investigation of an extraordinarily high rate of ALS and Parkinson's dementia complex (PDC) that occurred in Guam and other Western Pacific populations in the early-mid twentieth century (Spencer et al. 1986, Spencer et al. 2015). The most convincing instance of a causal link between ALS and toxin exposure is the case of the Chamorro population, in which L-BMAA has been identified in the brain tissue of patients who died of ALS-PDC (Murch et al. 2004). Indeed, since L-BMAA could be biomagnified/bioaccumulated in animals, such as flying foxes who fed on cycad seed, Chamorros who consumed these animals took a high dosage of the toxin (Cox et al. 2003). The extinction of both cycads and flying foxes correlated with a substantial decline in the prevalence of neurodegenerative disease on the island (Bradley and Mash 2009, Plato et al. 2003). The L-BMAA toxic effects may not be restricted just to Guam. Diverse taxa of ubiquitous cyanobacteria capable of producing L-BMAA are found in eutrophic freshwater and marine water bodies, and are an increasing environmental hazard also in several other parts of the world (Cox et al. 2005). These toxin-producing cyanobacteria are often noticed when they form

"blooms" on the surface of water bodies. However, the effects of acute exposure to L-BMAA through direct contact with blooms is still unknown.

The main observations about the mode of action of L-BMAA have emerged from *in vitro* toxicological studies carried out on different cellular models. L-BMAA has been found to over-activate glutamate receptors, leading to an excessive release of the neurotransmitter glutamate (Chiu et al. 2013).

The consequent over-stimulation of postsynaptic neurons, termed "glutamate excitotoxicity", found in ALS pathology, typically leads to neuronal death. Another hypothesis regarding the mechanism by which L-BMAA could exert its neurotoxic effects is its putative ability to get incorporated into proteins during protein synthesis (Glover et al. 2014, Karlsson et al. 2014). Indeed, once L-BMAA has been transported into neuronal cells by cystine/glutamate antiporter, "system xc^-", it is misincorporated in protein in exchange for L-serine (Albano et al. 2017). In addition, protein incorporation of L-BMAA could result in protein-refolding defects and a subsequent accumulation of misfolded proteins (Dunlop et al. 2013) associated with a massive Ca^{2+} entry leading to cell stress and to apoptotic cell death (Okle et al. 2013). Currently, although L-BMAA is associated with neurotoxicity, the relationship between cyanotoxins exposure and the development of neurodegenerative diseases remains unclear and further investigations are needed to determine potential environmental factors predisposing to neurodegeneration, and in particular ALS pathogenesis.

2. AIMS OF THE STUDY

It is well established that HIPK2 plays relevant role in many signaling and proliferation pathways through a multitude of different interactors acting as a pro-survival factor as well as an inductor of cell death in a cellular context-dependent manner. Given the involvement of HIPK2 in cell survival, it is not surprising that disruption of its physiological activity contributes to the development of several diseases ranging from cancer to neurodegeneration. In particular, neuronal cells are more sensitive to alteration of the cell signaling transducing machinery and HIPK2 protein appears to be critical for ensuring the correct homeostasis of nervous system.

Previously, in our lab, it has been generated a mouse model lacking *Hipk2* (HIPK2-KO): we found that HIPK2 loss leads to a strong reduction in number of Purkinje neurons due to the activation of a selective apoptotic process associated with a compromised proteasomal function followed by an unpredicted accumulation of ubiquitinated proteins. The alteration of protein homeostasis through the impairment of the ubiquitin-proteasome pathway plays a critical role in the pathogenesis of several neurodegenerative diseases. Indeed, accumulation of abnormally folded proteins is a distinct hallmark of Alzheimer's, Frontotemporal Dementia or Amyotrophic Lateral Sclerosis diseases. Since HIPK2-KO mice show motor symptoms and phenotypic features compatible with an ataxic-like phenotype overlapping ALS, we asked whether the lack of HIPK2 could contribute to the pathogenesis of ALS disease.

Therefore, the aim of my PhD project was to investigate HIPK2 involvement in the pathogenesis of neurodegeneration and to elucidate its potential implication in ALS pathology.

3. MATERIALS AND METHODS

3.1 Animals and Ethic Statement

HIPK2-KO mice were generated in a C57BL6/Sv129J genetic background through homologous recombination by eliminating the exon 3 coding for the entire catalytic domain at the N-terminus of the molecule, as previously reported (Valente et al. 2015). The study was conducted on homozygous mice (*Hipk2* $^{-/-}$) obtained by mating heterozygous mice (*Hipk2* $^{+/-}$). C57BL6/Sv129J WT and HIPK2-KO mice were housed under identical conditions of temperature (21 ± 1 °C), humidity (60 ± 5%), and light/dark cycle, with free access to normal mouse chow. To assess the genotype of the mice, DNA was extracted from a small piece of mouse tail as previously described (Gerlini et. al. 2019). The PCR products of *Hipk2* were separated on a 2% agarose gel. The following primers were used:

Hipk2-Fw 5′-TAGTACCCAGGTGAACCTTGGAGT-3′

*Hipk2*WT-Re 5′-GCTTCTCTCAAACTAAAGACCACGC-3′

*Hipk2*KO-Re 5′-CAAAGGGTCTTTGAGCACCAGA-3′

Each experimental protocol was approved by the Animal Care Committee of the "Federico II" University of Naples, and in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

3.2 L-BMAA administration

L-BMAA treatment was carried out by intraperitoneal injection. To preserve the normal development of the nervous system, we do not begin treatment before postnatal week 10 (weighing approximately 25g). The doses administered were 1-30-90 mg/kg daily over 5 consecutive days. L-BMAA (Sigma-Aldrich Cat. #B107) was dissolved in physiological saline solution. Overall, 48 mice were used, 38 WT mice and 10 KO mice were included in the

experimental groups for L-BMAA administration. During treatment, both mice groups were weighed to monitor health.

3.3 Motor behavioral tests

1. **Rotarod test**

Motor coordination and balance was assessed using a five-station mouse rotarod apparatus (Ugo Basile; Milan, Italy). In each station, the rod was 6 cm in length and 3 cm in diameter. Mice were trained to maintain balance at increasing speed up to a constant speed of 14 rpm for three consecutive trials. The test sessions were conducted by one rotarod trial administered once a week. In this session, the speed of rotation was increased from 4 to 14 rpm over 60 s. Mice had three trials on the rod, and the latencies to fall were measured once a week and then averaged. The maximum latency of 60 s was assigned to the mice that did not fall at all.

1. **Tail suspension test**

Hindlimbs clasping was assessed suspending mice by the base of the tail and monitoring posture of hindlimbs for three minutes. The test was performed once a week. Hindlimbs clasping was scored when the animal dragged its hindlimbs.

3.4 Cell cultures

Human neuroblastoma SH-SY5Y cells were grown in RPMI (Euroclone, Cat. #ECB2000) supplemented with 10% Fetal Bovin Serum (Cytiva HyClone™, Cat. #SH30070.03IH25-40) and L-glutamine (Euroclone, Cat. #ECB3000). Cells were maintained at 37 °C in a saturated humidity atmosphere containing 95% air and 5% CO_2.

3.5 Transfections and plasmids

Cells were transfected with plasmids by X-tremeGENE™ 9 DNA Transfection Reagent (Roche, Cat. #XTG9-RO) according to the manufacturer's instructions. Cells were transiently and stably transfected with the following plasmids: pCEFL-HA-WT or K221R/HIPK2; pRETROSUPER-shHIPK2 (kindly provided by Dr Rinaldo, IBPM CNR Rome, Italy).

