Ankrd2/ARPP is a novel Akt2 specific substrate and regulates myogenic differentiation upon cellular exposure to H_2O_2

Vittoria Cenni[a,b], Alberto Bavelloni[b], Francesca Beretti[c], Francesca Tagliavini[b,d], Lucia Manzoli[d], Giovanna Lattanzi[a], Nadir M. Maraldi[a,b], Lucio Cocco[d], and Sandra Marmiroli[a,c]
[a]IGM-CNR, Unit of Bologna c/o IOR, 40136 Bologna, Italy; [b]Laboratory of Musculoskeletal Cell Biology, IOR, 40136 Bologna, Italy; [c]Department of Anatomy and Histology, University of Modena and Reggio Emilia, 41124 Modena, Italy; [d]Cellular Signaling Laboratory, Department of Human Anatomical Sciences, University of Bologna, 40126 Bologna, Italy

ABSTRACT **Activation of Akt-mediated signaling pathways is crucial for survival, differentiation, and regeneration of muscle cells. A proteomic-based search for novel substrates of Akt was therefore undertaken in C_2C_{12} murine muscle cells exploiting protein characterization databases in combination with an anti–phospho-Akt substrate antibody. A Scansite database search predicted Ankrd2 (Ankyrin repeat domain protein 2, also known as ARPP) as a novel substrate of Akt. In vitro and in vivo studies confirmed that Akt phosphorylates Ankrd2 at Ser-99. Moreover, by kinase assay with recombinant Akt1 and Akt2, as well as by single-isoform silencing, we demonstrated that Ankrd2 is a specific substrate of Akt2. Ankrd2 is typically found in skeletal muscle cells, where it mediates the transcriptional response to stress conditions. In an attempt to investigate the physiological implications of Ankrd2 phosphorylation by Akt2, we found that oxidative stress induced by H_2O_2 triggers this phosphorylation. Moreover, the forced expression of a phosphorylation-defective mutant form of Ankrd2 in C_2C_{12} myoblasts promoted a faster differentiation program, implicating Akt-dependent phosphorylation at Ser-99 in the negative regulation of myogenesis in response to stress conditions.**

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INTRODUCTION

The 67-kDa serine/threonine kinase Akt was originally identified as the cellular homologue of the v-*akt* oncogene (Bellacosa *et al.*, 1993). Akt, also known as protein kinase B (PKB), is an essential enzyme in all eukaryotes and is involved in a wide variety of cellular functions, including proliferation, cell survival, differentiation, glucose mobilization, homeostasis, cell migration, and apoptosis.

Three isoforms of Akt exist, namely Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ (Brazil *et al.*, 2004), sharing a high degree of amino acid identity and the same ways of activation. Once activated, Akt is distributed in all cellular compartments (Andjelkovic *et al.*, 1997), where it phosphorylates its substrates. Groundbreaking experiments conducted in the past (Alessi *et al.*, 1996) with peptides containing variants of the first direct target of Akt, identified on a specific residue of the GSK3 kinase (Cross *et al.*, 1995), defined the minimal recognition motif of Akt as R-X-R-X-X-pS/T-B (Alessi *et al.*, 1996), where R is an arginine, X is any amino acid, and B represents bulky hydrophobic residues.

Akt-phosphorylated substrates are involved in a variety of cellular functions spanning from cell growth and survival (modulating proapoptotic and antiapoptotic transcription factors) to cell migration and invasion, from cellular metabolism to angiogenesis (reviewed in Manning and Cantley, 2007). By using a functional proteomic approach, recently our laboratory identified Lamin A/C as a novel Akt nuclear substrate, implicating the phosphorylation of Lamin A/C by Akt in the correct functioning of the nuclear lamina (Cenni *et al.*, 2008).

Skeletal muscle is a tissue highly responsive to Akt: Akt action has been linked to skeletal muscle development, regeneration, and

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Address correspondence to: Vittoria Cenni (vittoria.cenni@cnr.it, vcenni@gmail.com).

Abbreviations used: Ankrd2, Ankyrin repeat domain protein 2 (also known as ARPP); MARP, muscle ankyrin repeat protein; PI, isoelectric point; PKB, protein kinase B; ROS, reactive oxygen species; shRNA, short hairpin RNA; WT, wild type.

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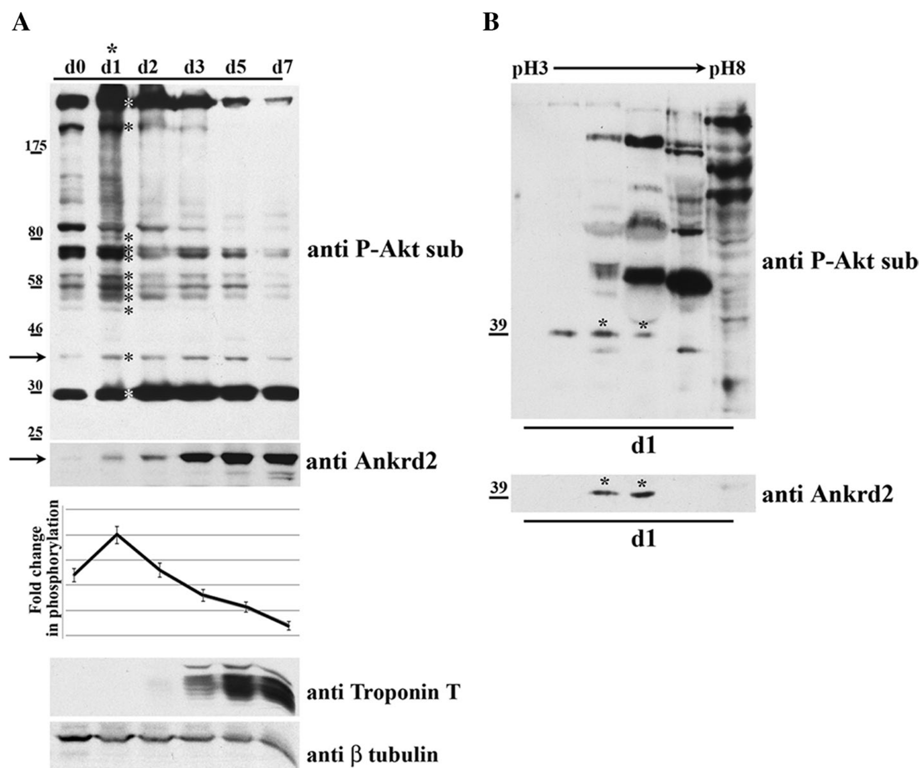

FIGURE 1: Akt phosphorylates Ankrd2. (A) Cycling C_2C_{12} cells were grown to confluence and allowed to differentiate from d0 to d7 in the presence of differentiating medium. Cell extracts were immunoblotted with the anti–phospho-Akt substrates (anti P-Akt sub) antibody. Anti-Troponin T and -β tubulin antibodies were used as differentiation and equal loading markers, respectively. Asterisks highlight bands significantly more phosphorylated at d1 of differentiation. Line graph demonstrates the average fold change in phosphorylation, determined as the ratio in the change of Akt-phosphorylated Ankrd2 vs. the amount of total Ankrd2 of the lysates of A and indicates that Ankrd2 phosphorylation by Akt peaks at d1. (B) Top, lysates from d1-differentiating C_2C_{12} myoblasts were focused with a MicroRotofor device and probed with the anti–phospo-Akt substrates antibody. Nitrocellulose was then stripped and incubated with an anti-Ankrd2 specific antibody (bottom panel).

hypertrophy through several pathways that culminate in stimulation of protein synthesis, inhibition of atrophy, and prevention of cell death (Glass, 2003; Hoffman and Nader, 2004). The function of the single Akt isoforms has been extensively studied in skeletal muscle also. This tissue revealed specialized roles for IRS-1/Akt2 and IRS-2/Akt1 in glucose and lipid metabolism (Bouzakri *et al.*, 2006). In 2002, Sumitani and colleagues demonstrated that Akt1 and Akt2 differently regulate muscle creatine kinase and myogenin gene transcription in differentiating C_2C_{12} myoblasts (Sumitani *et al.*, 2002). Later, by a small RNA interfering approach, Heron-Milhavet reached the conclusions that Akt1 only stimulates cell proliferation, whereas Akt2 appears to be involved in cell cycle exit (Heron-Milhavet *et al.*, 2006). This result had been extensively supported by other studies (Vandromme *et al.*, 2001; Al-Khalili *et al.*, 2004; Rochat *et al.*, 2004). More recently, however, Wilson and colleagues have demonstrated that both Akts are necessary for full myoblast differentiation and maturation. In particular, in C_2C_{12} myoblasts, the loss of Akt1 blocked muscular differentiation at its earliest stages, preventing induction of muscle-specific proteins and inhibiting formation of multinucleated myofibers. On the contrary, myoblasts lacking Akt2 differentiated normally, although resultant myofibers were thinner and incorporated fewer nuclei than controls (Wilson and Rotwein, 2007; Rotwein and Wilson, 2009).

