Oncogenic K-Ras suppresses IP₃-dependent Ca²⁺ release through remodeling of IP₃Rs isoform composition and ER luminal Ca²⁺ levels in colorectal cancer cell lines.

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Summary

The GTPase Ras is a molecular switch engaged downstream of G-protein coupled receptors and receptor tyrosine kinases that controls multiple cell fate-determining signalling pathways. Ras signalling is frequently deregulated in cancer underlying associated changes in cell phenotype. Although Ca²⁺ signalling pathways control some overlapping functions with Ras, and altered Ca²⁺ signalling pathways are emerging as important players in oncogenic transformation, how Ca²⁺ signalling is remodelled during transformation and whether it has a causal role remains unclear. We have investigated Ca²⁺ signalling in two human colorectal cancer cell lines and their isogenic derivatives in which the mutated K-Ras allele (G13D) has been deleted by homologous recombination. We show that agonist-induced Ca²⁺ release from intracellular stores is enhanced by loss of K-Ras G13D through an increase in the ER store content and a modification of IP₃R subtype abundance. Consistently, uptake of Ca²⁺ into mitochondria and sensitivity to apoptosis was enhanced as a result of K-Ras G13D loss. These results suggest that suppression of Ca²⁺ signalling is a common
response to naturally occurring levels of K-Ras$^{G13D}$ that contributes to a survival advantage during oncogenic transformation.

**Introduction**

Ras proteins serve as molecular switches downstream of receptor tyrosine kinases and upstream of the Raf protein kinases (Cully and Downward, 2008; Downward, 2003a; Downward, 2003b; Schulze et al., 2004). This pathway is frequently de-regulated in cancer due to mutation in RTKs (EGFR), Ras itself (~20% of all human cancers) and B-Raf (Downward, 2003b). These mutations elicit significant consequences for cell fate due to their position as upstream regulators of multiple pathways involved in regulation of cell cycle, metabolism and cell death – hallmarks of the transformed phenotype (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). This central role of Ras and the downstream pathways it engages have been targeted by the pharmaceutical industry in the development of cancer therapeutics. Indeed, drugs targeting the B-Raf-Mek-Erk pathway have now been approved in the clinic (Belden and Flaherty, 2012; Little et al., 2013).

As Ras lies upstream of multiple cellular pathways, redundancy in function between these signal transduction cascades allows transformed cells to overcome drug targeting and develop resistance (Little et al., 2013). Many of these downstream pathways are also dysregulated in cancer (Wu et al., 2013). Understanding the nature of interactions between Ras and other major cellular signalling pathways is therefore essential for development of effective strategies for suppression of Ras-driven cancer (Wu et al., 2013). A major, but as yet undefined signalling interaction in oncogenic transformation is that between Ras and Ca$^{2+}$.

Ca$^{2+}$ is a pleiotropic signalling messenger that, like Ras, plays key roles in life and death choices including the decision to proliferate or die by apoptosis (Berridge et al., 2003) (Berridge et al., 1998). Oscillations in cytoplasmic Ca$^{2+}$ are necessary to sustain the cell cycle via CaM (Cullen and Lockyer, 2002; Kahl and Means, 2003), whereas
Ca\textsuperscript{2+} overload by the mitochondria is an initiator of the intrinsic apoptotic cascade (Rizzuto et al., 2003). Not surprisingly therefore, deregulation of Ca\textsuperscript{2+} homeostasis has been reported in diseases associated with overt or diminished proliferation and increased or insufficient cell death. Cancer cells are characterized in part by uncontrolled proliferation and apoptosis evasion (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011); these characteristics may be supported by a remodeling of their Ca\textsuperscript{2+} signalling toolkit (Roderick and Cook, 2008). Indeed, alterations in the expression of a number of Ca\textsuperscript{2+}-handling proteins have been reported in various tumours (Monteith et al., 2007) but attempts to formulate general principles of Ca\textsuperscript{2+} signalling alterations in cancer have thus far failed. Little consistency in the alterations in Ca\textsuperscript{2+} protein expression is found among different tumours and between studies. Moreover, few studies have clarified whether altered Ca\textsuperscript{2+}-signalling contributes to the cancer phenotype or is a consequence.

Nowhere is this more apparent than in studies linking Ras to Ca\textsuperscript{2+} signalling, which go back over 25 years and reveal a complex interplay between these pathways. For example, Ras was shown to enhance agonist-regulated IP\textsubscript{3} production (Hashii et al., 1993; Lang et al., 1991; Wakelam et al., 1986), a result that may be due to the ability of Ras protein to bind to PLC\textepsilon (Bunney et al., 2006; Bunney and Katan, 2006). On the other hand, Ca\textsuperscript{2+} signalling can activate certain Ras guanine nucleotide exchange factors or Ras GTPase activating proteins to promote or inhibit activation of Ras and Ras-dependent signalling (Cook and Lockyer, 2006; Roderick and Cook, 2008). The normal interplay between these events is complex and is made all the more so in cells expressing mutant oncogenic variants of Ras such as those harbouring missense substitutions at Gly12, Gly13 or Gln61 (Barbacid, 1987), which prevent the hydrolysis of GTP by GAP proteins, resulting in Ras being permanently active (Bollag and McCormick, 1991). These de-regulated Ras oncoproteins activate several effector pathways and contribute to virtually all of the hallmarks of the cancer cell (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011) including promoting cell proliferation and survival. This pleiotropy of Ras undoubtedly contributes to some of the striking cell- and tissue-specific differences in the regulation of Ca\textsuperscript{2+} signalling seen in studies with oncogenic Ras mutants. In addition, however, it is apparent that mutant Ras proteins can elicit quite different effects depending on their
expression level. Most strikingly, conditional over-expression of oncogenic K-Ras elicits cell cycle arrest and senescence in primary mouse embryo fibroblasts whereas conditional expression at endogenous levels causes cell proliferation and oncogenic transformation (Tuveson et al., 2004). Thus, whilst studies employing conditional over-expression of mutant Ras proteins have merits, it is important to confirm results in cell systems with native expression levels of mutant Ras to avoid artefacts arising from over-expression.

To overcome this issue, we have taken advantage of isogenic cell line pairs in which the mutant K-Ras allele has been ablated by homologous recombination (Shirasawa et al., 1993). The parental cancer cell line harbouring the mutant allele can then be directly compared with an isogenic derivative that is identical apart from the lack of oncogenic Ras. This approach has the benefit of comparing the effects of a single copy of mutant Ras rather than using supra-physiological expression. Using this approach, we show that oncogenic K-Ras inhibits Ca\textsuperscript{2+} release from the ER, reduces ER Ca\textsuperscript{2+} levels and suppresses Ca\textsuperscript{2+} flux to the mitochondria. These results suggest that suppression of Ca\textsuperscript{2+} signalling is a common response to naturally occurring levels of K-Ras\textsuperscript{G13D} and may contribute to a survival advantage during oncogenic transformation.