3.6 RNA interference, RNA extraction and quantitative Real-Time PCR

RNA interference was obtained by specific siRNAs for HIPK2 (Life Technologies-Invitrogen siRNAs HSS120797, HSS120796, HSS178947, Cat. #5258224) using Lipofectamine RNAiMAX (Thermo Fisher Scientific, Cat. #13778100) according to manufacturer's instructions. As negative control, Ambion™ In Vivo Negative Control #1 siRNA (Invitrogen, Cat. #4457287) was used. Total RNA was isolated using TRI-reagent solution (Sigma-Aldrich, Cat. #T9424). Reverse transcription was performed using QuantiTect Rev. Transcription Kit (Qiagen, Cat. #205311) according to standard procedures. qRT-PCR analysis was performed using the FluoCycle Master Mix (Euroclone, Cat. # ERD002100BIM) with following primers:

human/mouse *Hipk2*-Fw 5'-GAGACACAGGCTCAAGATGG-3′
human/mouse *Hipk2*-Re 5'- TCTGCTCGTAAGGTAGGCTT -3'
human *TARDBP*-Fw 5'-CAGACAGCCTGATGTGCAGT-3'
human *TARDBP*-Re 5'-CAGTTGTTTTCCAGGGGAGA -3'
human *Actin*-Fw 5'- CTAAGGCCAACCGTGAAAAG -3'
human *Actin*-Re 5'- ACCAGAGGCA TACAGGGACA -3'

The relative expression levels were calculated by using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

3.7 Cell viability assay

SH-SY5Y cells were seeded in 24-well plates at $5x10^4$ cells per well, then interfered as above described. Cell viability of cells was quantified by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Invitrogen. Cat. #M6494) reagent MTT solution (0.5 mg/ml) for 1 h at 37◦C under 5% CO2. Cell viability was evaluated by measuring absorbance at 595 nm with a Microplate Reader (Biotek Synergy Microplate Reader), according to the MTT manufacturer's protocol.

3.8 Nuclear/ Cytoplasmic Fractionation

SH-SY5Y cells nuclear/cytoplasmic fractionation was obtained using ReadyPrep™ Protein Extraction Kit (Bio-Rad, Cat. #1632089) according to manufacturer instructions. Briefly, SH-SY5Y cells grown on petri dishes were scraped with CPEB buffer, leaved on ice for 30 min and centrifugated at 1000 *g* for 30 min. The supernatant containing the cytoplasmic fraction was collected. The pellet was solved in PSB buffer, vortexed and centrifuged at 12000 *g* for 20 min and the supernatant containing the nuclear fraction collected.

3.9 Western blotting and antibodies

Different brain sections were homogenized and total protein extracts were prepared with lysis buffer RIPA as previously described (Gerlini et al. 2019). Total protein extracts were separated by SDS-PAGE and transferred onto nitrocellulose transfer membranes (Perkin Elmer, Cat. #NBA085C001EA). Membranes were blocked with 5% BSA (bovine serum albumin protein in TBS 1% buffer, 0.02% sodium azide) and incubated with antibodies at the appropriate dilutions. The filters were incubated with horseradish peroxidase-conjugated secondary Antibodies, and the signals were detected with ECL (Thermo Fisher Scientific, Cat. #32106). The antibodies used for western blotting were as follows: rat-monoclonal anti-HIPK2 (kindly provided by Dr Schmitz, University of Giessen, Germany), polyclonal anti-TDP-43 (Proteintech, Cat. #10782-2-

AP), polyclonal anti-calnexin (Enzo Lifescience, Cat. #ADI-SPA-860-F), monoclonal anti-Sp3 (Santa Cruz, Cat. #365038), monoclonal anti-GAPDH (Santa Cruz, Cat. #32233).

3.10 Fluorescence microscopy

Animals were anesthetized and transcardially perfused with saline solution containing 0.01 ml heparin, followed by 4% paraformaldehyde in 0.1 mol/l PBS saline solution. Brains and spinal cord were processed as previously described (Anzilotti et al.2018). The sections were incubated with the following primary antibodies: monoclonal anti-HIPK2 (kindly provided by Dr Schmitz, University of Giessen, Germany), polyclonal anti-TDP-43 (Proteintech, Cat. #10782-2-AP), polyclonal anti-Ubiquitin (Santa Cruz Biotechnology, Cat. #sc-166553). The sections were then incubated with the corresponding florescent-labeled secondary antibodies, Alexa 488/Alexa 594 conjugated antimouse/antirabbit IgGs (Molecular Probes, Invitrogen). Nuclei were counterstained with Hoechst (Sigma-Aldrich, Cat. #33258). Three sections from each mouse were analyzed, with $n = 3$ mice per experimental group. Quantification of TDP-43, Ubiquitin, and HIPK2 fluorescence intensity on tissue sections, was quantified in terms of pixel intensity value by using the NIH image software. Cells stained with TDP-43 and HIK2 antibodies were fixed in PFA 4% and then processed as previously described (Fasano et al. 2018). Images were collected using confocal laser scanning microscope LSM 700. Fluorescence images were acquired using the ZEN Black software at a resolution of 1024 × 1024 pixels, with the confocal pinhole set to one Airy unit and then saved in the TIFF format.

3.11 Morphology, Histochemistry and Immunohistochemistry

For each animal, tissue sections were stained with hematoxylin and eosin (H&E) for a basic morphological evaluation. Images were acquired under a Nikon E600 optical microscope. Frozen sections of skeletal muscle were

subjected to a standard panel of histochemical staining, including hematoxylin and eosin (H&E), Engel's trichrome (ET), NADH-tetrazolium reductase (NADH-TR), succinate dehydrogenase (SDH), cytochrome oxidase (COX), non-specific esterase (NSE) as previously described (De Biase et al. 2021). Standard 3,3′-diaminobenzidine (DAB) staining was employed on sagittal spinal cord serial sections using antibody directed against TDP-43 protein. At least five fields at 40x magnification were evaluated for each section under an optical microscope (Nikon E600).

3.11 Statistical analysis

Data are expressed as mean ± SD. Data were analyzed using a two-sided unpaired Student's *t* test for western blotting, immunofluorescence, and real-time PCR analysis. Values of $P < 0.05$ were considered statistically significant. All animal experiments included at least three biological replicates. Data were evaluated as means ± SEM. Statistically significant differences were determined by two-way ANOVA followed by Bonferroni post-hoc used for motor performances test and body weight analysis.