The MARP (muscle ankyrin repeat protein) family consists of three proteins, namely Ankrd1 also known as cardiac ankyrin repeat protein, expressed in cardiac muscle, Ankrd2, also called ARPP expressed in skeletal and cardiac muscle, and Ankrd23 or DARP which is found in both skeletal and cardiac muscle but is also up-regulated in type 2 diabetes. All MARPs are primarily involved in the defense of the cells against injuries, following a prolonged muscle exercise, in response to acute exercise, or during work overload hypertrophy. A role for these proteins also has been described in pathological conditions, including tumors, diabetes, or a number of muscular dystrophies (Miller *et al.*, 2003). Our data establish Ankrd2 as a new signaling target of Akt2, and indicate this phosphorylation to be involved in muscle differentiation following oxidative stress.

RESULTS

Identification of Ankrd2/ARPP as an Akt substrate in C_2C_{12} muscle cells

To identify muscular proteins phosphorylated by Akt, crude lysates from C_2C_{12} myoblasts at different days of myogenic differentiation (from day 0 to day 7) were assayed with an antibody specifically raised against the Akt consensus phosphorylation motif, the specificity of which has been widely demonstrated (Alessi *et al.*, 1996; Zhang *et al.*, 2002). Through this biochemical approach, several differentiation-responsive proteins were detected (Figure 1A); however, we focused on proteins phosphorylated during the earliest stages of muscle differentiation, when the outcome of the entire differentiation process may be most affected. Indeed, it was especially during this stage that the highest activation of Akt was observed (Figure 1A; WB: anti–phospho-Akt substrates). In particular, at day 1 (d1), 11 proteins were evidently phosphorylated. Starting from the average molecular weight assigned to each of these phosphoproteins (calculated as described in *Materials and Methods*) a Scansite analysis (http://scansite.mit.edu/dbsequence_one.html) was performed. The search was carried out on a SWISS-PROT database specific for both *mus musculus* and muscular tissue proteins, querying the presence of a high-stringency Akt consensus phosphorylation motif, that is, [R][A-Z][R][A-Z][A-Z][S/T], phosphorylated at serine or threonine. This study resulted in a short list of potential Akt substrates, shown in Table 1.

To further narrow the number of potential Akt substrates, protein extracts from d1 differentiating C_2C_{12} myoblasts were fractionated by their isoelectric point (PI) by a MicroRotofor device, and the resultant protein fractions were probed for the presence of phospho-Akt substrates.

We reasoned that the phosphoprotein focusing at a PI value of approximately 6 and with a molecular weight of approximately 39 kDa, which correspond to Ankrd2, a muscle specific protein.

The membranes of the experiments just mentioned were therefore incubated with an anti-Ankrd2 specific antibody. Figure 1, A and B (bottom panels), shows that the 39-kDa band, recognized by

No.	Average Mass[1]	Acc. Numb[2]	Name[2]	Predicted Mass[2]	PI[3]	Akt motif*	Function[2]
1	272–301	Q8BTM8	FLNA	281.194	5.65	m/l motif	Actin binding protein
2	209–231	Q61879	MYH10	228.996	5.42	RQRHA**T1203**A	Cellular myosin
		O08638	MYH11	227.028	5.36	RVRKA**T1763**L	Myosin heavy chain, smooth muscle isoform
		Q6URW6	MYH14	228.586	5.48	m/l motif	Cellular myosin
		Q5SX40	**MYH1**	**223.342**	**5.58**	m/l motif	**Adult skeletal muscle myosin**
		A2AQP0	MYH7B	221.497	5.67	m/l motif	Cardiac myosin
		P13542	**MYH8**	**222.708**	**5.63**	m/l motif	**Muscle specific perinatal myosin**
		Q99104	MYH5A	215.595	8.79	RKRTS**S1650**I	Cellular myosin
		Q9JJV9	SCN5A	227.622	5.43	m/l motif	Cardiac specific voltage-gated sodium channel
3	77–86	P33146	CAD15	85.660	4.64	m/l motif	Muscle specific cell adhesion protein
		Q8BU25	PAMR1	80.378	6.79	RRRVL**S361**M	Inactive peptidase involved in muscle regeneration
		Q61321	**SIX4**	**77.203**	**5.76**	m/l motif	**DNA binding protein involved in skeletal muscle development**
				82.263	**5.55**	m/l motif	
4	68–79	Q9WTL8	BMAL1	69.452	6.04	RKRKG**S42**A RSRWF**S428**F	Transcription cofactor
5	59–65	Q2VPD4	BMAL2	64.399	7.96	m/l motif	Transcription coactivator
		Q3UZD5	PRDM6	64.503	7.55	RPRPA**S79**L	Smooth muscle specific histone methyltransferase
6	55–60	Q9JIF9	MYOTI	55.316	8.85	m/l motif	Component of a complex of actin cross-linkers
7	53–59	Q9JIF9	MYOTI	55.316	8.85	m/l motif	Component of a complex of actin cross-linkers
8	49–55	P46664	PURA2	50.021	5.85	m/l motif	Purine nucleotide biosynthesis enzyme
9	36–40	**Q9WV06**	**ANKRD2**	**39.858**	**6.1**	**RVRKTS98L**	**Structural and gene regulatory protein**
		Q9JLH8	**TMOD4**	**39.261**	**4.72**	m/l motif	**Actin capping protein**
		Q38HM4	TRI63	39.491	4.8	m/l motif	Muscle specific ubiquitin
10	28–31				No matching found		

[1]Estimated mass (expressed in kilodaltons) according to the Quantity One 1D Software, Bio-Rad. [2]Font: UniProtKB/Swiss-Prot. [3]Scansite calculation of PI following one phosphorylation event.
*High-stringency scan for Akt motifs performed on Scansite. Medium- or low-stringency Akt motifs (m/l motif) not reported. Entries in bold were obtained from musculoskeletal protein database and therefore indicate musculoskeletal proteins.

TABLE 1: *Mus musculus* and musculoskeletal specific proteins matching the phosphorylation motif profile for the Ser/Thr protein kinase Akt.

the anti–phospho-Akt substrate antibody, perfectly merges with that of the anti-Ankrd2. Moreover, although Ankrd2 expression increases with differentiation, as reported previously by other laboratories (Ishiguro *et al.*, 2002), it is worth noting that its phosphorylation peaks at d1 (Figure 1A, line graph). Finally, Figure 1B shows that Ankrd2 drops in two contiguous fractions, suggesting different phosphorylation degrees.

Ser-99 of Ankrd2 is a substrate of Akt both in vitro and in Akt-activated cultured cells

To identify the putative site(s) of Ankrd2 phosphorylated by Akt, Ankrd2 complete amino acidic sequences cloned so far were scanned by the Scansite databases for Akt consensus phosphorylation motifs. In good agreement with the results presented earlier in this article, this search produced a putative Akt phosphorylation site mapping at Ser-99 of the human sequence.

Therefore HEK 293T cells, which do not express endogenous Ankrd2, were transfected with either wild type (WT) or mutant Ser99Ala human flag-tagged Ankrd2. Then, pellets of immunoprecipitated flag-Ankrd2 were mixed with recombinant active Akt1 or Akt2 for in vitro kinase assay. As control, the activity of the kinases was assayed using myelin basic protein. The results show that both Akt1 and Akt2 failed to phosphorylate Ser99Ala Ankrd2, indicating Ser-99 as an authentic Akt phosphorylation site (Figure 2A).