**Results**

**IP\textsubscript{3}-induced Ca\textsuperscript{2+} release (IICR) is increased in K-Ras\textsuperscript{G13D} deleted cells**

Although alterations in expression of a number of proteins involved in Ca\textsuperscript{2+} regulation in various tumour types and tumour derived cell lines have been described (Monteith et al., 2007), few studies have analysed how Ca\textsuperscript{2+} signalling is altered as a result of transformation. Moreover, the nature and role of the effect of the presence of natively expressed driving oncogenes upon Ca\textsuperscript{2+} homeostasis has not been determined. As such, consensus regarding how Ca\textsuperscript{2+} signalling participates in cellular transformation is lacking (Roderick and Cook, 2008). Contributing to this great variability is the problem in identifying appropriate experimental controls for the cancer cell studied and the issues associated with use of experimental systems in which pleiotropic oncogenes are expressed at supra-physiological levels (Tuveson et al., 2004). In this
study, we sought to analyse the effects of a single oncogenic allele at physiological expression levels. To this end, we compared the HCT116 colorectal cancer cell line (K-RasG13D/WT) with its isogenic derivative HKH2 (K-RasWT) in which the mutated Ras allele has been deleted by homologous recombination. In contrast to HCT116 cells, HKH2 cells do not grow in soft agar and do not form tumours in nude mice (Shirasawa et al., 1993). We employed these cell lines to evaluate whether the presence of the endogenous oncogenic K-RasG13D allele modified the generation of Ca2+ signals. As shown in Fig. 1, as a consequence of loss of K-RasG13D in HKH2 cells Ca2+ signals induced following stimulation of purinergic receptors with ATP were enhanced when compared to HCT116 cells (Fig. 1Ai). This difference was evident in the percentage of responding cells (Fig. 1Aii), in the amplitude (Fig. 1Aiii) and in the integral (area under the curve; AUC) of the Ca2+ transients (Fig. 1Aiv).

To isolate the contribution of Ca2+ influx to the agonist-induced Ca2+ transient, experiments were performed in the absence of extracellular Ca2+. As observed in Ca2+-containing buffer, the AUC and responsiveness to agonist (applied at a concentration where the greatest differences in agonist responses were observed in Ca2+-containing buffer) remained greater in HKH2 than HCT116 cells when Ca2+ was omitted from the imaging buffer. These data therefore indicated that K-RasG13D in HCT116 cells was acting to suppress Ca2+ release from the ER (Fig. 1B).

To further probe the interaction between Ras and Ca2+ signaling in HCT116, Ras expression was also suppressed by siRNA. Using this approach, Ras expression was reduced by 85% when compared to HCT116 cells transfected with control, non-targeting siRNA (Fig. 2A). siRNA depletion of Ras in HCT116 cells resulted in a significant increase in ATP-stimulated Ca2+ signals in these cells (Fig. 2B). The increase in Ca2+ signalling was manifest as an increase in the percentage of responding cells and in the amplitude and AUC of the Ca2+ responses (Fig. 2Bii-iv). These data are consistent with that observed in HKH2 cells and support the conclusion that the difference between HCT116 and HKH2 cells was due to K-RasG13D ablation and not a phenotype developed as a result of HKH2 culture since their initial generation.

To test whether the enhanced Ca2+ responses observed as a result of K-RasG13D deletion in HCT116 cells was a general feature of GPCR signalling in these cells, we
examined histamine-induced Ca\textsuperscript{2+} responses, which proceed through a similar GPCR-G_q-PLC-IP\textsubscript{3} pathway. In response to this agonist, a greater percentage of HKH2 cells exhibited Ca\textsuperscript{2+} transients, which were of a greater amplitude and AUC than those observed in HCT116 cells (Fig. 3A). These data indicated that the alteration in Ca\textsuperscript{2+} fluxes was not specific to differences in purinergic receptor signalling but was a more general effect, involving signals downstream of GPCR engagement.

To directly address whether an alteration in IP\textsubscript{3} signalling contributed to the enhancement of ER Ca\textsuperscript{2+} release in cells lacking K-Ras\textsuperscript{G13D}, Ca\textsuperscript{2+} release was induced with a cell permeant esterified form of IP\textsubscript{3} (myo-inositol 1,4,5-trisphosphate hexakis(butyryloxy)methyl (IP\textsubscript{3}BM), which was perfused over the cells in Ca\textsuperscript{2+} free imaging buffer during the course of the experiment (Conway et al., 2006; Kasri et al., 2004; Thomas et al., 2000). Using this approach, IP\textsubscript{3}Rs are directly engaged, bypassing GPCR, G_q, PLC and endogenous IP\textsubscript{3}. As in experiments using ATP and histamine, Ca\textsuperscript{2+} signals induced by IP\textsubscript{3}BM were also greater in HKH2 cells that in their HCT116 counterparts (Fig. 3B).

Since K-Ras is frequently mutated in colorectal cancer, we investigated whether Ca\textsuperscript{2+} signalling was also remodeled as a result of loss of K-Ras\textsuperscript{G13D} in a second independent colorectal cancer cell line, DLD-1 (Shirasawa et al., 1993). As performed for HCT116 cells and their isogenic derivatives, experiments were carried out in Ca\textsuperscript{2+}-free imaging buffer to restrict our analysis to Ca\textsuperscript{2+} release from the ER. ATP-induced Ca\textsuperscript{2+} fluxes were greater in the K-Ras\textsuperscript{G13D}-deleted DKO4 cell line (K-Ras\textsuperscript{G13D/WT}) than in their parental isogenic DLD-1 cell line (K-Ras\textsuperscript{G13D/WT}) (Fig. 4). This was manifest as an increase in the percentage of responding cells and in the amplitude and AUC of the Ca\textsuperscript{2+} responses (Fig. 4ii-iv). Taken together, these data show that oncogenic K-Ras\textsuperscript{G13D} limits IICR in both HCT116 and DLD-1 cells.

**ER Ca\textsuperscript{2+} content is increased in K-Ras\textsuperscript{G13D}-deleted cells**

Since the magnitude of Ca\textsuperscript{2+} released from the ER is determined by its state of filling, we hypothesized that the enhanced Ca\textsuperscript{2+} release observed following K-Ras\textsuperscript{G13D} deletion in HKH2 cells was due to an increase in content of the IP\textsubscript{3}-releasing ER Ca\textsuperscript{2+} store. To assess ER Ca\textsuperscript{2+} levels, the magnitude of the Ca\textsuperscript{2+} mobilized from the ER by SERCA pump inhibitor thapsigargin (Tg) was analysed. Through inhibition of SERCA, Tg reveals the non-specific Ca\textsuperscript{2+} leak from the ER causing Ca\textsuperscript{2+}
accumulation in the cytosol. As store depletion with Tg leads to Ca\textsuperscript{2+} influx across the plasma membrane, measurements were performed in Ca\textsuperscript{2+}-free imaging buffer. Application of Tg induced an elevation in intracellular Ca\textsuperscript{2+} in both the HCT116 and HKH2 cell lines (Fig. 5A). The amplitude and AUC of the Tg-induced Ca\textsuperscript{2+} transient was however significantly greater in the HKH2 cell line compared to HCT116 cells (Fig. 5Aii, iii).

To complement these data and to accommodate for the indirect nature of using the Tg-induced elevation in cytosolic Ca\textsuperscript{2+} as a measure of ER luminal Ca\textsuperscript{2+} content, ER free Ca\textsuperscript{2+} was also measured directly using a genetically-encoded GFP-based Ca\textsuperscript{2+} indicator targeted to the ER (known as D1ER) (Palmer et al., 2004). This indicator relies upon a Ca\textsuperscript{2+}-dependent change in Förster resonance energy transfer (FRET) between cyan (CFP) and yellow (YFP) derivatives of green fluorescent protein (GFP). D1ER was expressed in a reticular pattern and co-localised with the ER protein calnexin in both cell types, confirming its ER localisation (Fig. 5Bi). In resting cells, greater FRET (YFP/CFP ratio) was observed in the HKH2 cell line than in the HCT116 cell line, indicating higher basal Ca\textsuperscript{2+} in the ER of this cell line (Fig. 5Bii). Application of the Ca\textsuperscript{2+} ionophore ionomycin to fully deplete Ca\textsuperscript{2+} from the ER store, resulted in a decline in FRET to a lower plateau, which was equivalent between the two cell types (Fig. 5Bii). The similar Ca\textsuperscript{2+}-free FRET between both cell types indicated that D1ER was behaving equivalently in the two cell types. A ratio of basal FRET to Ca\textsuperscript{2+}-free FRET was therefore used to normalize ER Ca\textsuperscript{2+} levels, which also indicated greater Ca\textsuperscript{2+} levels in the ER of HKH2 than HCT116 cells (Fig. 5Biii).