4. RESULTS

4.1 *Hipk2* deletion causes neuronal alterations and mild myopathy in mice

Recently, our research group analyzed the role of HIPK2 in nervous system physiology and, in particular, in cerebellar homeostasis. We demonstrated that HIPK2-KO (KO) mice show both morphological and functional cerebellar alterations that become more evident with aging (Anzilotti et al. 2015). To better characterize the role of HIPK2 deletion on the Central Nervous System (CNS), we performed morphological analysis on several sections of nervous tissues from Wild-Type (WT) and KO mice at different ages: 4, 12 and 18 months of age. By histological analysis performed by hematoxilyn and eosin staining, we found that the organs structure and development in KO mice are overall normal throughout the CNS, as previously reported (Anzilotti et al. 2015, Gerlini et al. 2019). However, we found mild to moderate anomalies in some areas of the brain and in the spinal cord of KO mice at 12 and 18 months of age. Indeed, as shown in Figure 6, we found: scattered neuronal loss in *cornu ammonis* (CA) of hippocampus (Figure 6 D) with several shrunken, hypereosinophilic, and pyknotic neurons; mild vacuolation of the neuropil, a large number of clearly defined and empty vacuoles of diverse size, and gliosis in cerebellar white matter (Figure 6 B); swollen neurons, vacuolated or with dispersed Nissl substance, surrounded by satellite cells in the ventral horns of the spinal cord (SC) (Figure 6 F). The brain of age-matched WT mice did not show any significant or specific histological alterations (Figures 6 A, C, E). All these alterations, in particular the presence of hypereosinophilic neurons associated with satellitosis, are indicative of diffuse neuronal necrosis compatible with an end-stage cellular response to irreversible injury, such as ischemia or metabolic dysfunction. Noteworthy, these alterations became remarkable in “old” KO mice (18-month-old), while they are absent in 4-month-old mice (not shown), and barely noticeable in 12-month-old mice (not shown) strongly suggesting that KO mice exhibit a neurodegenerative phenotype which

shows up in an age-dependent manner.

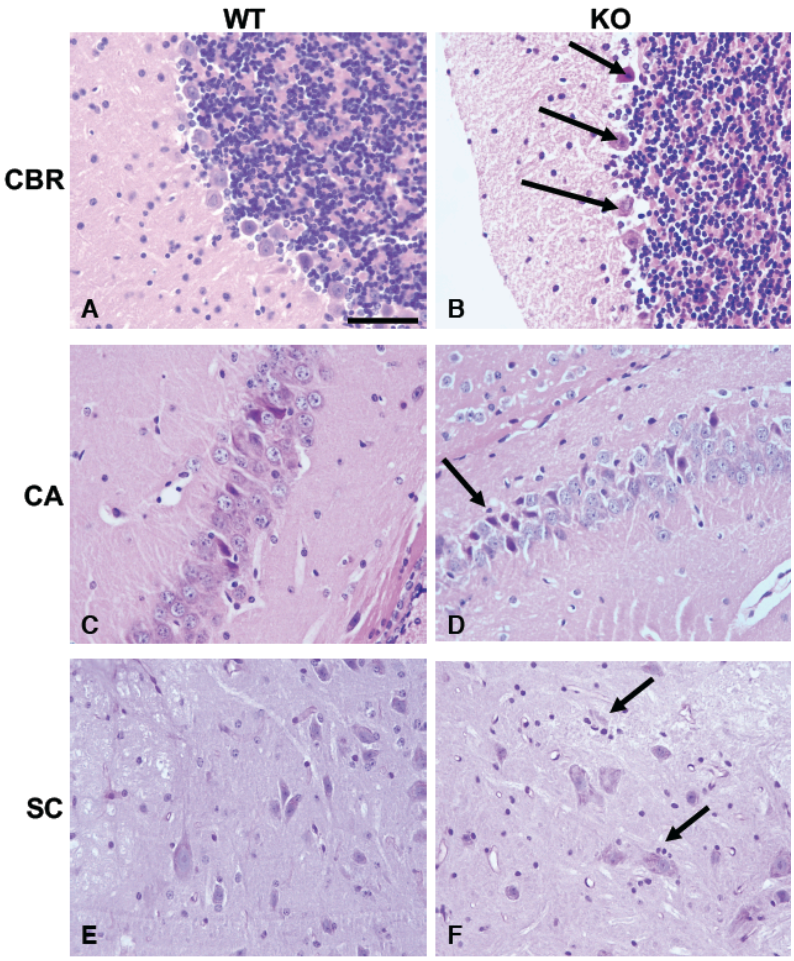

Figure 6. HIPK2-KO mice show neuronal alterations.
Representative images of H&E staining of cerebellum (CBR) (**A,B**), hippocampus (*cornu ammonis*) (CA) (**C,D**), spinal cord (SC) (**E,F**) from 18-month-old WT (left panel) and KO (right panel) mice. The arrows indicate neuronal loss (**B,D**) and satellite cells (**F**). Three sections from each mouse were analyzed, with $n = 10$ mice per experimental group. Original magnification, 40X. Scale bar = 50 μm. *From De Biase et al. 2021.*

On the basis of these observation and our previously data demonstrating the severe motor/balance impairment showed by KO mice (Anzilotti et al. 2015), we investigated whether some alteration could be present also at muscular level. Therefore, performing histological and histoenzymatic examinations on several muscle section from 18-month-old mice we found several muscular defects in KO mice respect to WT counterpart. In particular, by hematoxylin and eosin staining, we found different size of both angular and non-angular muscle fibers showing atrophy, several ring fibers with sarcoplasmic rimmed vacuoles and a variable increase of endomysium of connective tissue in KO skeletal muscle (Figure 7 B), whereas age-matching WT counterpart presented a normal morphology (Figure 7 A). Moreover, Engel's trichrome (ET) staining showed the presence of subsarcolemmal red deposits (ragged red fibers) in approximately 10% to 30% of the fibers (Figure 7 D) in KO skeletal muscle but not in the WT counterpart (Figure 7 C).

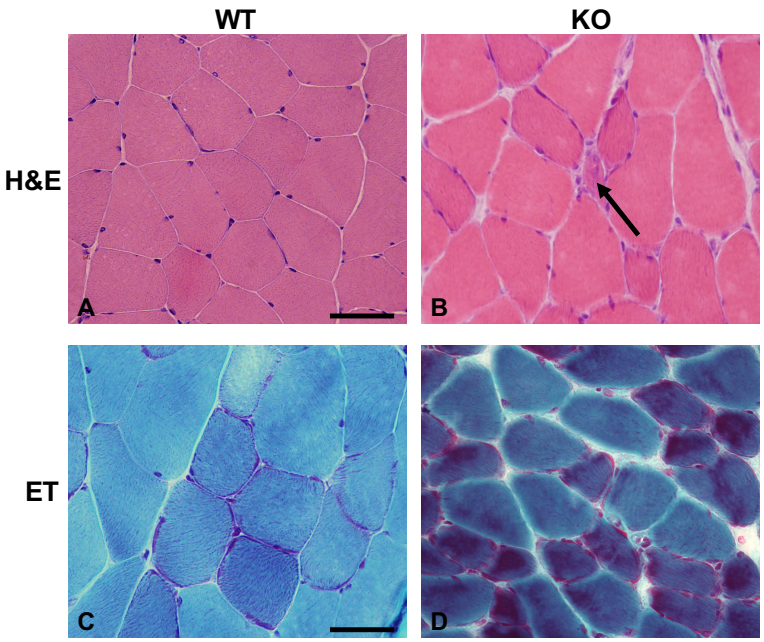

Figure 7. HIPK2-KO mice show skeletal muscle abnormalities.
Representative hematoxylin and eosin (H&E) and Engel's trichrome (ET) staining of sections of skeletal muscle from 18-month-old WT (**A**,**C**) and KO (**B**,**D**) mice. The arrow indicates an atrophic angular fiber (**B**). Three sections from each mouse were analyzed, with $n = 10$ mice per experimental group. Original magnification, 40X. Scale bar=20 μm. *From De Biase et al. 2021*

Furthermore, to assess whether these muscular abnormalities were associated with an impaired cell metabolism, we evaluated the distribution and activity of mitochondria in skeletal muscle cells by performing specific histoenzymatic staining. Indeed, NADH-tetrazolium reductase (NADH-TR), succinate dehydrogenase (SDH) and cytochrome oxidase (COX) staining performed on muscle sections from 18-month-old mice revealed the presence of mitochondria accumulation upon *Hikp2* loss in approximately 10% of the total fibers. In fact, we found positive sarcoplasmic and subsarcolemmal deposits (Figures 8 B, D, F) and negative cytoplasmic core (Figure 8 F) in KO mice. Notably, in one mouse, we also found several ring fibers suggestive of chronic myopathy (Figure 8 B, D). Moreover, neuromuscular junctions appeared moderately hypertrophic, bizarre in shape, and occasionally fragmented or moderately thicker than normal in KO mice, as revealed by non-specific esterase (NSE) staining (Figure 8 H). WT mice did not show any relevant pathological changes, either in mitochondrial activity and distribution or in shape and size of neuromuscular junctions (Figure 8 left panel).