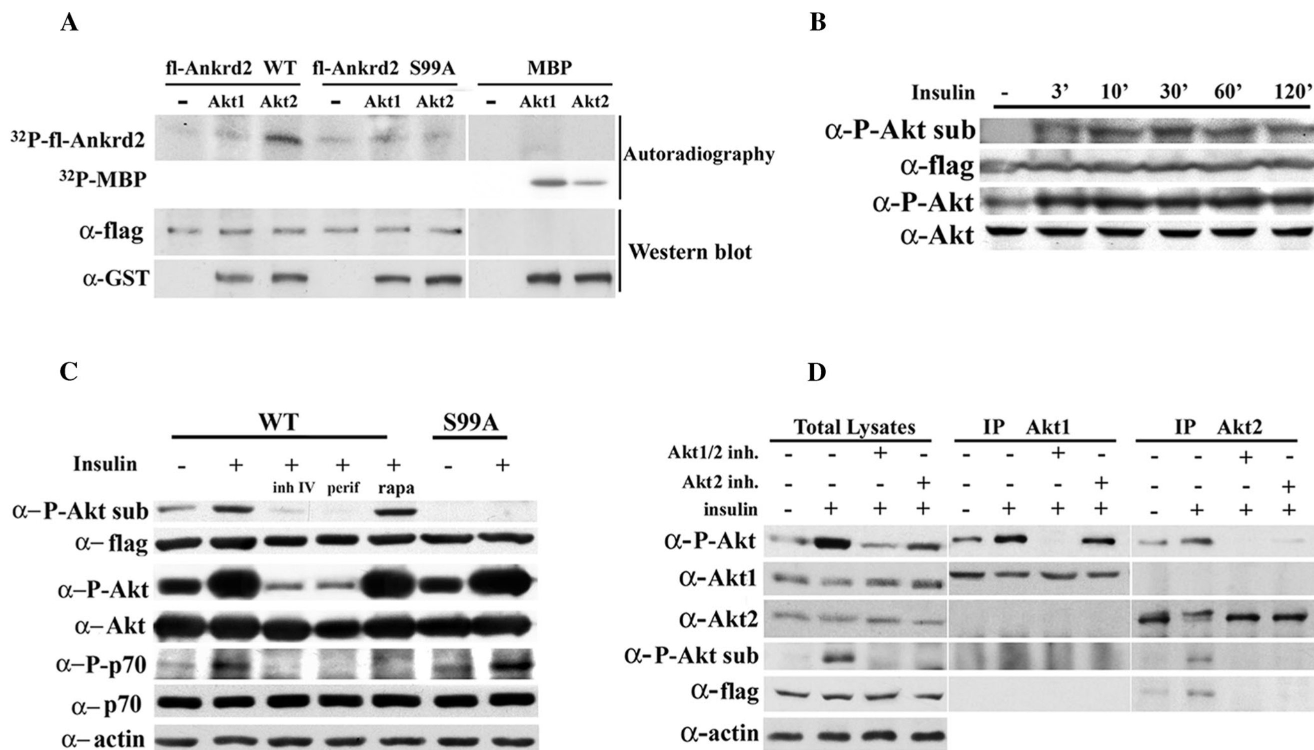

FIGURE 2: Akt phosphorylates Ankrd2 at Ser-99 in vitro and in HEK 293T cells. (A) In vitro Akt kinase assay. HEK 293T cells, which do not endogenously express Ankrd2, were transfected with flag-tagged WT or S99A Ankrd2 and then recovered in serum-free medium for 24 h. Overexpressed Ankrd2 forms were then immunoprecipitated by an anti-flag antibody. Immunoprecipitates were used as substrates for an in vitro kinase assay with [γ^{32}]P-ATP and recombinant active Akt1 (+Akt1) or Akt2 (+Akt2) or kinase buffer alone (–). As control, the activity of each Akt isoform was also monitored on myelin basic protein. Reactions were resolved on SDS–PAGE and blotted on nitrocellulose. (B) HEK 293T cells were transfected with flag-Ankrd2, serum starved for 24 h, and stimulated with 1.6 μM insulin for the indicated times. Cell extracts were resolved on SDS–PAGE and subjected to immunoblotting with the indicated antibodies. (C) HEK 293T cells were transfected with flag-Ankrd2 WT or S99A as indicated, serum starved for 24 h, and treated with insulin or vehicle for 30 min. Where indicated before insulin, cells were also pretreated with the inhibitors for Akt (perifosine and inhibitor IV) and p70 S6K (rapamycin). Lysates were resolved on SDS–PAGE and subjected to immunoblotting with the indicated antibodies. Activities of the inhibitors were checked by the use of antibodies directed against anti–-phospho Akt Ser473 (Cell Signaling) and -phospho p70 S6K Ser411 (Santa Cruz Biotechnology). (D) HEK 293T cells were transfected with flag-Ankrd2 WT, serum starved, and treated with insulin. Where indicated, cells were pretreated with Akt1/2 and Akt2 specific inhibitors. Akt1 and Akt2 were separately immunoprecipitated from cell lysates by the use of specific antibodies, and the corresponding immunocomplexes were resolved on SDS–PAGE, blotted, and probed with the indicated antibodies.

Conversely, recombinant Akt2 phosphorylates Ankrd2 to a much greater extent than does Akt1, indicating a preference of this substrate toward Akt2 (Figure 2A).

Next Ankrd2 phosphorylation by Akt was monitored in vivo. HEK 293T cells were transfected with WT or Ser99Ala–flag-Ankrd2. After 1 d of serum starvation, cells were treated with insulin to activate Akt, and the phosphorylation state of Ankrd2 was monitored by Western blot by the phospho-Akt substrate antibody. Figure 2, B and C, shows that insulin leads to phosphorylation of Ankrd2 by Akt. Confirming the data presented earlier in the text, and validating the antibody specificity, the immunoreactivity is undetectable in unphosphorylatable Ser99Ala samples (Figure 2C).

Remarkably, a recent proteomic study published by Moritz *et al.* in *Science Signaling* (Moritz *et al.*, 2010), in which the anti–phospho-Akt substrate antibody was used to capture peptides phosphorylated by this kinase, predicted Ser-99 of Ankrd2 as a phosphorylation site for Akt. This work provides further evidence that Ser-99 also is phosphorylated by Akt in in vivo cellular models.

To confirm the specificity of Akt-dependent Ankrd2 phosphorylation, HEK 293 cells were pretreated with specific inhibitors of selected kinases and then treated with insulin to activate Akt. As shown in Figure 2C, the use of the Akt broad inhibitors perifosine and Akt inhibitor IV completely prevented Ankrd2 phosphorylation. Conversely, the MEK1/2 pathway inhibitor PD-98059 did not interfere with Ankrd2 phosphorylation by Akt (unpublished data). Moreover p70 S6 kinase-1, an AGC kinase downstream of PI3K, which shares the Akt consensus phosphorylation motif, did not signal to Ankrd2, because treatment with rapamycin (a specific inhibitor of the S6K upstream kinase, mTORC1; Hong *et al.*, 2008), had no effect on Ankrd2 phosphorylation after insulin stimulation (Figure 2C).

To get more insight into the Akt isoform specificity of Ankrd2 phosphorylation, additional experiments were performed with specific inhibitors. Flag-Ankrd2–expressing HEK 293T cells were treated with Akt1/2 or Akt2 selective inhibitors (respectively, Akt inhibitors VIII and XII) and were stimulated with insulin. Then lysates were probed for phosphorylated Ankrd2. The results showed that pretreatment with either Akt1/2 or Akt2 specific inhibitors almost completely

prevented phosphorylation of Ankrd2 as detected in the cell lysate by anti–phospho-Akt substrates (Figure 3D, anti P-Akt sub), despite residual Akt1 activity in Akt2 inhibitor–treated cells (Figure 2D), supporting the finding that Ankrd2 is a specific substrate of Akt2. Indeed, it is worth noting that Ankrd2 was detectable only in pellets from Akt2, but not Akt1, immunoprecipitates (Figure 2D).

Akt interacts with Ankrd2 also in vivo

To further confirm the data just presented, short hairpin RNA (shRNA) targeting either Akt1 or Akt2 was introduced into HEK 293T cells expressing flag-Ankrd2. After activating Akt by insulin stimulation, Ankrd2 was immunoprecipitated and the immunocomplexes searched for the presence of phosphorylated Ankrd2. As shown in Figure 3A, the knock down of Akt2 nearly abrogated insulin-induced Ankrd2 phosphorylation. Conversely, depletion of Akt1 had minimal effects. Next HEK 293T cells were cotransfected with WT Ankrd2 together with each Akt isoform, followed by immunoprecipitation with anti-flag antibody. The results in Figure 3B reveal that Ankrd2 robustly associates with Akt2. Of note, Ankrd2 does not bind Akt3 and weakly binds Akt1 (Figure 3B, lane 1).