**IP\textsubscript{3}R isoform expression is remodeled and SERCA2b expression is increased in K-Ras\textsuperscript{G13D} deleted cells.**

Having identified that an increase in ER Ca\textsuperscript{2+} contributed to the enhanced Ca\textsuperscript{2+} signalling in K-Ras\textsuperscript{G13D} deleted cells, an analysis of proteins involved in ER Ca\textsuperscript{2+} signalling was carried out. In these experiments, as we have employed elsewhere when analyzing ER proteins of a high molecular weight (Drawnel et al., 2012; Harzheim et al., 2009), the ER membrane protein calnexin was used as a loading control for normalization of the protein of interest between cell types. Expression of calnexin was found to exhibit a similar expression profile between HCT116 and HKH2 cells as two other proteins - GAPDH and β-actin – that are routinely used for
normalization in immunoblotting (Fig S1). The expression of SERCA2b, which is primarily responsible for ER Ca$^{2+}$ sequestration, was increased in HKH2 cells (Fig. 5C), whereas the expression of calreticulin, the major Ca$^{2+}$ storage protein in non excitable cells, was not altered between the two cell types (Fig. 5D). Since SERCA3 upregulation has been reported in cancer (Brouland et al., 2005), its expression was also investigated but found to be unchanged in cells lacking K-Ras$^{G13D}$ (Fig. S1). Notably, the expression profile of IP$_3$Rs was significantly different between HKH2 and HCT116 cells. Specifically, IP$_3$R3 expression was increased and IP$_3$R1 expression reduced in the HKH2 K-Ras$^{G13D}$-deleted cells when compared to HCT116 cells (Fig. 5E). IP$_3$R2 expression was not detectable in either cell type (Fig. S1).

**K-Ras$^{G13D}$-deleted cells exhibit increase mitochondrial Ca$^{2+}$ uptake and sensitivity to apoptosis.**

Mitochondrial Ca$^{2+}$ uptake is a low affinity process that occurs in a privileged manner at microdomains of high Ca$^{2+}$ generated by IP$_3$Rs located at sites where the ER and mitochondria are in close proximity (Csordas et al., 2006; Duchen, 2000; Rizzuto et al., 1998; Rizzuto and Pozzan, 2006). Ca$^{2+}$ flux from ER-localized IP$_3$Rs to the mitochondria has been shown to play an important role in regulation of cell death and metabolism (Cardenas et al., 2010; Pinton et al., 2001) – cell properties remodeled during oncogenic transformation (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). Given the enhancement in IP$_3$-mediated Ca$^{2+}$ signalling observed in K-Ras$^{G13D}$-deleted HKH2 cells, we hypothesized that mitochondrial Ca$^{2+}$ uptake would also be enhanced and might contribute to greater sensitivity to death-inducing stimuli in the K-Ras$^{G13D}$-negative cells. The effect of K-Ras deletion upon mitochondrial Ca$^{2+}$ uptake during IP$_3$-stimulated Ca$^{2+}$ release from the ER was therefore analysed. To induce equivalent Ca$^{2+}$ signals between cell types and to restrict the source for mitochondrial Ca$^{2+}$ sequestration to Ca$^{2+}$ arising from IP$_3$Rs, experimental conditions were used in which a maximal concentration of ATP was applied and cells were imaged in Ca$^{2+}$-free imaging buffer. Mitochondrial matrix Ca$^{2+}$ was measured by confocal imaging of mitochondrially-compartmentalised rhod-2 AM (Fig. 6A). Cytoplasmic Ca$^{2+}$ responses were detected by measuring the residual non-compartmentalised rhod-2 fluorescence in the nucleus. In this way, a mitochondrial free region of the cell can be analysed and used as a surrogate for bulk cytosolic Ca$^{2+}$ (Collins et al., 2001; Szado et al., 2008). ATP induced an increase in Ca$^{2+}$ in the
majority of mitochondria of both cell types that remained elevated for the duration of
the recording (Fig. 6Bi). Although a minor difference in the percentage of responding
mitochondria was observed between cell types (Fig. 6Bii), the integrated Ca^{2+}
response of the K-Ras-deleted HKH2 cells was significantly greater than in HCT116
cells (Fig. 6Biii).

Experiments were next carried out to determine whether the differences in the
mitochondrial Ca^{2+} uptake between the two cell types were a consequence of altered
IP_{3} signalling in ER-mitochondrial microdomains or due to an intrinsic alteration in
the Ca^{2+} uptake properties of the mitochondria. To this end, mitochondrial Ca^{2+}
uptake during the increase in cytosolic Ca^{2+} associated with store-operated Ca^{2+} influx
was also monitored. Ca^{2+} influx was initiated by readdition of Ca^{2+} to the imaging
buffer following depletion of intracellular ER Ca^{2+} stores with thapsigargin in Ca^{2+}-
free imaging buffer (Collins et al., 2001; Giacomello et al., 2010; Hanson et al.,
2008b). Under these conditions, a minor difference in the percentage of responding
mitochondria was observed and no difference in the integrated Ca^{2+} response of
mitochondria was observed between HCT116 and HKH2 cells (Fig. 6C). These
observations are consistent with the reported properties of Ca^{2+} uptake from bulk
cytosol rather than from the microdomains of high Ca^{2+} at the ER-mitochondrial
interface (Collins et al., 2001; Giacomello et al., 2010; Hanson et al., 2008b).
Together these data indicated that ER-mitochondrial Ca^{2+} flux is enhanced as a result
of K-Ras^{G13D} deletion in a manner independent of an alteration in the intrinsic Ca^{2+}
uptake properties of the mitochondria.

As a measure of the functional consequences of enhanced ER-mitochondrial Ca^{2+} flux
following K-Ras^{G13D} deletion, the sensitivity of HCT116 and HKH2 cells to apoptosis
induced by a stimulus that acts via Ca^{2+} was assessed. We and others have previously
shown that menadione induces apoptosis through ROS-dependent activation of IP_{3}Rs
and an elevation in mitochondrial Ca^{2+} (Baumgartner et al., 2009; Szado et al., 2008).
To this end, cytochrome c loss from mitochondria and DNA fragmentation were used
as hallmarks of apoptosis. Cytochrome c distribution was assessed by confocal
imaging of cells immunolabelled cells with antibodies againsts cytochrome c
following exposure to menadione for 20 hrs (Fig. 7Ai). While untreated HKH2 and
HCT116 cells displayed a typical mitochondrial distribution of cytochrome c, this
mitochondrial distribution was lost following treatment with menadione and became
diffusely distributed throughout the cytosol (Fig. 7Ai). HKH2 cells however exhibited a greater sensitivity to menadione treatment than HCT116 cells with cytochrome c being lost from the mitochondria in a significantly greater number of cells at 50 µM menadione. (Fig. 7Aii). DNA fragmentation was assessed as the percentage of the cell population with DNA content lower than that observed in the G1 phase of the cell cycle (SubG1). DNA content was determined by flow cytometric analysis of cells stained with propidium iodide (PI) (Hanson et al., 2008a). Basal levels of cell death were detected in both HCT116 and HKH2 cells (Fig. 7B). Application of menadione induced apoptosis in both HCT116 and HKH2 cells. However, the percentage of the cell population with a subG1 DNA content was significantly greater in the K-RasG13D deleted cells than in their HCT116 counterparts (Fig. 7B). Caspase activation, a further hallmark of apoptosis, was also observed following menadione treatment in HCT116 and HKH2 cells by imaging (Fig. 7Aii) and immunoblot (Fig. S2).

Taken together, these data indicate that decreased flux of Ca2+ to the mitochondria contributes to oncogenic phenotype of HCT116 cells.