The presence of sarcoplasmic inclusions, deficient mitochondrial proliferation, and morphological alterations of the neuromuscular junctions found in KO mice is strongly suggestive that loss of *Hipk2* is responsible for the manifestation of a myopathic phenotype in adult KO mice.

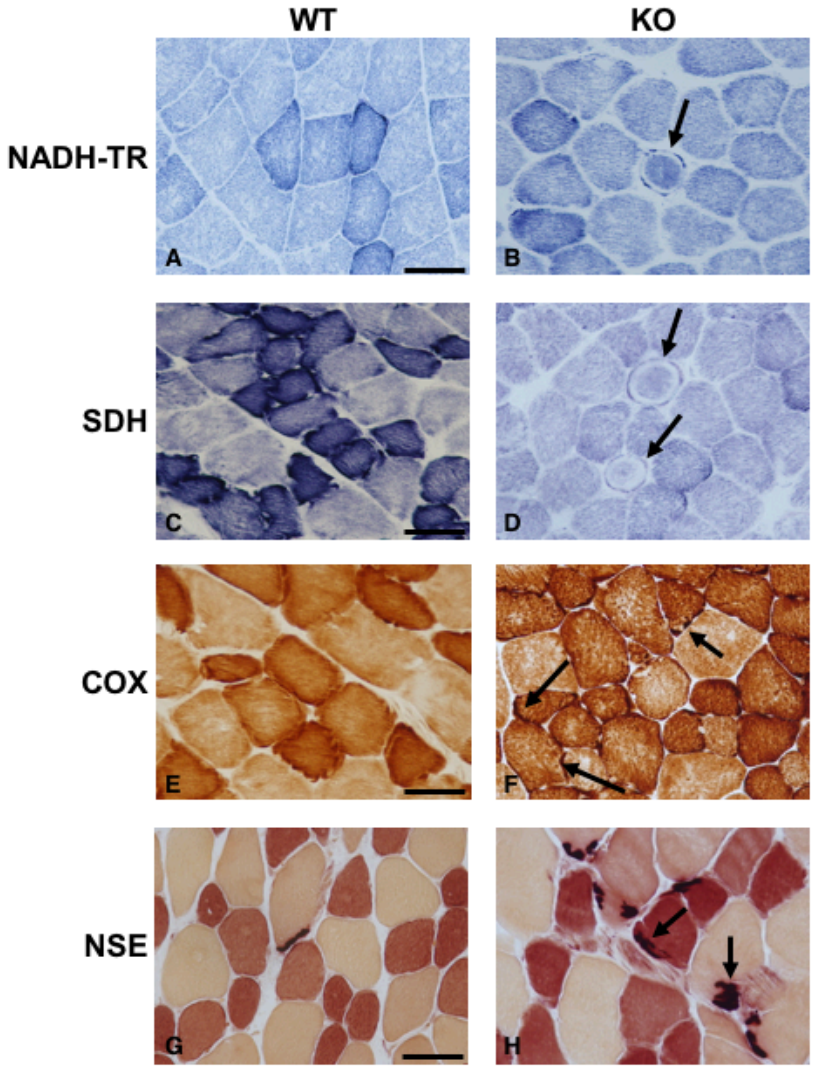

Figure 8. HIPK2 lack induces a mild myopathic phenotype in mice.
Histochemical analysis of NADH-TR (**A**,**B**), SDH (**C,D**), COX (**E,F**), NSE (**G,H**) on skeletal muscle sections from 18-month-old WT and KO mice are shown. Arrows in (**B,D**) indicate ring fibers with subsarcolemmal positive deposits; arrows in (**F**) indicate negative cytoplasmic core; arrows in (**H**) indicate hypertrophic and fragmented neuromuscular junctions. Original magnification, 40X. Scale bar =20 μm. Three sections from each mouse were analyzed, with $n = 10$ mice per experimental group. *From De Biase et al. 2021.*

6. CONCLUSIONS

The data we collected enlightened a crucial role of HIPK2 in the homeostasis of motor neurons. Its depletion may be involved in the pathogenesis of ALS disease by inducing an altered neuromuscular physiology and motor behavioral abnormalities associated with the dysregulation of TDP-43 protein homeostasis, which represent one of the main pathological hallmarks of ALS (Neumann et al. 2006, Mori et al. 2008, Ling et al. 2013).

Although ALS was first described in 1869 and the first disease-associated gene was discovered almost 20 years ago, the disease etiology is still not fully understood and treatment options are limited to Riluzole and Edavarone approved by the US Food and Drug Administration (Rowland et al. 1994, Study Writing Group 2020). Our study uncovered novel aspects of HIPK2 activity involved in neurodegeneration, suggesting its central role in ALS pathogenesis through the regulation of TDP-43 protein accumulation. These results would implicate that downregulation of HIPK2 may be a cause of MNs vulnerability. Hence, restoring of HIPK2 activity may represent a novel strategy to enhance survival of motor neurons for therapeutic intervention in ALS pathology.

7. LIST OF PUBLICATIONS

1. Fusco F, **Valente V**, Fergola D, Pescatore A, Lioi MB, Ursini MV. The Incontinentia Pigmenti Genetic Biobank: study design and cohort profile to facilitate research into a rare disease worldwide. Eur J Hum Genet. 2019 Oct;27(10):1509-1518.

2. Gerlini R, Amendola E, Conte A, **Valente V**, Tornincasa M, Credendino SC, Cammarota F, Gentile C, Di Guida L, Paladino S, De Vita G, Fusco A, Pierantoni GM. Double knock-out of Hmga1 and Hipk2 genes causes perinatal death associated to respiratory distress and thyroid abnormalities in mice. Cell Death Dis. 2019 Oct 3;10(10):747.

3. Cammarota F, Conte A, Aversano A, Muto P, Ametrano G, Riccio P, Turano M, **Valente V**, Delrio P, Izzo P, Pierantoni GM, De Rosa M. Lithium chloride increases sensitivity to photon irradiation treatment in primary mesenchymal colon cancer cells. Mol Med Rep. 2020 Mar;21(3):1501-1508.

4. De Biase D*, **Valente V***, Conte A, Cammarota F, Boccella N, D'Esposito L, d'Aquino I, Paciello O, Paladino S, Pierantoni GM. Phenotypic Effects of Homeodomain-Interacting Protein Kinase 2 Deletion in Mice. Int J Mol Sci. 2021 Aug 2;22(15):8294. (*Co-first authors)

Manuscripts in preparation

1. De Rosa L, Fasano D, and **Valente V** et al.
"*Alteration of endosomal trafficking is associated with trisomy for SYNJ".*

2. Zerillo L, Fasano D, **Valente V**, De Rosa A et al.
"Dysregulation of autophagy has a role in Synj1-associated early-onset parkinsonism".