Finally, to assess whether Akt activity is essential for its binding to Ankrd2, Akt2 association to Ankrd2 was monitored in insulin-treated cells. Confirming the findings presented earlier in the text, the interaction is indeed strongly increased upon Akt activation (Figure 3C).

Although Akt3 is expressed in HEK 293T (and C_2C_{12} cells), it is unlikely to contribute to Ankrd2 regulation, because overexpressed Akt3 does not bind to Ankrd2 and Akt2 shRNA significantly reduces Ankrd2 phosphorylation.

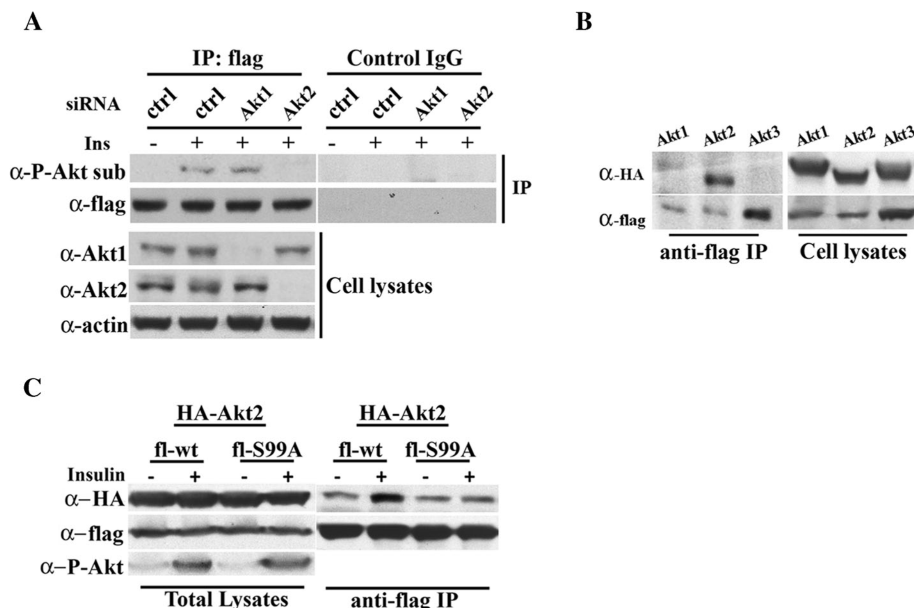

FIGURE 3: Ankrd2 is an Akt2 specific substrate. (A) HEK 293T cells expressing flag-wild-type (wt) Ankrd2 were infected with Akt1 or Akt2 shRNA lentiviral vector or empty vector for 24 h. Cells were then serum starved and stimulated with insulin for 30 min before addition of lysis buffer. Ankrd2 was then immunoprecipitated from total cell lysates by anti-flag antibody, and pellets were resolved on SDS–PAGE and subjected to immunoblot analysis. Western blot directed against Akt –1 and –2 of total lysates confirms Akt specific isoform silencing. (B) HEK 293T cells were cotransfected with flag-Ankrd2 wt and HA-Akt –1, –2, or –3. Cellular extracts were subjected to immunoprecipitation by an anti-flag antibody. Obtained precipitates and corresponding lysates were resolved on SDS–PAGE and immunoblotted with an anti–HA antibody to check the Akt isoform bound to Ankrd2. (C) HEK 293T cells were cotransfected with HA-Akt2 and wt or S99A forms of flag-Ankrd2. Cells were serum starved for 24 h and treated with 1.6 μM insulin for 30 min. Cells extracts were immunoprecipitated with anti-flag antibody and immunoblotted with the indicated antibodies.

H_2O_2 is a physiological activator of Ankrd2 phosphorylation by Akt in C_2C_{12} cells

Ankrd2 belongs to the MARP family, which members are all involved in the cellular response to stress conditions (Proske and Morgan, 2001). Therefore we asked whether reactive oxygen species (ROS), which can signal through the PI3K/Akt axis (Shaw *et al.*, 1998), might affect Ankrd2 phosphorylation by Akt.

C_2C_{12} myotubes were exposed to H_2O_2 (450 μM final concentration) from 5 min to 5 h, and the phosphorylation of Ankrd2 by Akt was probed by Western blot in total cellular lysates. Figure 4A reveals that the arrival of H_2O_2 induces a huge and sustained Akt activation, concomitantly to an increase of Akt-dependent Ankrd2 phosphorylation. This last observation came after probing the anti–phospho-Akt substrate antibody on total cell lysates and on Ankrd2-immunoprecipitates, both obtained from myotubes exposed to H_2O_2 (Figure 4A, WB: P-Akt sub, and Figure 4B, IP: α-Ankrd2, WB: α-P-Akt sub). To further support these findings, the phospho-Akt substrates immunoprecipitated from the lysates just mentioned were resolved by SDS–PAGE and then revealed with anti-Ankrd2. In agreement with the data just presented, Figure 4B demonstrates that the amount of Ankrd2 phosphorylated by Akt is robustly increased by H_2O_2 (Figure 4B, IP: α-P-Akt sub, WB: α-Ankrd2). Furthermore, Ankrd2 immunoprecipitates from H_2O_2-treated C_2C_{12} myotubes were probed with anti-Akt, demonstrating that oxidative stress conditions induced by H_2O_2 also promote association of endogenous Ankrd2 to Akt (Figure 4B, IP: α-Ankrd2, WB: α-Akt and vice versa).

Of note, Figure 4A also demonstrates that, concomitantly with Ankrd2 phosphorylation, cyclin D3, a key regulator of the differentiated phenotype (Cenciarelli *et al.*, 1999), is strongly down-regulated following H_2O_2 stimulation.

C_2C_{12} myotubes were then pretreated with selective inhibitors of Akt1/2 and Akt2 (Figure 4C) and exposed to H_2O_2 for 15 min. Ankrd2 was immunoprecipitated from C_2C_{12} myotube lysates, and the pellets were probed with the phospho-Akt substrate antibody. As previously observed, both Akt1/2 and Akt2 inhibitors were able to abrogate Ankrd2 phosphorylation (Figure 4C, IP: α-Ankrd2, WB α-P-Akt sub), despite the residual Akt1 activity in cells treated with Akt2 specific inhibitor (Figure 4C, IP: α-Akt1, WB: α-P-Akt).

We conclude therefore that, following H_2O_2 stimulation, also in C_2C_{12} myotubes, Ankrd2 is specifically phosphorylated by Akt isoform 2.

Oxidative stress induces nuclear translocation of Ankrd2

An analysis from the wolfpsort data set (http://www.wolfpsort.org) predicts Ankrd2 localization as 11.5% nuclear, 11.5% cytoplasmic, and 14.5% cytonuclear, thus indicating the possibility of localizing to both the cytosol and/or the nucleus without distinguishing between conditional and constitutive dual localization. To monitor Ankrd2 localization following stress stimulation, cytoplasmic and nuclear fractions obtained from C_2C_{12} cells exposed to H_2O_2 were checked for