Discussion

The impact of oncogenic K-Ras on Ca2+ signals, particularly in the context of oncogenic transformation is poorly understood. Here, we provide the first demonstration of an interaction of natively expressed oncogenic K-Ras with Ca2+ signalling and how this signalling crosstalk may affect cell fate. By comparing isogenic colon cancer cell line pairs expressing either a single mutant K-RasG13D or no mutant K-Ras we have determined that K-RasG13D deletion enhances IP3-dependent Ca2+ signals and ER-mitochondrial Ca2+ flux and this may sensitise cells to cell death stimuli. From these data, we propose that suppression of IP3 signalling from the ER and Ca2+ uptake by the mitochondria may contribute to the pro-survival properties of K-RasG13D associated with the oncogenic phenotype.

Cytosolic Ca2+ signals are generated by Ca2+ entry across the plasma membrane, Ca2+ release from intracellular stores or a combination of both (Berridge et al., 2003; Berridge et al., 2000; Bootman et al., 2003). Through manipulation of these Ca2+ signalling pathways in transformed cells, specific roles for each of these Ca2+ sources
in controlling aspects of cancer cell biology including regulation of cell proliferation, migration and death have been described (Crepin et al., 2007; Humez et al., 2004; Legrand et al., 2001; Lipskaia et al., 2009; Szatkowski et al., 2010; Yoshida et al., 2012). Altered expression of a number of Ca$^{2+}$ handling proteins in tumour tissue has also been determined (Arbabian et al., 2012; Korosec et al., 2006; Monteith et al., 2012; Monteith et al., 2007; Motiani et al., 2013). Although these findings are suggestive of an important role of certain Ca$^{2+}$ signalling pathways in transformed cells, causality has not been demonstrated. However, somatic mutations in SERCA have been identified in patients with colon cancer leading to the proposal that altered Ca$^{2+}$ signalling predisposes to oncogenic transformation (Korosec et al., 2006).

Cancers arise through mutations in oncogenes such as Ras or tumour suppressors that serve to promote or suppress their activity respectively (Hanahan and Weinberg, 2000). How the native expression of a specific oncogene in transformed cells affects Ca$^{2+}$ signalling and whether this contributes to the phenotype of the transformed cell is however not clear. A particular issue when investigating Ca$^{2+}$ signalling pathways is that the analysis of Ca$^{2+}$ signalling is only possible in live cells. The availability of appropriate controls for the cell line expressing the driving oncogene is also essential (Roderick and Cook, 2008). For analysing the effects of activated Ras isoforms, this is a particular concern since, oncogenic K-Ras can induce senescence or cell proliferation depending on the level of overexpression (Serrano et al., 1997; Tuveson et al., 2004). These issues are minimised through the use of isogenic cell line pairs in which studies are performed upon a cancer cell line harbouring a single allele of an activating oncogene and a second cell line in which the driving oncogene has been deleted by homologous recombination (Shirasawa et al., 1993). By comparing hormone-agonist induced Ca$^{2+}$ signalling between this pair of cell lines, we found that loss of K-Ras$^{G13D}$ enhanced cytosolic Ca$^{2+}$ signals. The elevated Ca$^{2+}$ responses in K-Ras$^{G13D}$ deleted cells persisted in Ca$^{2+}$-free imaging buffer, indicating that Ca$^{2+}$ release from the ER was important in defining the properties of the Ca$^{2+}$ response. Importantly, Ca$^{2+}$ signals were greater in two different colorectal cancer cell lines in which K-Ras$^{G13D}$ had been deleted (HKH2 and DKO4), as well as in HCT116 cells when K-Ras expression was reduced by siRNA, indicating that suppression of hormone-stimulated Ca$^{2+}$ signalling is a common response to K-Ras$^{G13D}$ in colorectal cell lines.
The greater magnitude of hormone-induced Ca\textsuperscript{2+} release from the ER in K-Ras\textsuperscript{G13D}-deleted cells could have arisen through a number of mechanisms. For example, increased GPCR expression or coupling to downstream effectors, modification in inositol phosphate metabolism, changes in IP\textsubscript{3}R expression or through a greater Ca\textsuperscript{2+} content of the ER. Given its pleiotropic nature, we speculated that Ras could interfere with any or all of these processes. Increased PLC activity and IP\textsubscript{3} levels have been reported in a number of transformed cell lines and in breast, ovarian and colonic carcinoma, suggesting that basal signalling is enhanced as a result of transformation (Weber, 2005). Oncogenic K-Ras may also increase signalling activity through stimulating PLC\textsubscript{ε}, which in turn, by increasing IP\textsubscript{3} levels would promote Ca\textsuperscript{2+} release from stores (Kelley et al., 2001). However, our observations that Ca\textsuperscript{2+} signals are negatively correlated with Ras abundance suggests that enhanced basal signalling possibly involving PLC\textsubscript{ε} does not contribute to Ras regulation of Ca\textsuperscript{2+} signalling in cells harbouring mutated K-Ras\textsuperscript{G13D}. As Ca\textsuperscript{2+} signals induced by stimulation of the histamine receptor, another GPCR, were also greater in K-Ras\textsuperscript{G13D}-deleted cells, it is unlikely that modifications in purinergic receptor expression contributed to the effects of K-Ras\textsuperscript{G13D} deletion. Similarly, Ca\textsuperscript{2+} responses induced by a cell permeant analogue of IP\textsubscript{3} were greater in K-Ras\textsuperscript{G13D}-deleted cells than in the isogenic parental cell line. As this cell-permeant IP\textsubscript{3} directly activates IP\textsubscript{3}Rs, circumventing GPCR, G-proteins and PLC, our data indicated that native levels of K-Ras\textsuperscript{G13D} had a direct effect on IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} release from the ER.

Notably, although the combined expression of IP\textsubscript{3}R isoforms was unaltered by K-Ras\textsuperscript{G13D} deletion, the relative abundance of the expressed IP\textsubscript{3}R subtypes was altered in the K-Ras\textsuperscript{G13D}-deleted cells. In particular, in K-Ras\textsuperscript{G13D}-deleted cells, IP\textsubscript{3}R3 expression was increased and IP\textsubscript{3}R1 expression suppressed, indicating that in colorectal cancer cell lines, K-Ras\textsuperscript{G13D} represses IP\textsubscript{3}R3 expression. Changes in the expression of IP\textsubscript{3}Rs in cancer cells have been reported previously. Most notably, an increase in IP\textsubscript{3}R3 expression at the mRNA level has been detected in a recent microarray analysis of K-Ras-deleted cells lines (Vartanian et al., 2013). In gastric cancer cells, an increase in IP\textsubscript{3}R3 was observed in the ascites, but not in cancer cells established from primary tumours. In the ascites, IP\textsubscript{3}R3 inhibition by 2-APB induced apoptosis (Sakakura et al., 2003). IP\textsubscript{3}R3 expression is also increased in MCF-7 cells.
induced to proliferate with estradiol (Szatkowski et al., 2010). An increase in IP₃R2 expression together with K-Ras has been observed in non-small cell lung cancer (NSCLC) cells (Heighway et al., 1996). Given the differing properties of each IP₃R isoform, this change in isoform composition following K-Ras deletion may have important consequences. As different IP₃R isoforms are differentially regulated by IP₃ and Ca²⁺, giving rise to distinct Ca²⁺ signalling fingerprints (Hattori et al., 2004; Miyakawa et al., 1999), the change in IP₃Rs isoform could contribute to the differences in Ca²⁺ signalling observed between the two cell types. A notable feature of IP₃R3 is that it is least sensitive to Ca²⁺-dependent inhibition (Hagar et al., 1998). As a result, whilst expression of IP₃R1 supports regular Ca²⁺ oscillations, monophasic Ca²⁺ transients are observed in IP₃R3 expressing cells (Almirza et al., 2010; Hattori et al., 2004; Miyakawa et al., 1999). Indeed, siRNA reduction in IP₃R3 expression in MCF-7 breast cancer cells transformed Ca²⁺ responses from a peak/plateau to a more oscillatory profile (Szatkowski et al., 2010). These different Ca²⁺ signatures likely allow signalling from each receptor to participate in different cell fate choices. Ca²⁺ oscillations arising from IP₃R1 may be optimised for controlling cytokinesis (Kittler et al., 2004) and gene expression (Dolmetsch et al., 1998) whereas the sustained Ca²⁺ signals arising from IP₃R3 may promote cell death (Blackshaw et al., 2000; Khan et al., 1996; Mendes et al., 2005; Szatkowski et al., 2010). Thus, a potential outcome of these different Ca²⁺ signatures is that the Ca²⁺ oscillations that arise from IP₃R1 in HCT116 cells sustain their rapid proliferation whereas the reduction in IP₃R3 protects the cells from cytotoxic Ca²⁺ signals.