3. Fasano D, **Valente V**, De Rosa L et al.
"FIG4 regulates the homeostasis of endosomal compartments in different cell types".

4. **Valente V**, Picciocchi M, De Rosa L et al.
"Analysis of dominant Erlin 2 mutation in spastic paraplegia pathogenesis".

5. **Valente V**, Paladino S, Conte A and Pierantoni GM
"HIPK proteins in cancer biology". Invited by Cellular Signaling.

8. REFERENCES

- Anzilotti, S., Tornincasa, M., Gerlini, R., Conte, A., Brancaccio, P., Cuomo, O., Bianco, G., Fusco, A., Annunziato, L., Pignataro, G., & Pierantoni, G. M. (2015). Genetic ablation of homeodomain-interacting protein kinase 2 selectively induces apoptosis of cerebellar Purkinje cells during adulthood and generates an ataxic-like phenotype. Cell death & disease vol. 6,12 e2004.
- Arai, T., Hasegawa, M., Akiyama, H., Ikeda, K., Nonaka, T., Mori, H., Mann, D., Tsuchiya, K., Yoshida, M., Hashizume, Y., & Oda, T. (2006). TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Biochemical and Biophysical Research Communications, 351(3), 602–611.
- Ayala, Y. M., Pantano, S., D'Ambrogio, A., Buratti, E., Brindisi, A., Marchetti, C., Romano, M., & Baralle, F. E. (2005). Human, Drosophila, and C. elegans TDP43: Nucleic acid binding properties and splicing regulatory function. Journal of Molecular Biology, 348(3), 575–588.
- Blaquiere, J. A., & Verheyen, E. M. (2017). Homeodomain-Interacting Protein Kinases: Diverse and Complex Roles in Development and Disease. In Current Topics in Developmental Biology (Vol. 123, pp. 73–103).
- Blokhuis, A. M., Groen, E. J. N., Koppers, M., van den Berg, L. H., & Pasterkamp, R. J. (2013a). Protein aggregation in amyotrophic lateral sclerosis. In Acta Neuropathologica (Vol. 125, Issue 6, pp. 777–794).
- Blokhuis, A. M., Groen, E. J. N., Koppers, M., van den Berg, L. H., & Pasterkamp, R. J. (2013b). Protein aggregation in amyotrophic lateral sclerosis. In Acta Neuropathologica (Vol. 125, Issue 6, pp. 777–794).
- Blokhuis, A. M., Groen, E. J. N., Koppers, M., van den Berg, L. H., & Pasterkamp, R. J. (2013a). Protein aggregation in amyotrophic lateral sclerosis. In Acta Neuropathologica (Vol. 125, Issue 6, pp. 777–794).
- Bradley, W. G., & Mash, D. C. (2009). Beyond Guam: The cyanobacteria/BMAA hypothesis of the cause of ALS and other neurodegenerative diseases. Amyotrophic Lateral Sclerosis, 10(SUPPL. 2), 7–20.
- Brettschneider, J., del Tredici, K., Toledo, J. B., Robinson, J. L., Irwin, D. J., Grossman, M., Suh, E., van Deerlin, V. M., Wood, E. M., Baek, Y., Kwong, L., Lee, E. B., Elman, L., McCluskey, L., Fang, L., Feldengut, S., Ludolph, A. C., Lee, V. M. Y., Braak, H., & Trojanowski, J. Q. (2013).

Stages of pTDP-43 pathology in amyotrophic lateral sclerosis. Annals of Neurology, 74(1), 20–38.

- Byrne, S., Walsh, C., Lynch, C., Bede, P., Elamin, M., Kenna, K., McLaughlin, R., & Hardiman, O. (2011). Rate of familial amyotrophic lateral sclerosis: A systematic review and meta-analysis. In Journal of Neurology, Neurosurgery and Psychiatry (Vol. 82, Issue 6, pp. 623–627).
- Calzado, M. A., Renner, F., Roscic, A., & Schmitz, M. L. (2007). HIPK2: A versatile switchboard regulating the transcription machinery and cell death. In Cell Cycle (Vol. 6, Issue 2, pp. 139–143). Taylor and Francis Inc.
- Chalazonitis, A., Tang, A. A., Shang, Y., Pham, T. D., Hsieh, I., Setlik, W., Gershon, M. D., & Huang, E. J. (2011). Homeodomain interacting protein kinase 2 regulates postnatal development of enteric dopaminergic neurons and glia via BMP signaling. Journal of Neuroscience, 31(39), 13746–13757.
- Chinnadurai, G. (2009). The transcriptional corepressor CtBP: A foe of multiple tumor suppressors. In Cancer Research (Vol. 69, Issue 3, pp. 731–734).
- Chiò, A., Logroscino, G., Traynor, B. J., Collins, J., Simeone, J. C., Goldstein, L. A., & White, L. A. (2013). Global epidemiology of amyotrophic lateral sclerosis: A systematic review of the published literature. In Neuroepidemiology (Vol. 41, Issue 2, pp. 118–130).
- Conte, A., & Pierantoni, G. M. (2018). Update on the Regulation of HIPK1, HIPK2 and HIPK3 Protein Kinases by microRNAs. MicroRNA, 7(3), 178–186.
- D'Orazi, G., Cecchinelli, B., Bruno, T., Manni, I., Higashimoto, Y., Saito, S., Gostissa, M., Coen, S., Marchetti, A., del Sal, G., Piaggio, G., Fanciulli, M., Appella, E., & Soddu, S. (2002). Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser 46 and mediates apoptosis. Nature Cell Biology, 4(1), 11–20.
- Dayton, R. D., Gitcho, M. A., Orchard, E. A., Wilson, J. D., Wang, D. B., Cain, C. D., Johnson, J. A., Zhang, Y. J., Petrucelli, L., Mathis, J. M., & Klein, R. L. (2013). Selective forelimb impairment in rats expressing a pathological TDP-43 25 kDa C-terminal fragment to mimic amyotrophic lateral sclerosis. Molecular Therapy, 21(7), 1324–1334.
- De Biase, D., Valente, V., Conte, A., Cammarota, F., Boccella, N., D'Esposito, L., d'Aquino, I., Paciello, O., Paladino, S., & Pierantoni, G. M. (2021). Phenotypic Effects of Homeodomain-Interacting Protein Kinase 2 Deletion in Mice. International journal of molecular sciences, 22(15), 8294.