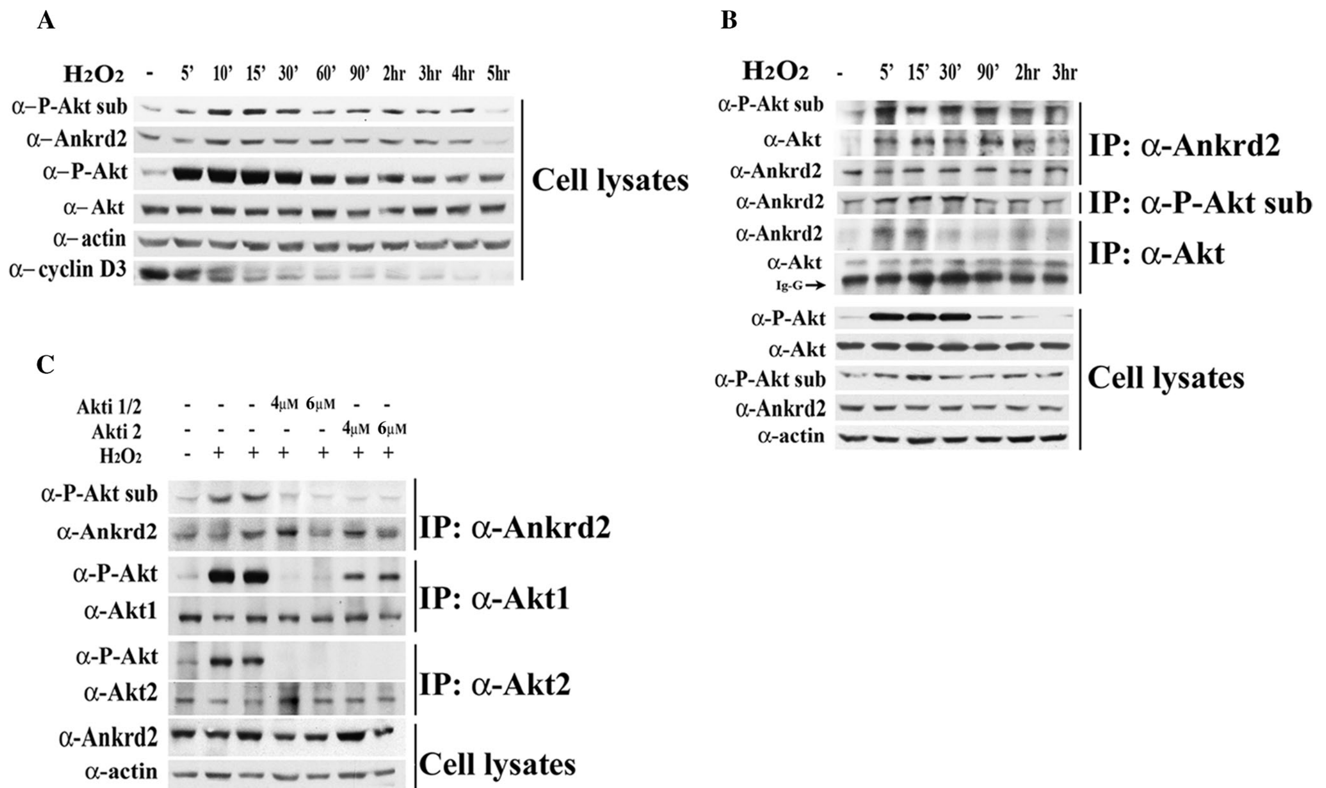

FIGURE 4: H_2O_2 is a physiological activator of Ankrd2 phosphorylation by Akt2 in C_2C_{12} cells. (A) Whole lysates from D1-differentiated C_2C_{12} cells exposed to H_2O_2 450 μM for the indicated times were resolved on a 12% SDS–PAGE and immunoblotted. Akt activation following H_2O_2 exposure was monitored by the anti Phospho-Akt Ser-473 antibody (α-P-Akt). Cell-cycle exit dependent from H_2O_2 exposure was examined by immunoblotting lysates with anti–cyclin D3. Anti-actin revealed equal loadings. (B) Day 1 (D1)-differentiated C_2C_{12} cells were exposed to H_2O_2 450 μM for the indicated times. Whole-cell lysates were immunoprecipitated with anti-Ankrd2, -Phospho-Akt substrate, or -Akt1/2 (N terminus) antibodies, as indicated. Immunocomplexes and corresponding lysates were resolved on 12% SDS–PAGE and immunoblotted as indicated. (C) D1-differentiated C_2C_{12} cells were left untreated or treated with 4 or 6 μM Akt1/2 or Akt2 inhibitors and then exposed to H_2O_2 450 μM for 15 min. Total cell lysates were divided and immunoprecipitated with anti–Ankrd2 antibody to check Ankrd2 phosphorylation by Akt, anti-Akt1 and -Akt2 antibodies to verify the efficacy of inhibitors.

the presence of endogenous Ankrd2. The results, shown in Supplemental Figure S1, indicate that Ankrd2 is located both in the cytoplasm and in the nuclei of differentiating C_2C_{12}. The arrival of the stimulus, however, promotes Ankrd2 to accumulate into the nucleus. In particular, Ankrd2 nuclear accumulation becomes increasingly evident over time, reaching a percentage of 70% at 3 h from the administration of H_2O_2.

Next cellular localization of both the WT and phosphorylation-defective mutant was checked. C_2C_{12} cells were transfected with flag-tagged WT or Ser99Ala Ankrd2, seeded on slides, and allowed to differentiate for 1 d. Slides were fixed and incubated with anti–flag antibody for immunofluorescence analysis. Figure 5B, top panel, shows that, in differentiating myoblasts, WT and Ser99Ala Ankrd2 have a similar localization, being distributed both in the cytoplasm and, to a much lesser extent, in the nucleus. We further analyzed the myotube formation by counting the multinucleated cells after the transfection. As shown in Figure 5C, transfection of the unphosphorylatable Ser99Ala mutant unexpectedly increased the number of myotubes having two to four nuclei, compared with untransfected or empty vector–transfected cells, or with WT Ankrd2–overexpressing cells in which the myotube formation was significantly lower (Figure 5C).

Coverslip-attached myotubes were then exposed for 3 h to 450 μM H_2O_2 and processed for immunocytochemical analysis. As shown in Figure 5A, the arrival of the stimulus induces Ankrd2 to concentrate in the nuclear and perinuclear region. In contrast, the phosphorylation-defective mutant was unable to redistribute into the nucleus upon H_2O_2 exposure (Figure 5A).

Next, by means of cell fractionation we tried to determine in which subcellular compartment Ankrd2 associates to and is phosphorylated by Akt. Nuclear and cytosolic lysates, obtained from differentiating C_2C_{12} myotubes exposed to H_2O_2 or left untreated, were subjected to immunoprecipitation with anti Ankrd2 antibody. Immunocomplexes and their corresponding lysates were resolved on SDS–PAGE and checked with antibodies against phospho-Akt substrates, Ankrd2, phospho-Akt or Akt. Confirming data in Supplemental Figure S1 and Figure 5C show that the amount of Ankrd2 in the nucleus increases upon H_2O_2 stimulation, being almost absent in unstimulated nuclei. Of note, Akt-phosphorylated Ankrd2 was found in both the nuclear and the cytosolic fractions.

Furthermore, a trace amount of Akt resides in the nucleus in the absence of stimulus and, similarly to its cytosolic counterpart, nuclear Akt activity is enhanced by H_2O_2. Despite this, only the cytosolic fraction of Ankrd2 is able to bind to Akt, suggesting that