An increase in ER Ca²⁺ store content was also observed in K-Ras<sub>G13D</sub>-deleted cells. As the Ca²⁺ content of the ER is a dominant determinant of the magnitude of IP₃-stimulated Ca²⁺ transient (Berridge, 2006; Caroppo et al., 2003), it is likely that this alteration in ER-Ca²⁺ store content significantly contributes to the enhancement in Ca²⁺ signalling observed in K-Ras<sub>G13D</sub>-deleted cells. Our data are consistent with the view that a relatively low level of Ca²⁺ in the ER offers an advantage to the transformed cell (Bergner and Huber, 2008). Specifically, by limiting Ca²⁺ release, the effect of stimuli that serve to induce apoptosis is diminished. The cytotoxic effects of Ca²⁺ release from the ER are mediated through accumulation in mitochondria, which results in permeability transition and activation of apoptotic pathways (Pinton et al., 2008; Roderick and Cook, 2008). Elevated Ca²⁺ may also activate DNA
endonucleases, promote phosphatidylyserine exposure, lead to cellular ATP depletion, increase in reactive oxygen species and ER stress (Orrenius et al., 2003). Despite this well accepted view of the benefit of lower ER Ca\textsuperscript{2+} for survival of transformed cells, much of the supporting data has emerged through investigation into the mechanism of actions of proteins involved in regulation of cell death pathways that are dysregulated in cancer. For example, enhanced expression of Bcl-2 family members or knockout of BH3 only pro-apoptotic proteins (which result in increased abundance of the anti-apoptotic family members) result in a lowering of ER free Ca\textsuperscript{2+} levels and reduced flux of Ca\textsuperscript{2+} to the mitochondria (Pinton et al., 2008; Roderick and Cook, 2008). By increasing leak through the IP\textsubscript{3}R or suppressing their activity, Bcl-2 family members also reduce IP\textsubscript{3}-induced Ca\textsuperscript{2+} release (Chen et al., 2004; Oakes et al., 2005). No differences in the expression of Bak, Bax, Bcl-2, Bcl-XL or Mcl-1 was detected between the HCT116 and HKH-2 cell types analysed here (data not shown). Notably, when analysed in HCT116 cells, no prominent role for Bax in the MOMP was detected (De Marchi et al., 2004), supporting the hypothesis that Ca\textsuperscript{2+} signalling remodelling by oncogenic K-Ras activation in HCT116 cells does not involve alterations in the expression of Bcl-2 family members. IP\textsubscript{3}R activity is also reduced by phosphorylation by the pro-survival kinase PKB/Akt (Khan et al., 2006; Marchi et al., 2012; Szado et al., 2008); a kinase that is increased in activity in many cancers.

A decrease in ER Ca\textsuperscript{2+} content has been detected in a subset of lung cancer cell lines when compared to normal human bronchial epithelial cells (Bergner et al., 2009). Consistent with the reduction in ER Ca\textsuperscript{2+} in cancer, a reduction in expression of SERCA pump has been observed in cancer derived cell lines and in tumours. Mutations in SERCA that result in loss of expression or activity have also been detected in tumours (Monteith et al., 2007). The importance of SERCA expression is also demonstrated by the induction of squamous cell cancers in SERCA2b haploinsufficient mice (Prasad et al., 2005). In humans however, loss of one SERCA allele results in Darrier’s disease (Hovnanian, 2007). In line with these studies, SERCA2b expression was significantly increased in K-Ras\textsuperscript{G13D}-deleted cells showing that a reduction in SERCA2b activity contributes to the phenotype of K-Ras-transformed cells. The increase in ER Ca\textsuperscript{2+} in K-Ras\textsuperscript{G13D}-deleted cells may also be explained by the observed reduction in IP\textsubscript{3}R1, which has been reported to contribute to the Ca\textsuperscript{2+} leak from the ER (Kasri et al., 2006; Oakes et al., 2005).
correlation between ER Ca\(^{2+}\) levels and proliferation has been observed in prostate cancer cell lines (Legrand et al., 2001). However, since depletion of the ER Ca\(^{2+}\) store inhibits proliferation and induces cell death in transformed cells, it would be important to correlate the absolute Ca\(^{2+}\) content of the ER and cell proliferation in these studies.

The mitochondria are an important target of Ca\(^{2+}\) released from the ER (Rizzuto et al., 2012). Mitochondrial Ca\(^{2+}\) uptake is a low affinity process. As such mitochondria preferentially accumulate Ca\(^{2+}\) at sites of close apposition with the ER called MAMs (Mitochondrial Associated Membranes) enriched in Ca\(^{2+}\) release channels including IP\(_3\)Rs (Csordas et al., 2010; Hajnoczky and Csordas, 2010; Rizzuto et al., 2004). Through this preferred pathway, mitochondrial function including metabolism and induction of apoptotic cell death is acutely modulated by IP\(_3\)-mediated Ca\(^{2+}\) signals. Here we show the first evidence of modifications in mitochondrial Ca\(^{2+}\) uptake downstream of endogenous oncogenic K-Ras. The ablation of oncogenic K-Ras\(^{G13D}\) increased the accumulation of Ca\(^{2+}\) in the mitochondria following Ca\(^{2+}\) release from the ER. Notably, the difference between the HCT116 and HKH2 cells was lost when Ca\(^{2+}\) uptake during Ca\(^{2+}\) influx from the extracellular space was analysed. Under these conditions Ca\(^{2+}\) uptake into the mitochondria is not restricted to the IP\(_3\)R containing MAMs. As such, microdomains of high Ca\(^{2+}\) at the ER–mitochondrial interface do not drive mitochondrial Ca\(^{2+}\) sequestration and the uptake observed is due to the properties of the mitochondrial uptake mechanisms alone (Collins et al., 2002; Rizzuto and Pozzan, 2006; Szabadkai and Duchen, 2008). Our data therefore suggest that the enhancement of ER-mitochondrial Ca\(^{2+}\) flux following K-Ras ablation is through modification of IICR. Consistent with increased IICR, this enhanced ER-to-mitochondria Ca\(^{2+}\) flux in HKH2 cells was mirrored by an increased sensitivity of these cells to Ca\(^{2+}\)-induced cell death. The increase in the ER-to-mitochondria Ca\(^{2+}\) transfer observed in HKH2 cells indicates that the expression of K-Ras\(^{G13D}\) acts to reduce this flux in HCT116 cells. In line with their lower ER-mitochondrial Ca\(^{2+}\) flux and apoptosis, the expression of IP\(_3\)R3, which has been proposed to specifically mediate pro-apoptotic Ca\(^{2+}\) fluxes at the MAM, is also reduced in HCT116 cells (Blackshaw et al., 2000; Khan et al., 1996; Mendes et al., 2005). More recently, the tumour suppressor PML localised at the ER was reported to specifically mediate the dephosphorylation of IP\(_3\)R3 by PP2a (Giorgi et al., 2010). Notably, the reintroduction
of ER-targeted PML in PML–/– cells restored the sensitivity to Ca\(^{2+}\)-dependent apoptosis (induced by Menadione and H\(_2\)O\(_2\)), but not that to Ca\(^{2+}\)-independent apoptosis (induced by the DNA-damaging agent Etoposide) (Giorgi et al., 2010). This finding is consistent with the increased sensitivity to menadione, observed in the K-Ras\(^{G13D}\)-ablated HKH2 cells.