- Designed Research; G, E. J. H. W. (2007). HIPK2 represses-catenin-mediated transcription, epidermal stem cell expansion, and skin tumorigenesis (Vol. 104, Issue 32).
- Dorazi, G., Rinaldo, C., & Soddu, S. (2012). Updates on HIPK2: A resourceful oncosuppressor for clearing cancer. In Journal of Experimental and Clinical Cancer Research (Vol. 31, Issue 1).
- Ewis, L., Owland, P. R., & Hneider, E. A. S. (2001). Medical Progress 1688 ·. In N Engl J Med (Vol. 344, Issue 22).
- Fan, Y., Wang, N., Chuang, P., & He, J. C. (2014). Role of HIPK2 in kidney fibrosis. In Kidney International Supplements (Vol. 4, Issue 1, pp. 97–101). Nature Publishing Group.
- Fritsche, M. K., Metzler, V., Becker, K., Plettenberg, C., Heiser, C., Hofauer, B., & Knopf, A. (2015.). Cisplatin fails to induce puma mediated apoptosis in mucosal melanomas. In Oncotarget (Vol. 6, Issue 12).
- Gerlini, R., Amendola, E., Conte, A., Valente, V., Tornincasa, M., Credendino, S. C., Cammarota, F., Gentile, C., di Guida, L., Paladino, S., de Vita, G., Pierantoni, G. M., & Fusco, A. (2019). Double knock-out of Hmga1 and Hipk2 genes causes perinatal death associated to respiratory distress and thyroid abnormalities in mice. Cell Death and Disease, 10(10).
- Gresko, E., Roscic, A., Ritterhoff, S., Vichalkovski, A., del Sal, G., & Schmitz, M. L. (2006). Autoregulatory control of the p53 response by caspase-mediated processing of HIPK2. EMBO Journal, 25(9), 1883–1894.
- Guo, Y., Sui, J. Y., Kim, K., Zhang, Z., Qu, X. A., Nam, Y. J., Willette, R. N., Barnett, J. v., Knollmann, B. C., Force, T., & Lal, H. (2019). Cardiomyocyte Homeodomain-Interacting Protein Kinase 2 Maintains Basal Cardiac Function via Extracellular Signal-Regulated Kinase Signaling. Circulation, 140(22), 1820–1833.
- Harada, J., Kokura, K., Kanei-Ishii, C., Nomura, T., Khan, M. M., Kim, Y., & Ishii, S. (2003). Requirement of the co-repressor homeodomain-interacting protein kinase 2 for Ski-mediated inhibition of bone morphogenetic protein-induced transcriptional activation. Journal of Biological Chemistry, 278(40), 38998–39005.
- Hardiman, O., Al-Chalabi, A., Chio, A., Corr, E. M., Logroscino, G., Robberecht, W., Shaw, P. J., Simmons, Z., & van den Berg, L. H. (2017). Amyotrophic lateral sclerosis. In Nature Reviews Disease Primers (Vol. 3).
- Hikasa, H., Ezan, J., Itoh, K., Li, X., Klymkowsky, M. W., & Sokol, S. Y. (2010). Regulation of TCF3 by Wnt-dependent phosphorylation during vertebrate axis specification. Developmental Cell, 19(4), 521–532.

- Hofmann, T. G., Jaffray, E., Stollberg, N., Hay, R. T., & Will, H. (2005). Regulation of homeodomain-interacting protein kinase 2 (HIPK2) effector function through dynamic small ubiquitin-related modifier-1 (SUMO-1) modification. Journal of Biological Chemistry, 280(32), 29224–29232. https://doi.org/10.1074/jbc.M503921200
- Hofmann, T. G., Mincheva, A., Lichter, P., Dröge, W., & Lienhard Schmitz, M. (2000). Human homeodomain-interacting protein kinase-2 (HIPK2) is a member of the DYRK family of protein kinases and maps to chromosome 7q32-q34. Biochimie vol. 82,12 (2000): 1123-7. Hofmann, T. G., Möller, A., Sirma, H., Zentgraf, H., Taya, Y., Dröge, W., Will, H., & Lienhard Schmitz, M. (2002). Regulation of p53 activity by its interaction with homeodomain-interacting protein kinase-2. Nature Cell Biology, 4(1), 1–10.
- Iacovelli, S., Ciuffini, L., Lazzari, C., Bracaglia, G., Rinaldo, C., Prodosmo, A., Bartolazzi, A., Sacchi, A., & Soddu, S. (2009). HIPK2 is involved in cell proliferation and its suppression promotes growth arrest independently of DNA damage. Cell Proliferation, 42(3), 373–384.
- Iguchi, Y., Katsuno, M., Niwa, J. I., Takagi, S., Ishigaki, S., Ikenaka, K., Kawai, K., Watanabe, H., Yamanaka, K., Takahashi, R., Misawa, H., Sasaki, S., Tanaka, F., & Sobue, G. (2013). Loss of TDP-43 causes age-dependent progressive motor neuron degeneration. Brain, 136(5), 1371–1382.
- Ikenaka, K., Katsuno, M., Kawai, K., Ishigaki, S., Tanaka, F., & Sobue, G. (2012). Disruption of axonal transport in motor neuron diseases. International Journal of Molecular Sciences, 13(1), 1225–1238.
- Inoue, T., Kagawa, T., Inoue-Mochita, M., Isono, K., Ohtsu, N., Nobuhisa, I., Fukushima, M., Tanihara, H., & Taga, T. (2010). Involvement of the Hipk family in regulation of eyeball size, lens formation and retinal morphogenesis. FEBS Letters, 584(14), 3233–3238.
- Inoue, T., Kagawa, T., Inoue-Mochita, M., Isono, K., Ohtsu, N., Nobuhisa, I., Fukushima, M., Tanihara, H., & Taga, T. (2010). Involvement of the Hipk family in regulation of eyeball size, lens formation and retinal morphogenesis. FEBS Letters, 584(14), 3233–3238.
- Isono, K., Nemoto, K., Li, Y., Takada, Y., Suzuki, R., Katsuki, M., Nakagawara, A., & Koseki, H. (2006). Overlapping Roles for Homeodomain-Interacting Protein Kinases Hipk1 and Hipk2 in the Mediation of Cell Growth in Response to Morphogenetic and Genotoxic Signals. Molecular and Cellular Biology, 26(7), 2758–2771.

- Jackson, K. L., Dayton, R. D., Orchard, E. A., Ju, S., Ringe, D., Petsko, G. A., Maquat, L. E., & Klein, R. L. (2015). Preservation of forelimb function by UPF1 gene therapy in a rat model of TDP-43-induced motor paralysis. Gene Therapy, 22(1), 20–28.
- Jonasson, S., Eriksson, J., Berntzon, L., Spáčcil, Z., Ilag, L. L., Ronnevi, L. O., Rasmussen, U., & Bergman, B. (2010). Transfer of a cyanobacterial neurotoxin within a temperate aquatic ecosystem suggests pathways for human exposure. Proceedings of the National Academy of Sciences of the United States of America, 107(20), 9252–9257.
- following neonatal administration in rats. Toxicology Letters, 226(1), 1–5.
- Karlsson, O., Jiang, L., Andersson, M., Ilag, L. L., & Brittebo, E. B. (2014). Protein association of the neurotoxin and non-protein amino acid BMAA (β-N-methylamino-l-alanine) in the liver and brain following neonatal administration in rats. Toxicology Letters, 226(1), 1–5.
- Kim, Y. H., Choi, Y., & Kim, Y. (n.d.). Covalent modification of the homeodomain-interacting protein kinase 2 (HIPK2) by the ubiquitin-like protein SUMO-1. Proceedings of the National Academy of Sciences of the United States of America vol. 96,22 (1999): 12350-5.
- Kondo, S., Lu, Y., Debbas, M., Lin, A. W., Sarosi, I., Itie, A., Wakeham, A., Tuan, J., Saris, C., Elliott, G., Ma, W., Benchimol, S., Lowe, S. W., Wah Mak, T., & Thukral, S. K. (n.d.). Characterization of cells and gene-targeted mice deficient for the p53-binding kinase homeodomain-interacting protein kinase 1 (HIPK1). Proceedings of the National Academy of Sciences of the United States of America vol. 100,9 (2003): 5431-6.
- Kraemer, B. C., Schuck, T., Wheeler, J. M., Robinson, L. C., Trojanowski, J. Q., Lee, V. M. Y., & Schellenberg, G. D. (2010). Loss of Murine TDP-43 disrupts motor function and plays an essential role in embryogenesis. Acta Neuropathologica, 119(4), 409–419.
- Kuwano, Y., Nishida, K., Akaike, Y., Kurokawa, K., Nishikawa, T., Masuda, K., & Rokutan, K. (2016). Homeodomain-interacting protein kinase-2: A critical regulator of the DNA damage response and the epigenome. In International Journal of Molecular Sciences (Vol. 17, Issue 10).
- Lavra, L., Rinaldo, C., Ulivieri, A., Luciani, E., Fidanza, P., Giacomelli, L., Bellotti, C., Ricci, A., Trovato, M., Soddu, S., Bartolazzi, A., & Sciacchitano, S. (2011). The loss of the p53 activator HIPK2 is responsible for galectin-3 overexpression in well differentiated thyroid carcinomas. PLoS ONE, 6(6).