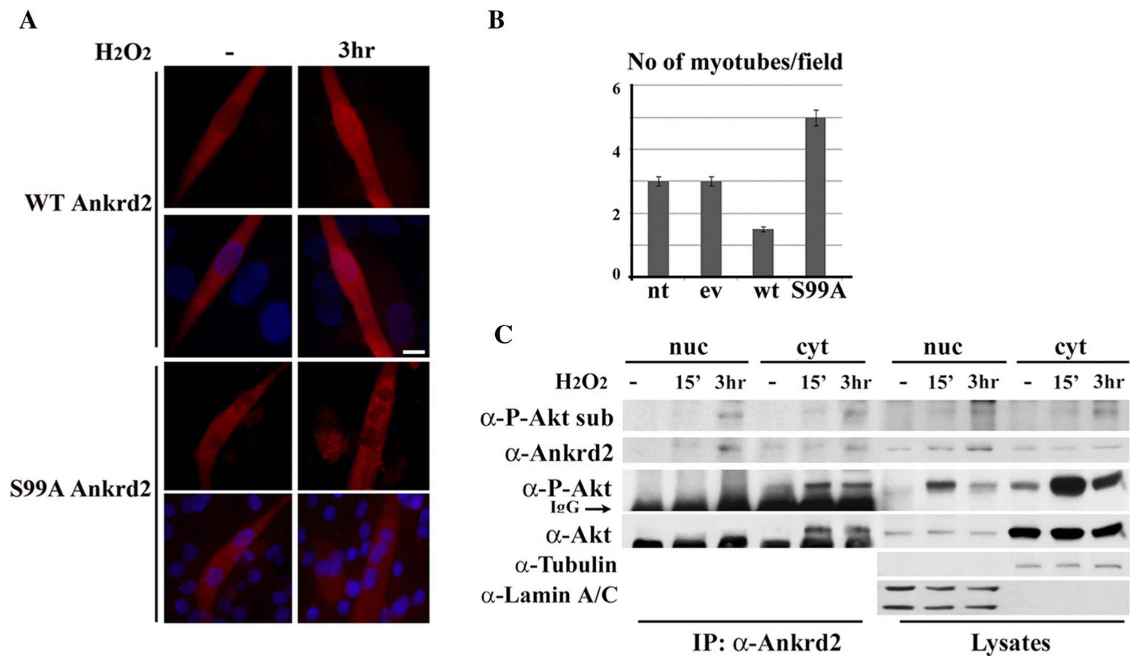

FIGURE 5: Oxidative stress induces nuclear translocation of Ankrd2. (A) Day 1 (D1)-differentiating C_2C_{12} myotubes were transfected with WT or S99A forms of flag-tagged Ankrd2 and exposed to H_2O_2 450 μM for 3 h. Immunofluorescence was performed using an anti-flag TRITC-conjugated antibody. Cell nuclei were counterstained with DAPI. Bar, 10 μM. (B) Statistical analysis (mean values ± SD of counts performed in three different samples). C_2C_{12} multinucleated myotube counts were performed after 2 d of serum deprivation of cells not transfected (nt), transfected with empty vector (ev), wild type (wt), or S99A-mutant Ankrd2. (C) D1-differentiating C_2C_{12} cells were treated with H_2O_2 450 μM for 15 min or 3 h or were left untreated, then nuclear and cytoplasmic fractions were purified. Endogenous Ankrd2 was next immunoprecipitated by anti–Ankrd2 antibody, run on SDS–PAGE, blotted, and checked for the presence of Phospho Akt substrates, Ankrd2, activated and total Akt. Lamin A/C and β-tubulin antibodies were used to exclude nuclear or cytoplasmic contamination from cytoplasmic and nuclear extracts, respectively.

binding and phosphorylation occur in the cytoplasm and precede nuclear translocation (Figure 5C, IP: α-Ankrd2, WB: α- Akt).

Phosphorylation at Ser-99 prevents the outcome of muscle differentiation

Recent studies from Bean and colleagues demonstrate that Ankrd2 is a negative regulator of muscle differentiation (Bean *et al.*, 2008). According to these authors, the overexpression of a WT form of Ankrd2 is responsible for a retardation of muscle differentiation. Therefore, based on these and on our observation of H_2O_2 effect on cyclin D3 (Figure 4A, anti-cyclin D3), we analyzed whether Ankrd2 phosphorylation at Ser- 99 affects the outcome of muscle differentiation.

Differentiating C_2C_{12} cells were transfected with empty vector and WT or Ser99Ala Ankrd2. Then differentiation of the culture was monitored from d1 to d3 of transfection by means of differentiation and muscle-specific markers. As shown in Figure 6, cells overexpressing unphosphorylatable Ser99Ala-Ankrd2 showed a significant increase in the expression of the muscle-specific transcription factor myogenin, with respect to cells transfected with WT Ankrd2 or vector alone. This result was confirmed by both the up-regulation of cyclin D3, which withdraws postmitotic cells from cell cycle, and the down-regulation of cyclin A, which is instead required for S-phase progression. These data were all corroborated by PCNA (Proliferating Cell Nuclear Antigen) profile, which is expressed in proliferating cells and decreases along with differentiation levels. Moreover, the absence of the cleaved form of poly ADP-ribose polymerase (PARP) excluded the activation of apoptotic pathways confirming cell viability.

It is worth noting that Akt phosphorylation/activity (Figure 6, anti–P-Akt/Akt) remains comparable among all samples and therefore cannot account for the change of the expression of myogenic and differentiation markers considered.

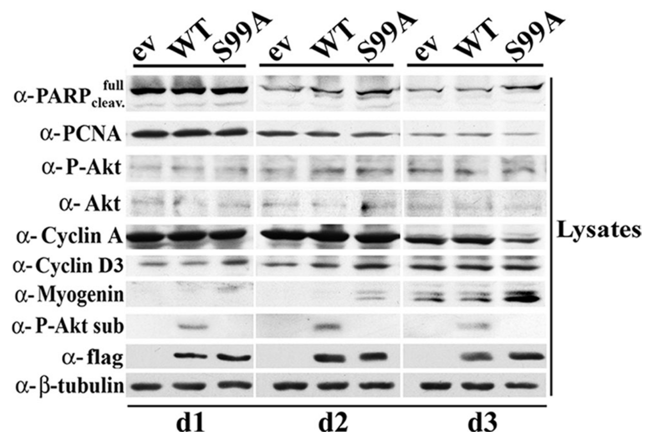

FIGURE 6: Failure of phosphorylation of Ankrd2 at Ser-99 promotes myogenic differentiation. C_2C_{12} cells at 95% confluence were transfected with wild type (wt), S99A mutant flag-Ankrd2, or empty vector and allowed to differentiate in differentiating medium for 1, 2, and 3 d (d1, d2, and d3). Cell lysates were resolved on SDS–PAGE, and the outcome of muscle differentiation was monitored by differentiation and cell cycle specific markers myogenin, cyclin A and D3, and PCNA (Proliferating Cell Nuclear Antigen). The absence of cleaved PARP confirmed cell viability. β-tubulin was shown for equal loading. Akt activity was monitored during differentiation by anti–phospho Akt Ser-473 (anti P-Akt).

DISCUSSION

Ankrd2 is a protein specifically expressed in skeletal and cardiac muscle. It plays both structural and regulatory roles. Indeed, in mature muscle, Ankrd2, which is mainly localized in the I-band of the fibers, physically interacts with many structural proteins as titin (Miller *et al.*, 2003), myopalladin (a nebulin-anchoring protein+, and telethonin (Kojic *et al.*, 2004). It has also been reported, however, that Ankrd2 can interact with regulatory nuclear proteins as promyelocytic leukemia protein, p53, and with transcription factor YB-1 in vitro (Kojic *et al.*, 2004). In muscle stressed by cardiotoxin injection or by dry ice, Ankrd2 accumulates in the nuclei of sarcomere-damaged myofibers (Tsukamoto *et al.*, 2008). Consistent with Ankrd2 nuclear accumulation in euchromatin, where genes are activated, and with its binding to transcription factors, it has been suggested that Ankrd2 may act as a transcriptional regulator in the nuclei of injured muscles (Tsukamoto *et al.*, 2008). Of note, Ankrd2 transcripts and protein increase in response to muscle injury, lengthening contractions, and after physical exercise (Barash *et al.*, 2004; Lehti *et al.*, 2009), underscoring the importance of the role of Ankrd2 in the rescue of stressed or damaged muscle cells.

A recent study, performed on C_2C_{12} myoblasts, suggests that Ankrd2 contributes to the proliferation and apoptosis machinery during myogenic differentiation mainly through the p53 network (Bean *et al.*, 2008).

Altogether, these data suggest a dual role for Ankrd2: During muscle differentiation, Ankrd2 contributes to regulate the balance between proliferation and apoptosis of differentiating myoblasts and stabilizes forming myotubes; in addition, Ankrd2 participates in the machinery that regulates protection of fibers after muscle injury.

By using a proteomic approach, we have identified Ankrd2 as a novel Akt substrate in C_2C_{12} muscle cells. Running the human Ankrd2 sequence into the Scansite database predicted Ser-99 to be a putative, high-stringency site for Akt phosphorylation. This serine lies in a canonical Akt phosphorylation motif, highly conserved in all Ankrd2 sequences cloned so far. Indeed, Ser-99 of Ankrd2 is one of the more than 500 phospho-Akt motif sites identified in a recent large-scale phosphoproteomic analysis, using the phospho-Akt substrate antibody to capture peptides (Moritz *et al.*, 2010). By mutational analysis and in vitro kinase assays, we show here that Ankrd2 is selectively phosphorylated by Akt at Ser-99 (Figure 2A).