In conclusion, our data describes for the first time the alterations in Ca\(^{2+}\) regulation driven by a single oncogenic K-Ras\(^{G13D}\) allele in colorectal cells. The enhancement of Ca\(^{2+}\) handling, mitochondrial sequestration and cell death as a result of loss of K-Ras\(^{G13D}\) in two isogenic models indicates that suppression of Ca\(^{2+}\) signalling is a common response to K-Ras\(^{G13D}\). Due to its pleitropic actions, modulation by K-Ras\(^{G13D}\) in colorectal cell is likely also to contribute to other aspects of cell physiology that may serve to promote cell transformation including enhancement of cell proliferation or modulation of metabolism. The importance of Ras activation and downstream pathways in cancers where mutated Ras is not the primary cause such as mutations in EGFR or B-Raf raises the possibility that the modulation of Ca\(^{2+}\) fluxes observed in this study due to K-Ras\(^{G13D}\) is a common feature of many cancers and is thus a target for intervention.

Materials and Methods

Cell culture

HCT116 and DLD-1 cells (both K-Ras\(^{G13D/WT}\)) and their respective isogenic derivatives HKH2 and DKO4 (both K-Ras\(^{WT}\)) were a kind gift of S. Shirasawa (Fukuoka University, Japan) and have been previously described (Shirasawa et al., 1993). Cells were cultured in DMEM (Life Technologies, Carlsbad, CA, USA), containing 10% heat-inactivated foetal bovine serum (FBS) (Invitrogen), 1% penicillin/streptomycin solution (5 units penicillin, 55 µg streptomycin) (Sigma, Dorset, UK). Cells were maintained at 37°C with 5% CO\(_2\) in saturated humidity and passaged upon reaching 80-90% confluency. Coverslips were coated with poly-L-lysine prior to seeding of cells.

Imaging of cytosolic Ca\(^{2+}\)
Cytosolic Ca\textsuperscript{2+} was imaged as previously described (Peppiatt et al., 2003). Briefly, cells were seeded onto poly-L-lysine-coated coverslips at equivalent densities and imaged after 48 h. Prior to each experiment, coverslips were mounted into stainless steel imaging chambers and loaded with fura-2 AM (Life Technologies; 2 µM for 30 min, followed by de-esterification in imaging buffer for a further 30 min). Coverslips were imaged on the stage of a Nikon Eclipse TE200 inverted epifluorescence microscope equipped with a Nikon PlanFluor 20x/0.75 NA multi immersion objective (Nikon, Kingston Upon Thames, Surrey, UK). Excitation light at 340 and 380 nm was selected using a motorised filter wheel (Sutter Industries, Novato, CA, USA) at a frequency of 1 image pair every 3 s with an exposure of 200 ms and emitted light was selected using a 400 nm dichroic mirror and filtered through a 460 nm long pass filter. Images were captured using a Hamamatsu ORCA ER Charge-Coupled Device (CCD) camera. Three coverslips per cell type were imaged per day on 3 separate days, each coverslip containing at least 50 cells. Ca\textsuperscript{2+} concentration was calculated according to Grynkiewicz et al. (Grynkiewicz et al., 1985).

**Imaging of ER Ca\textsuperscript{2+}**

The FRET-based, genetically-encoded D1ER Ca\textsuperscript{2+} indicator was a kind gift of A. Palmer (University of Colorado, Boulder, USA). The affinity of the indicator for Ca\textsuperscript{2+} has been determined to be 60 µM, allowing its successful use to monitor resting and dynamic changes in ER luminal [Ca\textsuperscript{2+}] (Palmer et al., 2004). In experiments involving D1ER, cells were seeded as indicated for ratiometric imaging but transfected with the D1ER construct after 24 h using JetPei (PolyPlus Transfection, Ilkirch, France) according to manufacturer’s specification. Cells were imaged 24 h post transfection. Coverslips were mounted in stainless steel chambers and imaged on the stage of an Olympus IX81 inverted epifluorescence microscope equipped with an Olympus UPlanSApo 20x/0.75 NA air objective. Excitation light at 435/10 nm was selected using a Polychrome V monochromator (Olympus, Southend-on-Sea, UK). Emitted fluorescence of CFP and YFP was simultaneously captured using a Cairn Optosplit II image splitter (Cairn Research Limited, Graveney Road, Faversham/Kent). The image splitter unit was configured with a 515 nm dichroic mirror, which reflected the emission fluorescence of CFP (further filtered through a 485/40 nm band-pass filter), and passed the emission of YFP (further filtered through a 535/30 nm band-pass filter).
Imaging of mitochondrial Ca\textsuperscript{2+}

Mitochondrial Ca\textsuperscript{2+} was imaged as previously described using rhod-2 AM as a Ca\textsuperscript{2+} indicator (Szado et al., 2008). Prior to each experiment, cells were loaded with rhod-2 AM (4 µM for 30 min at room temperature) followed by de-esterification in imaging buffer for a further 30 min. Cells were imaged using a VisiTech VoxCell Scan spinning disc confocal configured on a Nikon TE2000 microscope equipped with a Nikon 60x/1.25 NA oil immersion objective. Rhod-2 was excited by illumination with the 568 nm line of an argon/krypton laser. Emitted fluorescence was filtered through a 575/50 nm band-pass filter. Images were captured using a Hamamatsu ORCA ER CCD camera controlled by the Visitech Voxcell Scan software. Ca\textsuperscript{2+} concentration was calculated as previously described (Collins et al., 2001).

Immunoblotting

Cells were harvested after 48 h post seeding and protein lysate was quantitated using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). An equivalent amount of each sample (15 to 50 µg) was loaded onto 7% self-poured polyacrilamyde gels or onto 4-12% gradient pre-cast gels (NUPAGE; Life Technologies). Proteins were transferred from the gels onto poly vinilidene fluoride (PVDF) membranes (for IP\textsubscript{3}Rs and SERCAs and their loading controls) or nitrocellulose. Non-specific protein binding sites were first blocked by incubation for 1 h in TBS-T containing 5% milk. Membranes were subsequently probed with primary antibodies (diluted as indicated below in TBS-T milk) for 1 h at room temperature or 4°C overnight. Details of primary antibodies are as follows: anti-K-Ras (dilution 1:500, AbdSerotec); anti-SERCA2b (dilution 1:1000, kind gift of F.Wuytack, University of Leuven (Wuytack et al., 1989)); anti-IP\textsubscript{3}R3 (dilution 1:1000, BD Biosciences); anti-IP\textsubscript{3}R1 antibody (dilution 1:1000, in-house generated, (Kasri et al., 2004)); anti-calnexin (dilution 1:20000, Sigma); anti-SERCA3 (dilution 1:1000, gift of F.Wuytack(Wuytack et al., 1994)); anti-IP\textsubscript{3}R2 antibody (dilution 1:500, in-house generated (Harzheim et al., 2009)); anti-calreticulin (1:1000; (Roderick et al., 1997); anti-active caspase 3 (1:1000, BD BioSciences); anti-GAPDH (1:5000, Sigma) and anti-β actin (1:5000, AbCam). Excess antibodies were removed by washing with TBS-T. Membranes were then probed with either horse radish peroxidase (HRP)-conjugated (Jackson ImmunoResearch; 1:10000 dilution) or fluorescently-labelled secondary antibodies.
(Life Technologies and LI-COR; both at 1:5000). All membranes were then washed in 5 exchanges of TBS-T and 1 of TBS before band detection. HRP conjugated antibodies were detected by chemiluminescence (ECL) (Thermo Scientific) and subsequent exposure to film and fluorescently-labelled secondary antibodies (Life Technologies and LI-COR) were detected by digital scanning (LI-COR Odyssey). For band quantification, intensity values were obtained either through analysis of digitized film (ImageJ, for ECL detection) or Image Studio (for LI-COR detection). Bands of the protein of interest were normalised against the corresponding loading control band. Following normalization, protein abundance in the experimental conditions of interest was normalized to the control.