- Lee, S., Shang, Y., Redmond, S. A., Urisman, A., Tang, A. A., Li, K. H., Burlingame, A. L., Pak, R. A., Jovičić, A., Gitler, A. D., Wang, J., Gray, N. S., Seeley, W. W., Siddique, T., Bigio, E. H., Lee, V. M. Y., Trojanowski, J. Q., Chan, J. R., & Huang, E. J. (2016). Activation of HIPK2 Promotes ER Stress-Mediated Neurodegeneration in Amyotrophic Lateral Sclerosis. Neuron, 91(1), 41–55.
- Lee, S., Shang, Y., Redmond, S. A., Urisman, A., Tang, A. A., Li, K. H., Burlingame, A. L., Pak, R. A., Jovičić, A., Gitler, A. D., Wang, J., Gray, N. S., Seeley, W. W., Siddique, T., Bigio, E. H., Lee, V. M. Y., Trojanowski, J. Q., Chan, J. R., & Huang, E. J. (2016). Activation of HIPK2 Promotes ER Stress-Mediated Neurodegeneration in Amyotrophic Lateral Sclerosis. Neuron, 91(1), 41–55.
- Lee, W., Andrews, B. C., Faust, M., Walldorf, U., & Verheyen, E. M. (2009). Hipk is an essential protein that promotes Notch signal transduction in the Drosophila eye by inhibition of the global co-repressor Groucho. Developmental Biology, 325(1), 263–272.
- Lehman, N. L. (2009). The ubiquitin proteasome system in neuropathology. In Acta Neuropathologica (Vol. 118, Issue 3, pp. 329–347).
- Li, X. L., Arai, Y., Harada, H., Shima, Y., Yoshida, H., Rokudai, S., Aikawa, Y., Kimura, A., & Kitabayashi, I. (2007). Mutations of the HIPK2 gene in acute myeloid leukemia and myelodysplastic syndrome impair AML1- and p53-mediated transcription. Oncogene, 26(51), 7231–7239.
- Ling, S. C., Polymenidou, M., & Cleveland, D. W. (2013). Converging mechanisms in als and FTD: Disrupted RNA and protein homeostasis. In Neuron (Vol. 79, Issue 3, pp. 416–438).
- Ling, S. C., Polymenidou, M., & Cleveland, D. W. (2013). Converging mechanisms in als and FTD: Disrupted RNA and protein homeostasis. In Neuron (Vol. 79, Issue 3, pp. 416–438). https://doi.org/10.1016/j.neuron.2013.07.033
- Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods, 25(4), 402–408.
- Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods, 25(4), 402–408. https://doi.org/10.1006/meth.2001.1262
- Mackenzie, I. R. A., Bigio, E. H., Ince, P. G., Geser, F., Neumann, M., Cairns, N. J., Kwong, L. K., Forman, M. S., Ravits, J., Stewart, H., Eisen, A., McClusky, L., Kretzschmar, H. A., Monoranu, C. M., Highley, J. R., Kirby, J., Siddique, T., Shaw, P. J., Lee, V. M. Y., & Trojanowski, J. Q.

(2007). Pathological TDP-43 distinguishes sporadic amyotrophic lateral sclerosis from amyotrophic lateral sclerosis with SOD1 mutations. Annals of Neurology, 61(5), 427–434.

- Manning, G., Whyte, D. B., Martinez, R., Hunter, T., & Sudarsanam, S. (2002). The Protein Kinase Complement of the Human Genome Authors. In New Series (Vol. 298, Issue 5600).
- Möller, A., Sirma, H., Hofmann, T. G., Staege, H., Gresko, E., Lüdi, K. S., Klimczak, E., Dröge, W., Will, H., & Schmitz, M. L. (2003). Sp100 is important for the stimulatory effect of homeodomain-interacting protein kinase-2 on p53-dependent gene expression. Oncogene, 22(54), 8731–8737.
- Mori, F., Tanji, K., Zhang, H. X., Nishihira, Y., Tan, C. F., Takahashi, H., & Wakabayashi, K. (2008). Maturation process of TDP-43-positive neuronal cytoplasmic inclusions in amyotrophic lateral sclerosis with and without dementia. Acta Neuropathologica, 116(2), 193–203.
- Murch, S. J., Cox, P. A., & Banack, S. A. (2004). A mechanism for slow release of biomagnified cyanobacterial neurotoxins and neurodegenerative disease in Guam." Proceedings of the National Academy of Sciences of the United States of America vol. 101,33 (2004): 12228-31.
- Neumann, M., Sampathu, D. M., Kwong, L. K., Truax, A. C., Micsenyi, M. C., Chou, T. T., Bruce, J., Schuck, T., Grossman, M., Clark, C. M., McCluskey, L. F., Miller, B. L., Masliah, E., Mackenzie, I. R., Feldman, H., Feiden, W., Kretzschmar, H. A., Trojanowski, J. Q., & M-Y Lee, V. (2006). Ubiquitinated TDP-43 in Frontotemporal Lobar Degeneration and Amyotrophic Lateral Sclerosis. Science (New York, N.Y.) vol. 314,5796: 130-3.
- Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., & Tanaka, N. (2000). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science, 288(5468), 1053–1058.
- Okle, O., Stemmer, K., Deschl, U., & Dietrich, D. R. (2013). L-BMAA induced ER stress and enhanced caspase 12 cleavage in human neuroblastoma SH-SY5Y cells at low nonexcitotoxic concentrations. Toxicological Sciences, 131(1), 217–224.
- Petrov, D., Mansfield, C., Moussy, A., & Hermine, O. (2017). ALS clinical trials review: 20 years of failure. Are we any closer to registering a new treatment? In Frontiers in Aging Neuroscience (Vol. 9, Issue MAR). Frontiers Research Foundation.
- Pierantoni, G. M., Fedele, M., Pentimalli, F., Benvenuto, G., Pero, R., Viglietto, G., Santoro, M., Chiariotti, L., & Fusco, A. (2001). High mobility

group I (Y) proteins bind HIPK2, a serine-threonine kinase protein which inhibits cell growth. Oncogene. (43): 6132-41