Akt1 and Akt2 are closely related protein kinases with a conserved phosphorylation motif. They are all activated through similar mechanisms involving upstream kinases PI3K and PDK1. The function of each Akt isoform has been studied in knockout mice as well as in isoform-specific knockdowns in cell cultures by specific siRNA. These studies reveal the existence of both redundant and distinct functions of Akt isoforms. In particular, recent findings demonstrated that Akt1 and Akt2 can even have opposing roles. There is little information, however, about the isoform-specific downstream substrates of Akt. Interestingly, our model provides a further confirmation of the existence of distinct substrates for Akt1 and Akt2. Indeed, by both in vitro kinase assays using recombinant active Akt1 or Akt2, and by targeting each isoform with siRNAs, we demonstrated that Ankrd2 is an Akt2 selective substrate (Figures 2A and 3A). These findings are in good agreement with the stronger binding affinity shown by Ankrd2 toward Akt2 (Figure 3B), with respect to Akt1 (lower) or Akt3 (absent); moreover, Akt2 activity is a requisite for its binding to Ankrd2 (Figures 2D and 3C). Similar results were obtained by means of Akt inhibitors VIII and XII (selective for Akt1/2 and Akt2, respectively) both in HEK 293T cells stimulated with insulin (Figure 2D), and in C_2C_{12} myoblasts exposed to H_2O_2 (Figure 4C). All in all, these data represent new, important advances in the issue of Akt substrate isoform specificity as they establish Akt2 as the Ser99-Ankrd2 kinase. This conclusion does not exclude the possibility, however, that other AGC kinases, with similar substrate specificity, such as SGK (Brunet *et al.*, 2001), might phosphorylate Ankrd2 at Ser-99 or at a different epitope, possibly in a different subcellular compartment or in varied biological conditions.

A large body of evidence supports the activation of the PI3K/Akt signaling axis upon the arrival of ROS in several cellular models, as fibroblasts (Shaw *et al.*, 1998; Wang *et al.*, 2000), vascular smooth muscle cells (Ushio-Fukai *et al.*, 1999), and rat primary astrocytes (Salsman *et al.*, 2001). The activation of the PI3K/Akt pathway might have a pivotal role in controlling several downstream antiapoptotic and prosurvival cascades (Mercurio and Manning, 1999; Storz and Toker, 2003). Here we demonstrate that the PI3K/Akt pathway is also activated by stress in C_2C_{12} myoblasts. Moreover, in differentiating C_2C_{12}, stress promotes Ankrd2 phosphorylation by Akt and binding to the kinase (Figure 4, B and C). In muscle cells, ROS release occurs in response to mechanical stress, contraction, and, after a prolonged muscle exercise, as a consequence of aging and pathological conditions, as primary or secondary myopathies. Oxidative stress activates various signal transduction pathways, culminating with the activation of transcription factors nuclear factor κB and Jun N-terminal kinase. Through these pathways, ROS stimuli lead to the expression of proteins involved in the detoxification of ROS themselves, driving in turn to the rescue of the cells. As the stress becomes too long or too intense, however, cells respond by triggering protein damage, peroxidation of lipids and nucleotides, membranes break (Konat, 2003), and eventually apoptosis occurs (Oldham and Bowen, 1998; Orzechowski *et al.*, 2002).

In our model, we report that short-term H_2O_2 stimulation triggers a down-regulation of cyclin D3 (Figure 4A), confirming that oxidative stress can delay muscle differentiation (Langen *et al.*, 2002; Ardite *et al.*, 2004; Hansen *et al.*, 2007; Zaccagnini *et al.*, 2007).

What is more, the forced expression of an Akt-phosphorylation–defective Ankrd2 mutant in C_2C_{12} cells, induced to differentiate, robustly increased both myotube formation (as assessed by multinucleated cell counting, Figure 5B) and the amount of differentiation and muscle specific markers as cyclin A, cyclin D3, and myogenin (Figure 6), suggesting that phosphorylation of Ankrd2 at Ser-99 can hamper expression of genes essential for myogenic differentiation. Altogether, we conclude that Akt-phosphorylated Ankrd2 contributes to the oxidative stress–dependent slackening of muscle differentiation.

In unstimulated conditions, Ankrd2 is prominently located in the cytoplasm of differentiating cells. H_2O_2, however, induces Ankrd2 to massively redistribute into the nuclear compartment, where its binding partners are (Kojic *et al.*, 2004). Conversely, the phosphorylation-defective form of Ankrd2 is unable to relocalize into the nucleus upon H_2O_2 exposure, indicating that Ankrd2 phosphorylation regulates its cellular distribution and, in turn, modulates Ankrd2 binding to target proteins, including transcription factors implicated in muscle differentiation (Kojic *et al.*, 2004).

Possibly, upon the arrival of the stimulus, following Ankrd2 binding to Akt and its subsequent phosphorylation, Ankrd2 dissociates from this complex to migrate to the nucleus and interact with its effectors. Indeed, although a small amount of Akt resides in the nucleus and responds to H_2O_2 stimulation similarly to its cytosolic counterpart, our results demonstrate that Ankrd2 associates to Akt in the cytoplasm (Figure 5, A–C, and Supplemental Figure S1).

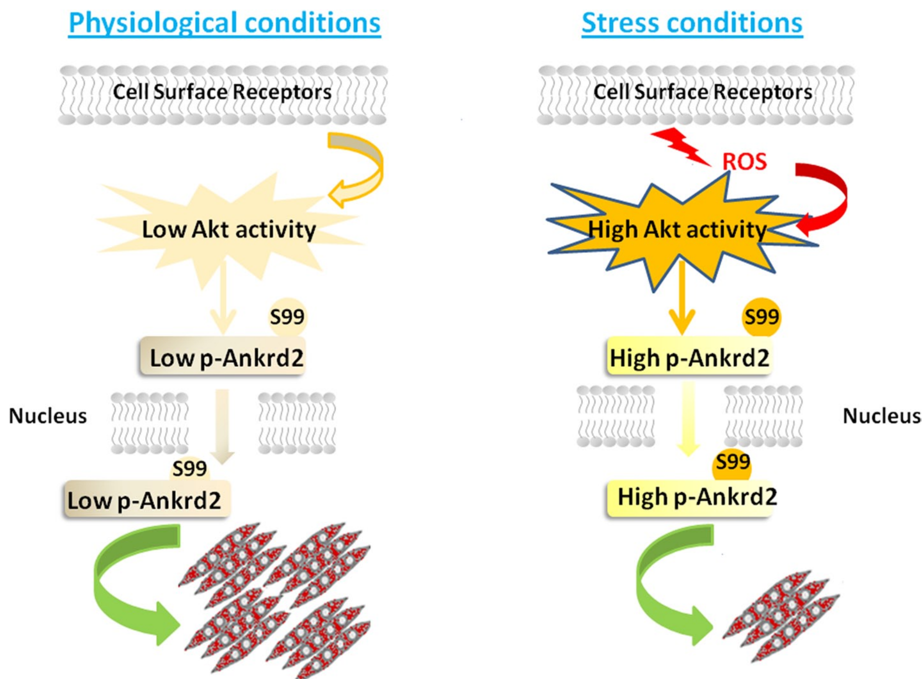

FIGURE 7: Model for inhibition of myogenesis following Ankrd2 phosphorylation by Akt2 induced by stress conditions. During normal physiological conditions, Ankrd2, which is weakly constitutively phosphorylated at Ser-99, might concur to control muscle differentiation rate, ensuring the time needed to allow proper assembly of the whole differentiation machinery. Under stressful conditions that trigger the production of ROS as H_2O_2 Akt2 induces a huge increase in Ankrd2 phosphorylation at Ser-99, inducing its nuclear translocation and resulting in a negative regulation of muscle differentiation pathways.

All together, Ankrd2 phosphorylation by Akt should be considered a limiting factor to muscle differentiation pathways. Indeed, the basal phosphorylation of Ankrd2 in unstimulated conditions (Figures 1A and 4A) might concur to control muscle differentiation rate, ensuring the time needed to allow proper assembly of the whole differentiation machinery. Similarly, H_2O_2-induced Ankrd2 phosphorylation negatively interferes with muscle differentiation pathways. Therefore, during stress, differentiating myoblasts might sense the extent of the stress and exploit the negative effects of phospho-Ankrd2 to decide whether to drive apoptotic or rescue pathways. A model for this mechanism has been proposed in Figure 7.

As recently another MARP, Ankrd1, has been identified to be point mutated in four different dilated cardiomyopathies (Arimura *et al.*, 2009; Moulik *et al.*, 2009), it will be interesting in the future to check the phosphorylation of Ser-99 in patients affected by different types of musculoskeletal pathologies.