Immunofluorescence

Immunofluorescence was performed as previously described (Higazi et al., 2009). Briefly, 48 h post seeding, cells were fixed with fixation buffer (2% paraformaldehyde, 0.05% glutaraldehyde in PBS) and permeabilised with 0.2% TRITON X-100/PBS. After incubation in blocking solution (0.1% TRITON X-100, 5% donkey goat serum diluted in PBS), cells were probed with primary antibodies (diluted in blocking solution) for 1 h at room temperature. Details of primary antibodies are as follows: anti-cytochrome c (dilution 1:200, Santa Cruz); anti-active caspase 3 (1:200, BD BioSciences); anti-calnexin (1:500, Sigma). After removal of excess antibodies by washing in 0.1% TRITON X-100-PBS, cells were incubated with AlexaFluor fluorescently labelled secondary antibodies (Life Technologies) for 1 h at room temperature. Excess antibody was subsequently removed with 5 washes in 0.1% TRITON X-100-PBS and 2X in PBS. Coverslips were mounted in Vectashield containing DAPI to counterstain nuclei. Cells were imaged by point scanning confocal microscopy using appropriate laser lines for excitation of the dyes (Olympus FV1000 confocal configured on an Olympus IX81 inverted microscope and using 60x/1.35 NA oil immersion objective for calnexin and YFP imaging and Nikon A1R confocal configured on a Nikon Ti inverted microscope and using 60x/1.4 NA oil immersion objective for imaging of cytochrome c and activated caspase). Images were processed and analysed using Image J.

Cell cycle analysis
Cells in the media were collected and then pooled with cells that that remained attached to their substrate that were harvested by trypsinisation. After washing in PBS, cells were fixed with 70% ethanol prior to RNase treatment and staining with propidium iodide (PI). Stained cells were analysed with a Becton Dickinson FACSCalibur machine (Oxford, UK). Single cells in suspension were excited at 488 nm by an argon laser and analysed according to the intensity of emitted fluorescence through a 585/42 band pass (Hanson et al., 2008a).

**Menadione treatment of cells**

24 h post seeding, cells were exposed to menadione diluted in culture medium at concentrations between 25 and 100 µM. Control samples cultured in parallel were also analysed. Cells were harvested 20 h after exposure to menadione and processed for flow cytometric analysis of immunofluorescence.

**Statistical analysis**

Where data was compared to normalized control a one-sample \( t \)-test was employed. Other experiments were analysed by Student’s \( t \)-test or two way ANOVA. Significance was accepted at \( p<0.05 \).

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References


Hanson, C. J., Bootman, M. D., Distelhorst, C. W., Wojcikiewicz, R. J. and Roderick, H. L. (2008b). Bcl-2 suppresses Ca(2+) release through inositol 1,4,5-trisphosphate receptors and inhibits Ca(2+) uptake by mitochondria without affecting ER calcium store content. Cell Calcium.


Aminoethoxydiphenyl borate (2-APB) antagonises inositol 1,4,5-trisphosphate-induced calcium release, inhibits calcium pumps and has a use-dependent and slowly reversible action on store-operated calcium entry channels. Cell Calcium 34, 97-108.


Fig. 1. Agonist (ATP)-induced Ca\textsuperscript{2+} signals are influenced by K-Ras\textsuperscript{G13D} in the HCT116 colorectal cancer cell line. (Ai) Representative ATP-stimulated Ca\textsuperscript{2+} responses of HCT116 and HKH2 cells. Cells were stimulated with ATP at the concentrations indicated and experiments performed in the presence of extracellular Ca\textsuperscript{2+}. (Aii) Percentage of responding cells. (Aiii) Peak amplitude of Ca\textsuperscript{2+} response. (Aiv) Integrated Ca\textsuperscript{2+} response (AUC) of responding cells. Bar graphs represent the means \pm SEM of data from four days of experiments, where 3 coverslips per cell type were imaged on each day (n=12). At least 60 cells per coverslip were analysed. (Bi) Representative ATP-stimulated Ca\textsuperscript{2+} responses in HCT116 and HKH2 cells. Cells were stimulated with 2 \mu M ATP in absence of extracellular Ca\textsuperscript{2+}. (Bii) Percentage of responding cells. (Biii) Peak amplitude of Ca\textsuperscript{2+} response. (Biv) AUC of responding cells. Bar graphs represent the means \pm SEM of data from three days of experiments, where 3 coverslips per cell type were imaged on each day (n=9). At least 60 cells per coverslip were analysed. *P<0.05, **P<0.01, ***P<0.001 (Student’s t-test).

Fig. 2. ATP-stimulated Ca\textsuperscript{2+} responses are enhanced by siRNA depletion of K-Ras in HCT116 cells. (Ai) Representative immunoblot of K-Ras in HCT116 cells transfected with control or K-Ras targeting siRNA. (Aii) Quantitation of K-Ras knockdown in HCT116 cells (n=3). (Bi) Representative Ca\textsuperscript{2+} responses of siRNA-transfected HCT116 cells stimulated with 2 \mu M ATP in Ca\textsuperscript{2+}-free imaging buffer. (Bii) Percentage of responding cells. (Biii) Peak amplitude of Ca\textsuperscript{2+} response. (Biv) AUC of responding cells. All bar graphs represent the means \pm SEM of data from three days of experiments, where 3 coverslips per cell type were imaged on each day (n=9). At least 60 cells per coverslip were analysed. *P<0.05, **P<0.01, ***P<0.001 (Student’s t-test).

Fig. 3. Ca\textsuperscript{2+} responses induced by histamine and IP\textsubscript{3} ester are enhanced in the K-Ras\textsuperscript{G13D}-deleted HKH2 cell line. (Ai) Representative Ca\textsuperscript{2+} traces of HCT116 and HKH2 cells exposed to 1 \mu M Histamine. Histamine was applied in the Ca\textsuperscript{2+} free imaging buffer. (Aii) Percentage of responding cells. (Aiii) Peak amplitude of Ca\textsuperscript{2+} response. (Aiv) AUC of responding cells. Bar graphs represent the means \pm SEM of data from four days of experiments, where 3 coverslips per cell type were imaged on each day (n=12). At least 30 cells per coverslip were analysed. (Bi) Representative Ca\textsuperscript{2+} traces of HCT116 and HKH2 cells exposed to IP\textsubscript{3} ester (10 \mu M). (Bii) Percentage of responding cells. (Biii) Peak amplitude of Ca\textsuperscript{2+} response. (Biv) AUC of responding cells. Bar graphs represent the means \pm SEM of data from four days of experiments, where 3 coverslips per cell type were imaged on each day (n=12). At least 60 cells per coverslip were analysed. **P<0.01, ***P<0.001 (Student’s t-test).