- Pierantoni, G. M., Pentimalli, F., Fedele, M., Santoro, M., Fusco, A., Bulfone, A., Ballabio, A., Iuliano, R., Chiariotti, L., & Ballabio, A. (2002). The homeodomain-interacting protein kinase 2 gene is expressed late in embryogenesis and preferentially in retina, muscle, and neural tissues. Biochemical and Biophysical Research Communications, 290(3), 942–947.
- Plato, C. C., Garruto, R. M., Galasko, D., Craig, U. K., Plato, M., Gamst, A., Torres, J. M., & Wiederholt, W. (2003). Amyotrophic lateral sclerosis and parkinsonism-dementia complex of Guam: Changing incidence rates during the past 60 years. American Journal of Epidemiology, 157(2), 149–157.
- Polymenidou, M., & Cleveland, D. W. (2011). The seeds of neurodegeneration: Prion-like spreading in ALS. In Cell (Vol. 147, Issue 3, pp. 498–508).
- Reeves, R. (2001). Molecular biology of HMGA proteins: hubs of nuclear function Gene, 277 (1-2): 63-81
- Rinaldo, C., Prodosmo, A., Siepi, F., & Soddu, S. (2007). HIPK2: A multitalented partner for transcription factors in DNA damage response and development. In Biochemistry and Cell Biology (Vol. 85, Issue 4, pp. 411–418).
- Rinaldo, C., Siepi, F., Prodosmo, A., & Soddu, S. (2008). HIPKs: Jack of all trades in basic nuclear activities. In Biochimica et Biophysica Acta - Molecular Cell Research (Vol. 1783, Issue 11, pp. 2124–2129).
- Rochat-Steiner, V., Becker, K., Micheau, O., Schneider, P., Burns, K., & Tschopp, J. (2000). FIST/HIPK3: a Fas/FADD-interacting Serine/Threonine Kinase that Induces FADD Phosphorylation and Inhibits Fas-mediated Jun NH 2-terminal Kinase Activation. In J. Exp. Med (Vol. 192, Issue 8).
- Roscic, A., Möller, A., Calzado, M. A., Renner, F., Wimmer, V. C., Gresko, E., Lüdi, K. S., & Schmitz, M. L. (2006). Phosphorylation-Dependent Control of Pc2 SUMO E3 Ligase Activity by Its Substrate Protein HIPK2. Molecular Cell, 24(1), 77–89.
- Rui, Y., Xu, Z., Lin, S., Li, Q., Rui, H., Luo, W., Zhou, H. M., Cheung, P. Y., Wu, Z., Ye, Z., Li, P., Han, J., & Lin, S. C. (2004). Axin stimulates p53 functions by activation of HIPK2 kinase through multimeric complex formation. EMBO Journal, 23(23), 4583–4594.

- Sakamoto, K., Karelina, K., & Obrietan, K. (2011). CREB: A multifaceted regulator of neuronal plasticity and protection. In Journal of Neurochemistry (Vol. 116, Issue 1, pp. 1–9).
- Saul, V. v., & Schmitz, M. L. (2013). Posttranslational modifications regulate HIPK2, a driver of proliferative diseases. In Journal of Molecular Medicine (Vol. 91, Issue 9, pp. 1051–1058).
- Sawada, H. (2017). Clinical efficacy of edaravone for the treatment of amyotrophic lateral sclerosis. Expert Opinion on Pharmacotherapy, 18(7), 735–738.
- Schmid, B., Hruscha, A., Hogl, S., Banzhaf-Strathmann, J., Strecker, K., van der Zee, J., Teucke, M., Eimer, S., Hegermann, J., Kittelmann, M., Kremmer, E., Cruts, M., Solchenberger, B., Hasenkamp, L., van Bebber, F., van Broeckhoven, C., Edbauer, D., Lichtenthaler, S. F., & Haass, C. (2013). Loss of ALS-associated TDP-43 in zebrafish causes muscle degeneration, vascular dysfunction, and reduced motor neuron axon outgrowth. Proceedings of the National Academy of Sciences of the United States of America, 110(13), 4986–4991.
- Schmitz, M. L., Rodriguez-Gil, A., & Hornung, J. (2014). Integration of stress signals by homeodomain interacting protein kinases. In Biological Chemistry (Vol. 395, Issue 4, pp. 375–386).
- Sephton, C. F., Good, S. K., Atkin, S., Dewey, C. M., Mayer, P., Herz, J., & Yu, G. (2010). TDP-43 is a developmentally regulated protein essential for early embryonic development. Journal of Biological Chemistry, 285(9), 6826–6834.
- Shang, Y., Doan, C. N., Arnold, T. D., Lee, S., Tang, A. A., Reichardt, L. F., & Huang, E. J. (2013). Transcriptional Corepressors HIPK1 and HIPK2 Control Angiogenesis Via TGF-β-TAK1-Dependent Mechanism. PLoS Biology, 11(4).
- Sombroek, D., & Hofmann, T. G. (2009). How cells switch HIPK2 on and off. In Cell Death and Differentiation (Vol. 16, Issue 2, pp. 187–194).
- Swarup, V., Phaneuf, D., Dupré, N., Petri, S., Strong, M., Kriz, J., & Julien, J. P. (2011). Deregulation of TDP-43 in amyotrophic lateral sclerosis triggers nuclear factor κB-mediated pathogenic pathways. Journal of Experimental Medicine, 208(12), 2429–2447.
- Tsermentseli, S., Leigh, P. N., & Goldstein, L. H. (2012). The anatomy of cognitive impairment in amyotrophic lateral sclerosis: More than frontal lobe dysfunction. In Cortex (Vol. 48, Issue 2, pp. 166–182).
- Valente, D., Bossi, G., Moncada, A., Tornincasa, M., Indelicato, S., Piscuoglio, S., Karamitopoulou, E. D., Bartolazzi, A., Pierantoni, G. M.,

Fusco, A., Soddu, S., & Rinaldo, C. (2015). HIPK2 deficiency causes chromosomal instability by cytokinesis failure and increases tumorigenicity. Oncotarget, 6(12), 10320–10334.

- Van der Laden, J., Soppa, U., & Becker, W. (2015). Effect of tyrosine autophosphorylation on catalytic activity and subcellular localisation of homeodomain-interacting protein kinases (HIPK). Cell Communication and Signaling, 13(1).
- Wales, S., C Kiernan DSc, A. M., Cheah MBiostat, B. C., Burrell MBBS, J., Zoing BNurs, M. C., Kiernan, M. C., Vucic, S., Cheah, B. C., Turner, M. R., Eisen, A., Hardiman, O., Burrell, J. R., & Zoing, M. C. (2011). Seminar Amyotrophic lateral sclerosis. The Lancet, 377, 942–955.
- Wang, I. F., Wu, L. S., & Shen, C. K. J. (2008). TDP-43: an emerging new player in neurodegenerative diseases. Trends in Molecular Medicine, 14(11), 479–485.
- Wiggins, A. K., Wei, G., Doxakis, E., Wong, C., Tang, A. A., Zang, K., Luo, E. J., Neve, R. L., Reichardt, L. F., & Huang, E. J. (2004). Interaction of Brn3a and HIPK2 mediates transcriptional repression of sensory neuron survival. Journal of Cell Biology, 167(2), 257–267.
- Wook Choi, D., & Yong Choi, C. (2014). HIPK2 modification code for cell death and survival. In Molecular and Cellular Oncology (Vol. 1, Issue 2). Taylor and Francis Ltd.
- Zhang, J., Pho, V., Bonasera, S. J., Holzmann, J., Tang, A. T., Hellmuth, J., Tang, S., Janak, P. H., Tecott, L. H., & Huang, E. J. (2007). Essential function of HIPK2 in TGFβ-dependent survival of midbrain dopamine neurons. Nature Neuroscience, 10(1), 77–86.
- Zhang, Q., Nottke, A., & Goodman, R. H. (2005). Homeodomain-interacting protein kinase-2 mediates CtBP phosphorylation and degradation in UV-triggered apoptosis.