MATERIALS AND METHODS

Cell culture and transfection

C_2C_{12} mouse myoblasts and HEK 293T cells were cultured in DMEM high glucose (Invitrogen–Life Technologies, Groningen, The Netherlands) supplemented with 10% fetal calf serum (EuroClone, Leeds, UK) at 37°C and 5% CO_2. Differentiation of C_2C_{12} was induced by culturing 100% confluent cells in a differentiating medium consisting of DMEM high glucose plus 2% horse serum (EuroClone). Differentiation was quantified by monitoring muscle and cell-cycle specific markers and by counting the number of myotubes (with more than two nuclei) present in a field under a phase-contrast microscope (Nikon Instruments, Calenzano, Italy).

Transient transfections of HEK 293T cells were performed by using the calcium phosphate method. C_2C_{12} cells were transfected using INGENIO reagent (MirusBio, Madison, WI), with the Amaxa electroporator, according to the manufacturer's instructions. Where indicated, cells were treated with 450 μM H_2O_2, 1.6 μM insulin, and eventually pretreated 30 min before stimulus with 10 μM LY294002, 10 μM Akt inhibitor IV, 5 μM PD 98059, 5 μM rapamycin (all obtained from Sigma, St. Louis, MO), 10 μM perifosine (Æterna Zentaris, Frankfurt, Germany) or with 6 μM Akti 1/2 and Akti 2 (inhibitor VIII and XII, obtained from Merck-Millipore, Darmstadt, Germany).

Plasmids

WT, flag-tagged human Ankrd2, cloned in pMe-18S, was a gift of K. Shomori (Faculty of Medicine, Tottori University, Japan; described in Shomori *et al.*, 2007). The cDNA was used as template to generate Ser99Ala mutant by means of the QuikChange strategy (Stratagene, LaJolla, CA). The following mutation primers were used: forward primer, GTG CGC AAG ACG *g*CC CTG GAC CTG C; reverse primer, G CAG GTC CAG GG*c* CGT CTT GCG CAC. The mutagenesis was verified by a DNA sequencing service (BMR, Padua, Italy). Mammalian expression vectors containing all the Akt isoforms (HA -Akt1, -Akt2, and -Akt3 in pCDNA 3), Akt1 and Akt2 specific shRNAs in pLKO were donated by A. Toker (Harvard Medical School, Boston, MA).

RNA interference

To produce lentiviral supernatants, 293T cells were cotransfected with control or Akt1 or Akt2 shRNA-containing pLKO vectors, VSVG, and psPAX2 for 48 h. Where indicated, cells were infected with Akt-1 and -2 shRNA lentiviral vectors or empty vector. Forty-eight hours after infection, cells were exposed to 2 μg/ml puromycin (Sigma), serum starved overnight, and treated with insulin for 30 min. Total cell lysates were subjected to immunoblot analysis.

Protein fractionation by MicroRotofor isoelectric focusing

Pellets of C_2C_{12} cells (~2.5 mg of protein) were dissolved in 2.5 ml of isoelectric focusing buffer (7 M urea, 2 M thiourea, 5 mM dithiothreitol, 4% [wt/vol] CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate), and 2% [vol/vol] Pharmalyte, pH 3–10), applied to MicroRotofor (Bio-Rad Laboratories, Hercules, CA), and electrophoresed according to the manufacturer's instructions for 3 h at a constant power of 1 W at 20°C. After electrophoresis, protein fractions from each compartment were harvested, denatured in SDS sample buffer, boiled, and subjected to monodimensional electrophoresis.

Preparation of whole-cell extracts and subcellular fractions

Whole cell lysates were prepared by the addition of AT lysis buffer (20 mM Tris-HCl, pH 7.0, 1% Nonidet P-40, 150 mM NaCl, 10% glycerol, 10 mM EDTA, 20 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml pepstatin)

at 4°C. Cellular lysates were next sonicated and cleared by centrifugation.

C_2C_{12} nuclei were purified as follows. Briefly, to 5×10^6 cells was added 400 µl of nuclear isolation buffer (10 mM Tris-HCl, pH 7.8, 1% Nonidet P-40, 10 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin and leupeptin, and 5 mM NaF) for 8 min on ice. MilliQ water (400 µl) was then added to swell cells for 3 min. Cells were sheared by passages through a 22-gauge needle. Nuclei were recovered by centrifugation at $400 \times g$ at 4°C for 6 min and washed once in 400 µl of washing buffer (10 mM Tris-HCl, pH 7.4, and 2 mM $MgCl_2$, plus inhibitors as described earlier in the text). Supernatants (containing the cytosolic fractions) were further centrifuged for 30 min at $4000 \times g$. Isolated nuclear and cytoplasmic extracts were finally lysed in AT lysis buffer, sonicated, and cleared by centrifugation. Before being used for Western blot or immunoprecipitation experiments, total and single cellular fractions were quantified using a Bradford protein assay (Bio-Rad). Purity of isolated nuclei and cytoplasms were analyzed by detection of ß-tubulin and Lamin A/C, respectively.

Immunochemical analysis and immunoblotting

Eighty micrograms of proteins from the whole, nuclear, or cytoplasmic extracts were separated on 10–12% SDS–PAGE gels. Proteins were blotted onto nitrocellulose for subsequent immunodetection with the specific antibodies and detected by using the enhanced chemiluminescence method (Amersham Biosciences, Arlington Heights, IL). The expression of specific proteins was analyzed by the use of the following antibodies: polyclonal anti-Ankrd2, donated by S. Labeit (University of Mannheim, Germany) and polyclonal anti-Ankrd2 (purchased from PTGlab, Chicago, IL), anti: -total Akt (N-term) SC-1618 and -total Akt (C-20) SC-1619, -Akt1 SC-7126, -Akt2 SC-7127, -Lamin A/C sc-6215, -actin C-20, -PARP, -Cyclin D3, -Cyclin A (Santa Cruz Biotechnology, Santa Cruz, CA), anti–Phospho-Akt Substrate and -Phospho-Akt Ser473 (from Cell Signaling Technology, Danvers, MA, respectively: #9611 and #4051), anti-HA, -Flag, and -ß-tubulin, from Sigma.

For immunoprecipitation studies, ~1–6 mg of precleared lysates were incubated with 3 µg of antibody or with host immunoglobulin G to provide a negative control for 3 h. Then, immunocomplexes were pelleted by 35 µl of 50% (vol/vol) of protein A/G agarose slurry (Santa Cruz Biotechnology) at 4°C with gentle rocking. Pellets were washed twice with phosphate-buffered saline containing 1% Nonidet- P40, twice in TNE (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA), and boiled in Laemmli sample buffer. Samples were resolved by SDS–PAGE and processed as described earlier in the text. Molecular weights (with an error of ±5%) of the proteins identified by the anti–phospho-Akt substrate antibody were calculated according to Quantity One 1D Analysis Software, following the acquisition of the images with a GS-800 Densitometer (all from Bio-Rad).

Immunofluorescence microscopy

Myoblasts and myotubes grown on glass coverslips were fixed in 4% paraformaldehyde at 4°C for 10 min or methanol at –20°C for 7 min. Cells were blocked in 4% bovine serum albumin–phosphate-buffered saline and incubated with primary antibodies overnight at 4°C. Coverslips were next washed and incubated with fluorescence-labeled secondary antibodies (DAKO, Glostrup, Denmark), for 1 h at room temperature. Slides were washed and mounted with an antifade reagent in glycerol and observed with a Nikon E600 fluorescence microscope equipped with a digital camera.

In vitro kinase assay

Pellets of flag Ankrd2 (WT or Ser99Ala mutant), obtained by immunoprecipitation from lysates of HEK 293T cells overexpressing their respective cDNAs, were separately combined with recombinant active Akt1 or Akt2 (SignalChem, Richmond, Canada) in the Akt kinase reaction buffer comprising 20 mM Tris-Cl, pH 7.5, 25 mM $MgCl_2$, 80 µM ATP, and 10 µCi/assay [γ-^{32}P]ATP (3000 Ci/mmol; Amersham GE Healthcare Europe, Milan, Italy) for 30 min at 30°C. The reactions were stopped by the addition of Laemmli sample buffer, resolved by SDS–PAGE, blotted onto nitrocellulose, and detected by both autoradiography and Western immunoblotting.

All the images shown in this article are representative of three independent experiments carried out under the same conditions. Images from immunochemical and immunofluorescence studies were processed using Adobe Photoshop CS 8.0 software (Adobe Systems).

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