Fig. 4. ATP-stimulated Ca\textsuperscript{2+} responses are controlled by K-Ras\textsuperscript{G13D} in the DLD-1 colorectal cancer cell line. (Ai) Representative Ca\textsuperscript{2+} responses of DLD-1 and DKO4 cells stimulated with 2 \mu M ATP in Ca\textsuperscript{2+}-free imaging buffer. (Aii) Percentage of responding cells. (Aiii) Peak amplitude of Ca\textsuperscript{2+} response. (Aiv) AUC of responding cells. All bar graphs represent the means \pm SEM of data from three days of experiments, where 3 coverslips per cell type were imaged on each day (n=9). At least 60 cells per coverslip were analysed. *P<0.05, **P<0.01, ***P<0.001 (Student’s t-test).

Fig. 5. ER Ca\textsuperscript{2+} levels are greater and expression of ER Ca\textsuperscript{2+} handling proteins remodeled in K-Ras\textsuperscript{G13D}-deleted HKH2 cells. (Ai) Representative Ca\textsuperscript{2+} responses
in HCT116 and HKH2 cells following application of thapsigargin in Ca^{2+}-free imaging buffer. (Ai) Peak Ca^{2+} response. (Aii) AUC. Bar graphs represent the means ± SEM of data from four days of experiments, where 3 coverslips per cell type were imaged on each day (n=12). At least 60 cells per coverslip were analysed. (Bi) Expression of the D1ER Ca^{2+} sensor in the ER. The expression and localization of D1ER determined by its excitation of YFP (in green) co-localises with the ER localized protein calnexin (in red; overlay image in yellow). (Bii) Representative FRET signals of D1ER-transfected cells. The Ca^{2+} level at the beginning of the experiment (represented by the YFP/CFP emission ratio) is higher in HKH2 cells compared to HCT116. (Biii) Baseline-subtracted Ca^{2+}-free fluorescence of the two cell types imaged. Bars represent the mean ± SEM of three days of experiments, where 3 coverslips per cell type were imaged on each day (n=9). At least 10 D1ER-expressing cells per coverslip was analysed. *P<0.05, **P<0.01, ***P<0.001 (Student’s t-test). (Ci) Representative immunoblot of SERCA2b. Calnexin is used as a loading control. (Cii) Fold change in SERCA2b expression in HKH2 cells with respect to HCT116 cells. Bar graph represents the mean ± SEM (n=21 for SERCA2b). (Di) Representative immunoblot of calreticulin. (Dii) Fold change in calreticulin protein expression in HKH2 cells with respect to HCT116 cells (mean ±SEM, n=8). (Ei, Eiii) Representative immunoblot of IP_3Rs detected with an anti-IP_3R1 antibody (Ei) and an anti IP_3R3 antibody (Eiii). IP_3R2 was not expressed in the cells. Calnexin (CLNX) was used as a loading control. (Eii, Eiv). Fold change in IP_3R1 (Eii, n=15) and IP_3R3 (Eiv, n=27) expression in HKH2 cells with respect to HCT116 cells. *P<0.05 and **P<0.01 (one-sample t-test).

**Fig. 6. Mitochondrial Ca^{2+} responses are enhanced by loss of K-Ras^{G13D} in HKH2 cells.** (A) Representative images of cells exhibiting mitochondrial Ca^{2+} responses to 100 µM ATP. Experiments are in Ca^{2+}-free imaging buffer. Time points of experiments are indicated. Scale bar = 10 µm. (Bi) Representative traces of mitochondrial Ca^{2+} responses in cells exposed to 100 µM ATP in the absence of extracellular Ca^{2+}. (Bii) Percentage of responding mitochondria. (Biii) AUC of mitochondrial Ca^{2+} response. (Ci) Representative mitochondrial Ca^{2+} responses during store operated Ca^{2+} entry, which was initiated by addition of Ca^{2+} to the imaging buffer following depletion of intracellular stores with TG. (Ci) Percentage of mitochondria exhibiting Ca^{2+} responses. (Ciii) AUC of mitochondrial Ca^{2+} response. All bar graphs represent the means ± SEM of data from three days of experiments, where 3 coverslips per cell type were imaged on each day. At least 10 cells per coverslip and at least 5 mitochondria per cell were analysed. *P<0.05, **P<0.01, ***P<0.001 (Student’s t-test).

**Fig. 7. Sensitivity to apoptosis induced by is enhanced by loss of K-Ras^{G13D} in HKH2 cells.** (Ai) Confocal images of cytochrome c (green) and activated caspase 3 (red) in HCT116 and HKH2 cells following 20 h exposure to 50 µM menadione. Scale bar represents 10 µ (Aii) Number of cells exhibiting diffuse cytochrome c. Upon treatment with menadione, a higher percentage of HKH2 cells displayed diffuse cytochrome c distribution in comparison with HCT116 (n=3 experiments). (B) Analysis of Sub-G_1 DNA content of HCT116 and HKH2 cells by flow cytometric analysis of PI-stained DNA. Percentages of cells in sub-G_1 are shown. An increase in the sub-G_1 DNA content of HKH2 cells with respect to HCT116 cells following
treatment with menadione is shown. All bar graphs represent means ± SEM (n=5).
*P<0.05 determined by two way ANOVA.
Fig. S1. (A) Expression of calnexin in lysates prepared from HCT116 and HKH2 cells. (i) Representative immunoblot of calnexin. GAPDH and beta-actin are used as a loading control. (ii) Fold change in calnexin expression in HKH2 cells with respect to HCT116 cells. Bar graph represents the mean ± SEM (n=6). Expression of calnexin was not significantly different between HCT116 and HKH2 (one-sample t-test).

(B) Expression of SERCA3 in lysates prepared from HCT116 and HKH2 cells. (i) Representative immunoblot of SERCA3. Calnexin is used as a loading control. (ii) Fold change in SERCA3 expression in HKH2 cells with respect to HCT116 cells. Bar graph represents the mean ± SEM (n=8). Expression of SERCA3 was not significantly different between HCT116 and HKH2 (one-sample t-test).

(C) Expression of IP$_3$R2 in lysates prepared from HCT116 and HKH2 cells. Representative immunoblot of IP$_3$R2 in HCT116, HKH2 and Rat1 fibroblasts. Calnexin is used as a loading control. An equivalent amount of protein lysate to that used for detection of IP$_3$Rs in HCT116 and HKH2 cells prepared from the Rat-1 fibroblast cell line was used as a positive control for detection of IP$_3$R2.

Fig. S2. Immunoblot of activated caspase 3 in HCT and HKH2 cells exposed to menadione for 20 h. Lysates prepared from naïve and menadione exposed HCT116 and HKH2 cells were resolved by SDS PAGE and transferred to nitrocellulose. Blots were probed with antibodies directed against activated (cleaved) caspase and against β-actin and GAPDH, which were used as controls for equivalent loading. Fluorescently labeled secondary antibodies were detected using the LI-COR Odyssey imaging system.
**A**

- **i**
  - Graph showing ATP concentrations (2 μM, 10 μM, 100 μM) over time (s).
  - Traces for HCT116 and HKH2.

- **ii**
  - Bar graph showing responding cells (%) for ATP concentrations (2, 10, 100 μM).
  - Significance indicated: *** and *.

- **iii**
  - Graph showing peak $\left[Ca^{2+}\right]_{(nM)}$ over ATP concentrations (2, 10, 100 μM).
  - Significance indicated: ***.

- **iv**
  - Bar graph showing AUC for ATP concentrations (2, 10, 100 μM).
  - Significance indicated: *** and **.

**B**

- **i**
  - Graph showing ATP concentration (2 μM) in Ca$^{2+}$-free buffer over time (s).
  - Traces for HCT116 and HKH2.

- **ii**
  - Bar graph showing responding cells (%) for HCT116 and HKH2.

- **iii**
  - Bar graph showing peak $\left[Ca^{2+}\right]_{(nM)}$ for HCT116 and HKH2.

- **iv**
  - Bar graph showing AUC for HCT116 and HKH2.